Molecular Epidemiology of Livestock-Associated Methicillin Resistant *Staphylococcus aureus* (LA-MRSA) and Extended-Spectrum Beta-Lactamase (ESBL)-Producing *Enterobacteriaceae* in Pigs and Exposed Workers in Cameroon and South Africa

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A

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Submitted in fulfilment of the requirements for the degree of

Doctor of Philosophy (PhD) in Pharmaceutics

(Pharmaceutical Microbiology)

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By

Luria Leslie Founou

BSc.; MSc. in Clinical Microbiology

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Co-supervisor: Dr Cyrille Le Duc Finyom Djoko

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A thesis submitted to the School of Health Sciences, College of Health Science, University of KwaZulu-Natal, Westville Campus, for the degree of Doctor of Philosophy in Pharmaceutics (Pharmaceutical Microbiology).

This is a thesis in which the chapters are written as a set of discrete research publications, with an overall introduction and final summary.

This is to certify that the content of this thesis is the original research work of Mrs Luria Leslie Founou.

As the candidate's supervisor, I have approved this thesis for submission.

Supervisor:

Signed: ---------- Name: **Prof. Sabiha Y. Essack** Date: ----------

Signed: ---------- Name: **Dr Cyrille Le Duc F. Djoko** Date: ----20/11/2017----

DECLARATION

I, **Luria Leslie Founou** declare that

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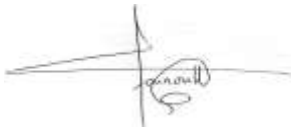
Signature: 

Date: 20th November 2017

DEDICATION

To all my loved ones and all those who have one day contributed to raising me up to this level.

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May God bless and protect you all!!!

LIST OF ABBREVIATIONS AND ACRONYMS

ABR	Antibiotic Resistance
AMR	Antimicrobial Resistance
ARB	Antibiotic Resistant Bacteria
ARGs	Antibiotic Resistant Genes
CC	Clonal Complex
CLSI	Clinical and Laboratory Standards Institute
CTX-M	Cefotaximase-München
DDST	Double Disk Synergy Test
ERIC-PCR	Enterobacterial Repetitive Intergenic Consensus- Polymerase Chain Reaction
ESBL-PE	Extended Spectrum Beta-Lactamase Producing *Enterobacteriaceae*
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FAO	Food and Agriculture Organization of the United Nations
HGT	Horizontal Gene Transfer
HIV/AIDS	Human Immunodeficiency Virus and Acquired Immune Deficiency Syndrome
MDR	Multi-Drug Resistant
MIC	Minimum Inhibitory Concentration
MRSA	Methicillin Resistant *Staphylococcus aureus*
OIE	World Organization for Animal Health
PBP	Penicillin Binding Protein
PCR	Polymerase Chain Reaction
PVL	Panton-Valentine Leucocidin
REP-PCR	Repetitive Element Palindromic-Polymerase Chain Reaction (REP-PCR)
SCC	Staphylococcal Cassette Chromosome
SHV	Sulfhydryl-variable enzyme
Spa	Protein A of *Staphylococcus aureus*
ST	Sequence Type
TEM	Temoneira Enzyme
TSST	Toxic Shock Syndrome Toxin
UN	United Nations
VGT	Vertical Gene Transfer
WHA	World Health Assembly
WHO	World Health Organization

ABSTRACT

Livestock-associated methicillin resistant *Staphylococcus aureus* (LA-MRSA) and extended-spectrum β-Lactamase (ESBL)-producing *Enterobacteriaceae* (ESBL-PE) are amongst the main resistant bacteria of serious public health concern worldwide. Between March and October 2016, MRSA and ESBL-PE were identified from 288 nasal and rectal pooled samples collected from 432 pigs as well as from nasal and hand swabs sampled from 82 humans in five abattoirs in both Cameroon and South Africa. Carriage, risk factors, antibiotic resistance profiles, genotypic characteristics and clonal relatedness of circulating MRSA and ESBL-PE in pigs and humans were investigated using various microbiological (selective agar, biochemical testing, VITEK 2 system) and molecular methods (REP-PCR, ERIC-PCR and whole genome sequencing) and compared with statistical tools.

Of the 288 pooled samples, methicillin resistant staphylococci (MRS) were isolated from 108/144 (75%) and 102/144 (70%) of the pooled nasal and rectal samples, respectively. Only one (0.07%) and four (16.66%) MRSA were detected from pigs in Cameroon and South Africa, respectively. None of the workers were colonized by MRSA. Genome analysis revealed that all circulating MRSA isolates belonged to the clonal lineage ST398.

ESBL-PE were isolated from 75% (108/144) and 71% (102/144) of the pooled nasal and rectal samples, respectively. ESBL-PE prevalence in animal samples from Cameroon was higher than for South Africa whereas human ESBL-PE carriage was observed in Cameroonian workers only. Some *E. coli* and *K. pneumoniae* isolates detected in humans were highly related to those isolated from pigs within and across countries. The circulating *K. pneumoniae* strains were dominated by the sequence types (ST) 14 and 39. The "high-risk" ST307 clone and two novel STs assigned ST2958 and ST2959 were also detected.

LA-MRSA ST398 is already present in both Cameroon and South Africa and is probably underestimated in the absence of molecular epidemiological studies. The high prevalence of ESBL-PE in pigs in both countries as well as in humans in Cameroon highlights their active dissemination in the farm-to-plate continuum. LA-MRSA and ESBL-PE are serious food safety and public health threats requiring the urgent implementation of multi-sectorial, multi-faceted, and, sustainable interventions among all stakeholders involved in this continuum to contain their emergence and zoonotic transmission.

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CHAPTER I
GENERAL INTRODUCTION

I. Introduction[1]

After saving countless lives and being available for the last 80 years, antibiotics are now facing extinction through the worldwide emergence of antimicrobial resistance (AMR). Included in the One Health Agenda as one of the most important priorities of the "Tripartite alliance", AMR has been recognized has a global health crisis which no government can counter alone (FAO et al., 2012; WHO, 2014a, 2015a; OIE, 2016). AMR kills, hampers the control of infectious diseases, increases the costs of health care, jeopardizes health care gains in the society, and has the potential to threaten health security, and weaken trade and economies. It also raises mortality, morbidity, duration of hospitalization, treatment and expenses (WHO, 2014a, 2015a). The occurrence and spread of AMR differs greatly with geographical distribution –between continents and inside countries. In developed countries, multi-drug resistant bacteria induce infections which lead to increased therapeutic cost whereas in developing nations, the same pathogens considerably enhance mortality and morbidity (WHO, 2014a). The emergence of AMR can occur in commensal bacteria as well as in pathogenic bacteria of humans, animals, and the environment (Marshall and Levy, 2011; Berendonk et al., 2015; Holmes et al., 2016). AMR is considered as a cross-sectoral issue because antibiotics are widely used in aquaculture, livestock production, and horticulture, where antibiotic resistant bacteria (ARB) and antibiotic resistant genes (ARGs) can easily spread into the human population via the food chain including farming, slaughtering processes, food storage and transportation.

Livestock-associated methicillin resistant *Staphylococcus aureus* (LA-MRSA) and extended-spectrum β-Lactamase (ESBL)-producing *Enterobacteriaceae* (ESBL-PE) are amongst the main resistant bacteria of serious public health concern globally (Chen, 2013, 2014). *S. aureus* became a worldwide health problem due to the emergence of multidrug-resistant strains named methicillin resistant *Staphylococcus aureus* (MRSA) (Chen, 2013). The recent detection of a new Clonal Complex (CC) MRSA CC398 in Dutch pigs has increased the concern (Voss et al., 2005; Price et al., 2012). This finding suggests pigs as the true reservoir of LA-MRSA even if other livestock animals –poultry, cattle, calves, rabbits, etc.- have also been identified as reservoirs of LA-MRSA. Generally, they act as reservoir of LA-MRSA and enhance the risks of zoonotic transmission among persons exposed to them as well as for other animals and the environment, contributing by this way, to the complex exchanges, variations and hosts

[1] The reference list of this chapter is combined with that of Chapter 8 at the end of the thesis.

adaptabilities of different strains (van Loo et al., 2007; van Den Broek et al., 2008; Price et al., 2012). Similarly, ESBL-PE have emerged following chromosomal mutation, acquisition of new resistance genes into the chromosome or by transferable genetic elements (Bradford, 2001; Liebana et al., 2013). ESBL-producing bacteria are resistant to the majority of the beta-lactam class of antibiotics with a few exceptions: cephamycins, carbapenems, and virtually all beta-lactamase inhibitors which are hydrolysed by other enzymes such as Amp-C enzyme; *Klebsiella pneumoniae* carbapenemase (KPC), metallo-beta-lactamase enzyme, OXA beta-lactamase and inhibitor-resistant enzymes respectively (Bradford, 2001; Liebana et al., 2013). They are found in commensal as well as in pathogenic strains of *Escherichia coli*, *Klebsiella spp*., *Proteus spp*., *Salmonella spp.*, etc., and can occur in non-fermentative organisms such as *Acinetobacter baumannii* and *Pseudomonas aeruginosa* (Liebana et al., 2013; Revathi et al., 2013). The spread of these resistant bacteria in human and animal health is a considerable problem not only because their increase treatment failures, mortality and treatment expenses, but also because resistance genes can be transferred among different bacteria and because they could ultimately lead to pre- or post- antibiotic era where minor infections and injuries could once again kill.

Cameroon and South Africa are peculiar countries with evidence of several factors that lead to the emergence and dissemination of AMR. Additionally, in these countries the sub-optimal health systems and programs able to control and lessen infectious diseases and resistant bacteria further increase difficulties for the prevention and monitoring of AMR in human and veterinary medicine as well as in agriculture. This study illustrates the zoonotic transmission of AMR in the One Health context.

II. Background and rationale

Given the recent isolation of LA-MRSA and ESBL-PE in pigs and exposed workers (including farmers, slaughterers and veterinarians who are highly associated with LA-MRSA and/or ESBL-PE colonization/infection), the high level of antibiotic consumption and prevalence of infectious diseases in Sub-Saharan African countries, together with sub-optimal monitoring, prevention and control measures it is inevitable that dissemination of ARB does occur at the human-animal interface on the continent. In Africa, the prevalence of ARB in food animals is not well known and may probably be underestimated due to the gaps encountered in their detection. The importance of Cameroonian and South African food safety is not only national but more importantly sub-regional and even continental due to their geographic location and

as they are both among principal pig producers in Africa. Despite the gaps evidenced and to the best of our knowledge, studies focusing on the direct exposure and zoonotic transmission of ARB, especially MRSA and ESBL-PE from animals to humans have not been undertaken concomitantly in human and animal populations in these two nations. This study was thus designed to correlate the genetic diversity of resistant bacteria associated with human and animal populations as well as to show the potential of zoonotic dissemination in the farm-to-plate continuum; this in order to elucidate the potential public health risk of these resistant pathogens. It will also provide evidence to agricultural practitioners, policy makers, scientific communities and health policy-makers, about the dangers posed by the emergence of ARB in the food chain. It will finally suggest effective prevention measures to contain AMR and behavioural changes for better antimicrobial usage in agricultural practice and in human health to successfully combat infectious diseases and preserve the efficacy of antibiotics around the world in general and particularly in Africa.

III. Literature review

The following sections discuss the role of animals as source of (resistant) human infectious diseases and the different initiatives promoting and improving human and animal health. The literature review is expanded further in Chapters Two and Three in the form of one published and one unpublished review papers.

1. Animal origin of infectious diseases

Malaria, highly pathogenic avian Influenza (HPAI H5N1), Human immunodeficiency virus infection and acquired immune deficiency syndrome (HIV/AIDS), Severe Acute Respiratory Syndrome (SARS) and Ebola haemorrhagic fever are known mainly for their worldwide devastating impact on human health but less so for their common characteristic of their animal origin (Woolhouse and Gowtage-Sequeria, 2005; Calistri et al., 2013; Gibbs, 2014; Travis et al., 2014). Since many decades, pathogens affecting the human population have been associated with animal exposure. About 60% of the global human infectious diseases and 75% of new emerging pathogens have an animal origin and are thus zoonotic infectious diseases (Woolhouse and Gowtage-Sequeria, 2005; Calistri et al., 2013; Gibbs, 2014; Travis et al., 2014). “Zoonosis”, “anthropo-zoonosis” and “zoo-anthroponosis” are general terms used to define any disease or infection naturally transmitted from vertebrate animals to humans and vice versa (Woolhouse and Gowtage-Sequeria, 2005; Messenger et al., 2014). Globally, “hot

spot" regions including the Congo Basin, Amazonia and Mexico, the Ganges plain and South-East Asia have principally been identified as high-risk areas for the emergence or re-emergence of zoonotic infectious diseases (Woolhouse and Gowtage-Sequeria, 2005; Mersha and Tewodoros, 2012; Messenger et al., 2014). The understanding of the ecology of emerging diseases forms the basis for strategies designed to contain new pathogens and pandemic situations (Woolhouse and Gowtage-Sequeria, 2005; Gibbs, 2014 ; Messenger et al., 2014; Travis et al., 2014). Modern public health issues are now becoming increasingly complex with the international transport systems. A resistant pathogen can easily go across the world via people, animals, plants and food products in less than 24 hours (Woolhouse and Gowtage-Sequeria, 2005; Mersha and Tewodoros, 2012; Messenger et al., 2014). The process of bringing a foreign disease into a new area due to human involvement has been termed "pathogen pollution" in an effort to describe the spread of pathogens around the world (Woolhouse and Gowtage-Sequeria, 2005; Mersha and Tewodoros, 2012; Messenger et al., 2014). The high level of mobility of microorganisms, and the worldwide emergence and dissemination of zoonotic infectious diseases led to several concerns for the global economy and especially for public health in both developed and developing countries. With the major implication of animals in this universal problem, there is a need to follow multidisciplinary and inter-sectorial strategies exhorted by the "One Health" approach which is crucial to the sustainable prevention of potentially pandemic infectious diseases to assure global health (Woolhouse and Gowtage-Sequeria, 2005; Mersha and Tewodros, 2012; Messenger et al., 2014; Gibbs, 2014; Travis et al., 2014).

For effective and efficacious actions, strong synergies amongst all sectors, good governance and political will are essential. In keeping with this, the World Health Assembly (WHA) of the World Health Organization (WHO) urged the revision of the International Health Regulations (IHR) (1995), considered a central element of the One Health approach (Nutall et al., 2014). The revised IHR (2005) offer a more supportive and legally binding framework for the assessment of public health emergencies in general, and infectious disease risk, in particular. The IHR (2005) are not limited to the human health sector, but encourage integrated and coordinated risk responses, and thereby represent an ideal environment for the implementation of the One Health approach towards the prevention and containment of disease outbreaks, including those of zoonotic nature (FAO et al., 2008; Nuttall et al., 2014).

The Global Health Security Agenda (GHSA) initiated in February 2014 by the United States (US), several partner nations, international organizations and civil society as a 5-year plan to:

- Increase global efforts for prevention, detection, and response to infectious disease threats.
- Promote global security as a worldwide priority
- Support progress towards complete implementation of the WHO IHR (2005) (Toner et al., 2015)

In 2014, 11 actions packages were developed and the emergence and spread of AMR was one of the key challenge recognized by the GHSA. The action packages on AMR form an inclusive and multipronged "prevent, detect, respond" 5-year framework with international public-private efforts to curb spread of resistance. The five-years action package on AMR seeks to develop activities to combat it across animal, human, agriculture, food and environmental aspects (Toner et al., 2015).

2. The "One Health" approach

An alliance of a few international organizations including the British Royal Society, Food and Agriculture Organization of the United Nations (FAO), WHO, World Organization for Animal Health (OIE), World Bank, United Nations System Influenza Coordination (UNSIC), World Small Animal Veterinary Association (WASAVA), the US Army Medical Department have mobilized to develop and promote One Health (Mersha and Tewodoros, 2012; Calistri et al., 2013; Gibbs, 2014; Travis et al., 2014). The One Health approach, defined as '...the collaborative effort of multiple disciplines –working locally, nationally, and globally – to attain optimal health for people, animals and our environment…''(AVMA, 2008) acknowledges that a human being is an entity which does not exist in isolation but is the central element of the total living ecosystem where activities and status of each member directly affect the others (Mersha and Tewodoros, 2012; Calistri et al., 2013; Gibbs, 2014; Travis et al., 2014). Indeed, healthy livestock animals and healthy environment, lead to healthy human population because humans, animals and environment are inexorably connected. Furthermore, livestock dynamics are considered a major determinant of global health. In fact, the potential emergence and spread of new pathogens from animals to human is favoured by changes in animal production. Thus, the key element of this approach which addresses a manifold of interconnected health risks is the animal-human-environment interface (Mersha and Tewodoros, 2012; Gibbs, 2014).

The One Health approach seeks to improve the diagnosis and prevention of zoonotic infectious diseases as well as the early detection of environmental health risks at this interface (Mersha and Tewodoros, 2012; Calistri et al., 2013; Gibbs, 2014; Travis et al., 2014). This original initiative has expanded to a set of holistic and integrated policies, programs and interventions, which improve the global health of humans, animals and the environment and promote the involvement, improved coordination and suitable cooperation between several scientific specialities, practitioners, scientists locally, nationally and internationally (AVMA, 2008; Mersha and Tewodoros, 2012; Calistri et al., 2013; Gibbs, 2014; Travis et al., 2014). It takes place in developed and developing countries, where the targets are populations subject to risk factors such as poor health, unsafe or limited water, lack of hygiene, insecure food, and proximate contact with animals. It has been recognized as the most productive method to prevent, identify, contain and control zoonotic infectious diseases (Mersha and Tewodoros, 2012; Calistri et al., 2013; Gibbs, 2014; Travis et al., 2014). One Health is strengthened by the coalition between the WHO, OIE and FAO with their specialized skills to ensure coordinated international efforts, to implement effective facilities, policies, and efficient behavioural change towards the mitigation of health threats related to the human-animal-environment interface (Mersha and Tewodoros, 2012; Calistri et al., 2013; Gibbs, 2014; Travis et al., 2014). The success of this action will be determined by our understanding of the different factors that drive the emergence and spread of emerging infectious diseases. Among those factors is the microbial adaptation with AMR as one of the most challenging consequence.

3. Antimicrobial resistance—a worldwide threat

AMR is the ability of pathogens to grow despite the presence of antimicrobial agents usually effective for treatment of infections caused by these pathogens (WHO, 2014a, 2014b). It is a general term including resistance to antibiotics that treat infections caused by bacteria (e.g. *E. coli*), parasites (e.g. *Plasmodium spp*.), viruses (e.g. HIV), and fungi (e.g. *Candida spp*.) while antibiotic resistance (ABR) is the specific term used to describe resistance to antibiotics that occurs in commensal, pathogenic and zoonotic bacteria (WHO, 2014a, 2014b). These resistance mechanisms are not a new finding but rather a natural evolutionary process that affects humans, animals, and the environment (plants, soil and water). Indeed, during his Nobel Prize allocution in 1945, Alexander Fleming, who discovered the first antimicrobial drug, had already mentioned the possible acquisition of resistance by bacteria (WHO, 2014a, 2014b). Microorganisms are becoming increasingly resistant to antibiotics with worldwide evolution

as a result of the selective pressure induced through extensive consumption of antibiotics whether appropriate or inappropriate use (Founou et al., 2016).

According to the Review on Antimicrobial Resistance chaired by O'Neill, AMR claims more than 50,000 deaths every year across US and Europe and is expected to prematurely kill 300 million people by 2050, with 2 to 3.5% losses of the world's Gross Domestic Product (GDP) by the same period (O'Neill, 2015). Sixty to 100 trillion USD worth of the economic output will be lost if AMR is not considerably contained (O'Neill, 2015). The WHO indicated "antimicrobial resistance" as the topic of its annual *World Health Day* in 2011 confirming AMR as a significant worldwide health concern (WHO, 2014a, 2014b) including AMR in food animal production throughout the "No action today, no cure tomorrow" WHO's slogan (WHO, 2014a, 2014b).

The occurrence and spread of AMR differ greatly with geographical distribution between continent and within countries (Laxminarayan et al., 2013). In developed countries, multi-drug resistant (MDR) bacteria cause infections which result in increasing cost of therapy whereas in developing ones, the same pathogens considerably enhance mortality. Antimicrobial usage is strictly controlled in human as well as in animal health in developed countries through several initiatives and the strategies established for the prevention and containment of AMR illustrate the significance of this threat in the developed world (Laxminarayan et al., 2013). The risk of emergence and spread of AMR is worsened in developing countries because treatment of bacterial infections is empirical and there is usually sub-optimal policy, monitoring and surveillance systems, diagnosis capability and control of infectious diseases, antibiotic consumption and AMR detection as well as numerous issues related to the quality and accessibility of antibiotics (Vila, 2010; Laxminarayan et al., 2013; WHO, 2014b). Indeed, several factors have been incriminated in the speedy emergence and spread of AMR. These include poverty, overcrowding, hygiene status, education level, self-medication, poor and limited supply chain, lack of knowledge about antibiotics, inappropriate use misuse of antibiotics and chronic diseases –such as tuberculosis, hepatitis, asthma, HIV/AIDS which enhance the proportion of immune-compromised population who are more prone to infection, requiring frequent antibiotic treatment resulting in selection pressure for the development or exacerbation of antibiotic resistance (Vila, 2010; Laxminarayan et al., 2013; WHO, 2014b). AMR is of paramount importance because many common infectious diseases in the developing world, especially malaria, HIV/AIDS, respiratory infections (influenza, asthma, tuberculosis,

etc.) and sexual transmissible infections raise the risk of acquisition of resistant pathogens (Vila, 2010; Laxminarayan et al., 2013; WHO, 2014a, 2014b). Given the important ability of microorganisms to adapt to their environment, a comprehensive strategy involving key stakeholders promoting the "One Health" approach is likely to succeed in containing AMR.

4. Tripartite Alliance in the containment of antimicrobial resistance

At the 2011's High Level Technical Meeting held in Mexico City, AMR was recognized, as one of the three priorities for the control of health risks under the auspices of the coalition between the WHO, OIE and the FAO, referred as the "Tripartite Alliance" (Mersha and Tewodoros, 2012; FAO et al., 2012; Gibbs, 2014). During this meeting, the need to improve multidisciplinary cooperation through recognized mechanisms and standards such as the IHR (2005), the FAO-WHO Codex Alimentarius and the FAO-WHO-OIE Global Early Warning System (GLEWS) platform was strengthened from both human and animal health sectors (FAO et al., 2012).

Following several initiatives such as the World Health Assembly resolution WHA 58.27, the global strategy for containment of AMR, the World Health Day policy package in 2011 and several international conference proceedings agree that essential systematic multi-sectoral and coordinated efforts, collaboration and cooperation as recommended by the One Health approach are required to curb the spread of AMR worldwide (WHO, 2014a, 2014b). By endorsing the One Health approach, the FAO, WHO and OIE with their specialized skills, reinforce, facilitate, and enhance international efforts, implement adequate framework, policies, and efficient behavioural change towards the mitigation of health threats related to the human-animal-environment interface (FAO et al., 2012; FAO, 2015; WHO, 2015a; OIE, 2016).

The recent Global Action Plan (GAP) on AMR developed by the WHO in collaboration with the FAO and the OIE, and published in 2015 in response to the resolution WHA 67.25 aims at ensuring sustainable efficacious treatment and prevention of infectious diseases using efficient, safe and proper quality of drugs, consumed appropriately and accessible to all needy persons (WHO, 2015a). It highlights the necessity to contain the emergence and spread of AMR across sectors and outline several measures under five strategic objectives (WHO, 2015a):

- Improve awareness and understanding of AMR using effective education, training, and communication

- Strengthen evidence base and knowledge via surveillance and research
- Decrease the incidence of infection by implementing effective sanitation, hygiene and infection prevention and control measures (IPC)
- Optimize rational antibiotic use in human and animal health
- Promote sustainable investment considering the needs of all countries, and increase investment in the creation of new medicines, vaccines diagnostic tools, and other alternatives to antibiotics (WHO, 2015a).

The GAP therefore underscores the importance of an effective One Health approach encompassing cross-sectoral and interdisciplinary coordination among all stakeholders (WHO, 2015a). The WHA 68.25 resolution further strengthened the Tripartite Alliance and made requisite for Member States to adapt this strategic document to their realities and develop and implement their own National Action Plans (NAPs) on AMR within the subsequent two years upon this endorsement viz. mid-2017.

Similarly, the FAO's 39th Conference held in June 2015, endorsed the Resolution 4/2015 on AMR recognizing it as a growing serious threat for public health and sustainable food production and consumption, and confirming that involvement of all sectors of government and society are required for its effective containment. The FAO released its "Action Plan on Antimicrobial Resistance 2016–2020" in September 2016 to assist Member States in the development and implementation of their multi-sectorial NAP by May 2017 and mitigate the impact of AMR. It supports the food and agricultural sectors to implement the WHO-led GAP and emphasizes the necessity to adopt the One Health approach, with the participation of veterinary and public health authorities, food and agriculture sectors, environmental specialists, financial planners, and consumers (FAO, 2016). The FAO Action Plan on AMR addressed four main focus areas including:

- Improvement awareness on AMR and related threats
- Development of capacity for monitoring and surveillance of AMR and antimicrobial use in food animals and agriculture
- Strengthening of governance associated with antimicrobial use and AMR in food and agriculture
- Promotion of good practices in food and agricultural systems and the prudent use of antimicrobials (FAO, 2016).

The OIE also calls for curbing AMR in keeping with the One Health approach and, during its 83rd general Assembly in 2015, all 180 Member States committed themselves to support the WHO GAP on AMR, and develop NAPs. The Resolution 36, mandating OIE to compile AMR activities into a strategy was unanimously adopted at its 84th General Assembly (OIE, 2016). Its "Strategy on Antimicrobial Resistance and the Prudent Use of Antimicrobials" which is aligned with the WHO GAP objectives and recognize the importance of the One Health approach was published in November 2016 (OIE, 2016). The OIE's strategy on AMR outlines strategies and goals to support Member States and encourage national implementation and ownership. It further reflects the mandate of the OIE as outlines in its Basic Texts and Strategic Plans, through four principal objectives:

- Improve awareness and understanding
- Strengthen knowledge using surveillance and research
- Support capacity building and good governance
- Encourage implementation of international standards (OIE, 2016).

Finally, in September of the same year, the foremost advancement in the fight against AMR, was the adoption of a resolution at the 71st Session of the United Nations General Assembly to strengthen implementation of NAP in line with the One Health concept and as advocated by the Tripartite partners (UN, 2017). Consequently, an Intergovernmental Ad Hoc Group encompassing Tripartite Partners and others international agencies was thereafter created in March 2017 for effective containment of AMR globally (UN, 2017).

5. Antimicrobial resistance in the food chain –a serious food safety issue

The emergence of ARB and ARGs and their spread in the farm-to-plate continuum (viz. from the farm to the end consumer) via direct and indirect contact, exacerbate health and socio-economic repercussions of AMR globally. Direct contact, that occurs through immediate exposure of humans with contaminated food animals and biological substances, enhances the rapid and easy dissemination of ARB and ARGs from host-to-host. It increases the likelihood of ARB and ARGs of animal origin to enter and spread into the human communities and hospital settings where substantial exchanges are possible, thereby jeopardizing healthcare systems (Marshall and Levy, 2011; Woolhouse et al., 2015; Founou et al., 2016). The human population may also be indirectly exposed to ARB and ARGs via contact with or consumption of contaminated food products (Marshall and Levy, 2011; Founou et al., 2016). This indirect

transmission is often more dangerous and far-reaching contamination, allows the spread of AMR at each step in the food chain, and has been associated with several foodborne infection outbreaks globally (WHO, 2015b; EFSA and ECDC, 2016). Moreover, with the globalisation of trade in animals and food products as well as international travel, there are neither species, ecological nor geographical boundaries to contain AMR. Resistance emerging in one geographical location or bacterial species can easily spread or spill-over into several bacteria at each stage in the farm-to-plate continuum and AMR may thus similarly affect all countries, regardless of the income level (Founou et al., 2016; Holmes et al., 2016). In fact, the presence of these bacteria in commensal micro-flora and pathogenic bacteria of animals implies that they could act as reservoirs for AMR and be a source of contamination for the human population (Smet et al., 2010). There are multiple examples of resistant bacteria which are major public health concerns, but bacteria causing infections in humans including antibiotic-resistant infections and originating (partially) from food animals, are frequently *Staphylococcus aureus* and the members of the *Enterobacteriaceae* family.

5.1. Methicillin Resistant *Staphylococcus aureus*

S. aureus has become a worldwide health problem due to the emergence of drug-resistant strains named Methicillin Resistant *Staphylococcus aureus* (MRSA) (Price et al., 2012). MRSA has developed its resistance to methicillin following the acquisition of the mobile genetic element, *Staphylococcal Cassette Chromosome (SCCmec*), carriers of the mec resistance gene (IWG-SCC, 2009). *SCCmec* is a DNA fragment that has integrated the *S. aureus* chromosome near the origin of replication and has conferred to this bacterium a methicillin resistance as well as a large spectrum resistance to the beta-lactam family (IWG-SCC, 2009).

The first detection of MRSA Clonal Complex (CC) CC398 in Dutch pigs suggested that this animal is the true reservoir of LA-MRSA even if other livestock animals –poultry, cattle, calves, rabbits, etc.- have also been identified as reservoirs of LA-MRSA (Voss et al., 2005; van Loo et al., 2007; van den Broek et al., 2008; Price et al., 2012; Chen, 2013). Several reports have focused on the history and evolution of MRSA and its spread among the animal population as well as its transmission from animals to humans and vice-versa (van Loo et al., 2007; van Den Broek et al., 2008; Wulf and Voss, 2008; Wulf et al., 2008a; Price et al., 2012; Chen, 2013). In an international study including nine countries, Wulf et al., (2008) reported a 12.5% (34/272) prevalence of MRSA in professionals (other than farmers and veterinarians) in

contact with pigs. They showed that 91% (31/34) of these strains were multilocus sequence type (ST) 398, and were previously isolated from Dutch pigs, pig farmers and veterinarians (Wulf et al., 2008a). They further concluded that if the spread of these strains is not considerably curbed, they could represent an important source of community-acquired MRSA (CA-MRSA) not only in Europe, but also worldwide (Wulf et al., 2008a). Price et al., (2012) revealed that livestock-associated MRSA CC398 emerged as methicillin susceptible *S. aureus* in humans. They argued that this CC398 strain underwent a jump from humans to food animals where it has subsequently acquired methicillin and tetracycline resistance. They further highlighted the public health risks associated with the widespread use of antibiotic in food animal production (Price et al., 2012). The rise of carriage of LA-MRSA could therefore threaten epidemiology, national economies and global strategy for MRSA containment in communities and hospitals. It is further important to consider the extent to which these strains may have disseminate in the livestock, community and hospital settings.

Animals are often colonized by LA-MRSA but clinical diseases linked to LA-MRSA have rarely been reported (van Loo et al., 2007; van Den Broek et al., 2008; Wulf et al., 2008a; Chen, 2013). Generally, they act as reservoirs of LA-MRSA and enhance the risks of zoonotic transmission among persons exposed to them as well as among other animals and the environment, contributing by this way to complex exchanges, variations and hosts adaptabilities of different strains (van Loo et al., 2007; van Den Broek et al., 2008; Wulf et al., 2008a; Chen, 2013).

Some risks factors have been associated with the colonization and spread of LA-MRSA in the human populations. These include pig exposure, living or working in farms with animals, recent hospitalization, living in rural areas, proximity to farm and human MRSA colonization or infection –individual carriers of MRSA are predisposed to LA-MRSA, since there is just a little difference between both strains (Lewis et al., 2008; Wulf and Voss, 2008; Graveland et al., 2011). These factors in addition to other elements such as lack of knowledge about good farming practices, limited education, poor hygiene, low income, easy accessibility to and extensive use (appropriate and inappropriate) of antibiotics facilitate the emergence and colonization of LA-MRSA (Van Cleef et al., 2011; Laxminarayan et al., 2013). Pig farmers, slaughterhouse workers, pig's carriers and veterinarians, have high occupational risks for LA-MRSA colonization and infection (Voss et al., 2005; van Loo et al., 2007; van Den Broek et al., 2008; Wulf et al., 2008a; Van Cleef et al., 2011; Price et al., 2012; Chen, 2013; van de

Vijver et al., 2014). LA-MRSA CC398 has been identified in humans in Dutch regions with a high density within animal farming areas (van Loo et al., 2007, van Den Broek et al., 2008; Van Cleef et al., 2011; van de Vijver et al., 2014). Several other clonal lineages of MRSA such as CC5, CC97, CC30, CC9 have been involved in livestock colonization, outbreaks and clinical illness in humans and identified as LA-MRSA (Wagenaar et al., 2009; Köck et al., 2013). This indicates that LA-MRSA could be associated with human infectious diseases and has a pandemic potential (Wagenaar et al., 2009; Price et al., 2012; Chen, 2013; Köck et al., 2013).

The zoonotic transmission of MRSA from animal to human and vice-versa is then possible through various pathways and the isolation of the ST398 from pigs and other species has demonstrated their transmission between different animal reservoirs (Voss et al., 2005; Price et al., 2012; Mehndiratta and Bhalla, 2014). To confirm this, several studies have established the predominance of LA-MRSA in livestock around Europe including the Netherlands, Denmark, Germany, Belgium, France, etc. and its emergence in pig farms in Northern America, Latin America, and Asia (van Duijkeren et al., 2007; van Loo et al., 2007; van den Broek et al., 2009; de Boer et al., 2009; Kluytmans, 2010). In a case-control study, van Loo et al. (2007) showed that LA-MRSA colonized more frequently pig and cattle farmers. The study revealed that 32 out of 35 patients were colonized by MRSA ST398 whereas the remaining harboured ST9, ST752 and ST753 that are closely related to ST398. They further concluded that MRSA from animal origin spread to the human population and was responsible for > 20% of all MRSA cases in the Netherlands (van Loo et al., 2007). When comparing the prevalence of animal and human MRSA in Dutch pig farms, van den Broek et al. (2009) identified 28 out of 50 (56%) farms had MRSA positive pigs, while 15 out of 50 (30%) humans were identified as MRSA carriers. MRSA strains isolated from humans share similar spa-type as those found in pigs and were ST398 (van den Broek et al., 2009). Working in pig stables (OR=40, 95% CI: 8-209) and the presence of sows and finishing pigs (OR=9, 95% CI: 3-30) were identified through multivariate analyses as main risk factors for human MRSA carriage (van den Broek et al., 2009).

Some reports have further confirmed the emergence of MRSA and particularly of LA-MRSA in food of animal origin. For instance, a Dutch survey revealed a 11.9% prevalence of MRSA in a variety of meat with predominance (85%) of LA-MRSA (de Boer et al., 2009). In this study, MRSA strains were isolated mainly from 16.0% of turkey, 15.2% of veal, 10.7% of pork, 10.7% of chicken, 10.6% of beef (de Boer et al., 2009). This observation suggests that

contamination may occur during several steps of the food production process. In Africa, there is a lack of information about MRSA in food animal as well as in food of animal origin. This is due to the lack of research studies conducted in the domain.

Besides the global dissemination and asymptomatic carriage, LA-MRSA have increasingly been incriminated in various human infections –such as endocarditis, necrotizing pneumonia, necrotising fasciitis, ocular infections, wound infections and even death- in Dutch and German hospitals particularly in regions with a high density of pig production activities (Wulf et al., 2008b, Köck et al., 2013). Although LA-MRSA virulence is almost null or likely lesser than other human MRSA clones, it is nevertheless important to notice the reports of outbreaks and clinical illness in humans in hospitals due to a variety of clonal lineages of LA-MRSA such as ST398, ST9, ST5 and ST97 (Wulf et al., 2008b, Wagenaar et al., 2009). For instance, two hospital-acquired outbreaks of MRSA of livestock origin have recently been recognized in Dutch health care settings and in nursing homes demonstrating the putative spreading of LA-MRSA in the general population (Wulf et al., 2008b).

The constant evolvement, versatility, and virulence mechanisms of *S. aureus* as well as the potential for genetic exchanges between LA-MRSA and other bacteria among several hosts are of further great concern. Many reports have underlined the origin and evolution of MRSA of animal origin in the human population, enlightening its adaptation, its ability to switch among host species and subsequent spread of new clones widely into the general population (van Loo et al., 2007; van Den Broek et al., 2008; Graveland et al., 2011; Köck et al., 2013). In addition, the acquisition and arrangement by LA-MRSA of human virulence mechanisms such as Panton-Valentine leucocidin (PVL) toxin as well as the ability of this highly virulent strain to spread easily and quickly (via skin-to-skin contact) among human population is a worrying scenario.

Resistant strains recognized as originating from animal, are serious threats to the world as they could lead to the emergence of new and more resistant, virulent, and mobile strains, unknown from the human immune system given their multiple hosts adaptability, virulence mechanisms and high genetic exchanges (Ewers et al., 2012; Price et al., 2012; Fernandes et al., 2016; Liu et al., 2016). This phenomenon termed “superbug” illustrates the main concern feared by the international scientific community (Wulf and Voss, 2008; Weese, 2010) and is a considerable worldwide issue.

Accordingly, studies are needed around the world and especially in low and middle-income countries (LMICs) where policies on antimicrobial use and antimicrobial stewardship programs are limited, to correlate the genetic diversity associated with the genetic exchange and spreading of resistant bacteria. The current study aims at exploring and monitoring the LA-MRSA at its origin viz. in livestock animal, as well as in the human population exposed to it, viz. in slaughterhouse workers and veterinarians; thus providing valuable source of information to understand the potential public health impact associated with these bacteria in Cameroon and South Africa.

5.2. Extended Spectrum Beta-Lactamase Producing *Enterobacteriaceae*

Among all antibiotics, the beta-lactam antibiotics are those which are most frequently used in the treatment of various infectious diseases caused by *Enterobacteriaceae*, that is a heterogeneous family and the most important bacteria encountered in human health clinically (Kayser, 2004). Indeed, the beta-lactam family involves numerous groups of antibiotics characterized by the presence of one special element, called the "β-lactam ring" (Bradford, 2001; Kayser, 2004; Liebana et al., 2013). Beta-lactamases are enzymes produced by several bacterial species able to inactivate beta-lactam antibiotics by the hydrolysis and opening of the beta-lactam ring. Extended spectrum β-lactamases (ESBLs) have emerged following chromosomal mutation or following acquisition of new resistance genes into the chromosome or by transferable genetic elements. Thus, beta-lactamase-encoding genes are located on the chromosome as well as on genetic elements such as plasmids, insertion sequences, genomic islands, integrons, transposons, and phage-related elements (Bradford, 2001; Kayser, 2004; Liebana et al., 2013). These can yield bacteria that are resistant to the majority of beta-lactam antibiotics through the hydrolysis and opening of the beta-lactam ring. Indeed, ESBL-producing bacteria are resistant to most of the beta-lactam class of antibiotics with a few exceptions: cephamycins, carbapenems, and virtually all beta-lactamase inhibitors which are hydrolysed by other enzymes such as Amp-C enzymes; class A, B and D carbapenemases and inhibitor-resistant enzymes respectively (Bradford, 2001; Liebana et al., 2013).

They are found in commensal as well as in pathogenic strains of *E. coli*, *Klebsiella spp.*, *Proteus spp.*, *Salmonella spp.*, and can occur in other *Enterobacteriaceae* species and in non-fermentative organisms (Bradford, 2001; Liebana et al., 2013). The most important ESBLs enzymes involved in human infections-related to *Enterobacteriaceae* are TEM-, SHV- and CTX-M- enzymes (Bradford, 2001; Liebana et al., 2013).

As with MRSA, the emergence and spread of ESBL-producing *Enterobacteriaceae* in food animal is a worldwide public health issue due to their ability to disseminate among the human population. The latest worrying scenario is the emergence of carbapenem-resistant *Enterobacteriaceae* (CRE) in both humans and animals; especially with the detection ESBL-producing *E. coli* resistant to carbapenems in German pigs. These strains harbour a carbapenemase enzyme called Verona integron-encoded metallo-β-lactamase (VIM)-1 carbapenemase resistant to the beta-lactam antibiotics family plus additional co-resistance (Fischer et al., 2012).

Emergence of ESBL-PE in food animal is a major public health issue worldwide since many studies have largely incriminated the most predominant species of *Enterobacteriaceae, E. coli* and *Klebsiella spp*., each one producing ESBL, in the colonization and infection of food producing animals (Cortés et al., 2010; Ben Sallem et al., 2012; Geser et al., 2012; Liebana et al., 2013; Blaak et al., 2015; Dahms et al., 2015; Dohmen et al., 2015; Iweriebor et al., 2015; Kilani et al., 2015; Chishimba et al., 2016). In human health, antibiotic use has been established as the most important risk factors for the colonization and spread of multidrug-resistant organisms, and the same evidence has been shown in animal health. In fact, the common use of antibiotics and particularly of broad spectrum cephalosporins in livestock as well as the administration of sub-therapeutic doses of these substances as growth-promoters to prevent rather than cure infections in different categories of food animal industries led to the emergence of ESBL-producers and increased their transmission risks to humans through various reservoirs (asymptomatic or sick humans and animals, and the environment) and through the food chain (Geser et al., 2012). The misuse and inappropriate use of antimicrobials agents, mainly broad-spectrum antibiotics, may therefore contribute to the emergence and spread of ESBL-producer in animals (Geser et al., 2012). The emergence of ESBL-PE in animals seriously threatens the global health as there are no geographic borders to impede their worldwide dissemination. Prevention and containment measures should be applied locally, nationally, and regionally, to not compromise the efficacy and endanger ABR containment policies implemented in other parts of the world, the best-managed high-resource countries included.

Different types of ESBLs (Class A, B, C and D) have been progressively found in food animal, food of animal origin and in the environment globally (Smet et al., 2008, 2010; Geser et al., 2012; Liebana et al., 2013; Brower et al., 2017). Since 2000, ESBLs of animal origin particularly from pigs, poultry, cattle, rabbits, have retained the worldwide attention (Smet et

al., 2010; Geser et al., 2012; Liebana et al., 2013). Smet et al. (2008) reported 70% of ceftiofur resistant *E. coli* isolated from 489 cloacal samples collected at five Belgian broiler farms. Out of these, 45% and 43% of *E. coli* isolates were classified as ESBL and AmpC β-lactamase producers, respectively (Smet et al., 2008). Different resistance mechanisms were identified, including TEM-106 (2%), CTX-M-15 (2%), CTX-M-14 (5.9%), CTX-M-2 (7.8%), TEM-52 (13.2%), CTX-M-1 (27,4%) and CMY-2 (49%) (Smet et al. 2008). The remaining isolates exhibited the combination of ESBL and AmpC β-lactamase phenotype (12%) in two farms. The authors concluded that the presence of ESBLs and AmpC genes in commensal *Enterobacteriaceae* of normal microbiota of food animals might pose a human health threat given the fact that they constitute an important reservoir of resistance genes for pathogenic bacteria (Smet et al., 2008). Likewise, A 2012 study, examining ESBL-PE in faecal samples at slaughterhouse and food products at dairy farms, revealed 1%, 8.6%, 13.7%, 15.3%, and 63.4% ESBL-PE prevalence in mastitis milk, sheep, cattle, pigs, and chicken, respectively (Geser et al., 2012). CTX-M group 1 (CTX-M-gr1) and CTX-M group 9 (CTX-M-gr9) genes were produced by 85.7% and 6.6% of strains, respectively; whereas 5.5% harboured SHV- and 2.2% TEM-type enzyme (Geser et al., 2012). In a survey on 18 poultry farms Punjab, India, Brower et al., (2017) detected 1,556 *E. coli* from 530 birds and reported that resistance profiles and prevalence of ESBL-PE significantly differed between farm types. They concluded that unregulated use of clinically important antibiotics in Indian broiler and layer farms might lead to the emergence of ABR and underline the need to limit the non-therapeutic use of these medically important substances in food animals (Brower et al., 2017).

In Africa, knowledge about the epidemiology of colonization or infections related to these resistant bacteria in animals is relatively limited. Few studies have recognized the faecal carriage of ESBL producers in food animals. ESBL-producing *E. coli* strains harbouring CTX-M-8 and SHV-5 have been detected in 13.8% of faeces of various healthy food animals in Tunisian farms (Ben Sallem et al., 2012). In Nigeria, 20.7% of bacteria mostly *Salmonella spp.*, were identified as producers of ESBL enzymes in commercial poultry feeds (Oyinloye Jr. and Ezekiel, 2012). In South Africa, Iweriebor et al. (2015) detected 31.7% of *E. coli* O157 from faecal samples collected from cattle and dairy farms. The majority (88.42%) of isolates were shiga toxin produced with high prevalence of MDR. TEM (27%), CTX-M (65%), CMY (70%) and AmpC (90%) were the main β-lactamase gene identified (Iweriebor et al., 2015). Similarly, 90 out of 150 chickens (60%) sampled from eight slaughterhouse/markets in Cameroon were

detected positive for *Salmonella spp.*, with almost 40% of strains being MDR (Wouafo et al., 2010).

However, studies ascertaining the molecular epidemiology and zoonotic transmission of ESBL-PE concomitantly in food animals and occupationally exposed workers are rare in developing countries, including Cameroon and South Africa. Substantial studies considering risk factors, prevalence, genetic diversity, and transferability of resistance genes are therefore required and should thus be carried out to determine potential reservoir and estimate public and animal health threat associated with ESBL-PE in these nations.

Given their high prevalence in human infections, and to their worldwide faecal carriage in healthy humans and animals, ESBL-PE could undoubtedly contaminate humans, food animals and products either during the farming (directly via person-to-person contact and indirectly via food, water, soil, air), slaughtering (stunning, cutting the throat, scalding, evisceration), storage or food transportation processes (Geser et al., 2012; Oyinloye Jr. and Ezekiel, 2012; Ewers et al., 2012; Liebana et al., 2013). Ewers et al., (2012) revealed that exposed humans and animals shared identical sequence type (ST) of ESBL/AmpC isolates suggesting that there is a potential transmission or parallel micro-evolution (Ewers et al., 2012). They also suggested that the role of animal ESBL/AmpC-producing isolates as major source of human infections is underestimated and that it is a highly serious issue (Ewers et al., 2012).

This study seeks therefore to consider, ascertain, and monitor the ESBL-PE in livestock animal and occupationally exposed workers in order to provide information on the current status, molecular epidemiology and potential public health repercussions associated with these bacteria in Cameroon and South Africa.

5.3. Concluding remarks

ABR is a worldwide public health issue with serious health and socio-economics repercussions that is significantly influenced by antibiotic use in food animals. The World Bank recently estimated in its 2016 report that the annual cost of AMR could be as high as those of the 2008's global financial crisis and that LMICs would be most affected with the largest economic shortfalls in economic growth (World Bank, 2016). It revealed that output and trade in food animals and products are especially vulnerable to AMR effects not merely because of reduced productivity associated with resistant infections, but also due to international trade disruption in the wake of disease outbreaks (World Bank, 2016). Indeed, consequences related to food

contamination will drain national economy as country's economic losses associated with resistant foodborne infections will range from increase of national medical expenses, outbreak investigations and food recalls. The report estimated that decline in global food production could range between 2.6-7.5% of food products and LMICs will be the most affected with 11% loss in a simulation of worst AMR impact scenario (World Bank, 2016).

These suggest that if nothing is done to significantly address this threat, the socio-economic development of several countries, particularly those in the developing world which relies mainly on agriculture and food production, and are not sufficiently implementing adequate measures to prevent and curb the spread of ABR from farm-to-plate, will be seriously hindered, along with the achievement of some world's sustainable development goals (Laxminarayan et al., 2013; Årdal et al., 2016; Jasovsky et al., 2016; O'Neill, 2016).

There is currently limited data on the molecular epidemiology of MRSA and ESBL-PE in Cameroon and South Africa, ascertaining risk factors, resistance and virulence mechanisms, in the food chain. This study provides a food safety and One Health perspective in selected abattoirs in Yaoundé, Cameroon and KwaZulu-Natal Province, South Africa, which could be useful in motivating the development of effective prevention and containment measures in these settings, and to a certain extent to other African countries as some frontier nations could face similar realities. The relative limitation observed in terms of prevalence, risk factors, resistance and virulence mechanisms, and clonality in pigs and exposed workers is addressed in this study within and between countries, to provide a holistic overview of the current status of resistance burden related to carriage isolates and underscore the urgent need for efficient containment measures in the food chain in Cameroon and South Africa.

IV. Research aims and objectives of the study

1. Overarching aim

The overarching aim of this study is set to estimate the prevalence and determine the phenotypic and genotypic characteristics including but not limited to the clonal relatedness, genetic diversity, virulence factors and resistance mechanisms of circulating MRSA and ESBL-PE in pigs and occupationally exposed humans in Cameroon and South Africa; this in order to improve knowledge about risks factors associated with the emergence and spread of MRSA and ESBL-PE as well as their zoonotic transmission in the food chain.

2. Specific objectives

More specifically, the study aims:

i. To evidence the danger of antibiotic resistance in the food chain and particularly in developing countries as a serious global public health threat by means of a review paper.

ii. To describe the distribution of ARB in food animals in Africa, in order to highlight the need to restrict the use of antibiotics in agriculture, and provide evidence for the implementation of the One Health approach to contain the emergence and spread of ABR on the continent by means of a systematic review and meta-analysis of published literature.

iii. To detect MRSA/ESBL-PE strains from nasal and rectal swabs of pigs from five selected slaughterhouses, using selective media, followed by delineating the bacterial characteristics (morphology after Gram staining, appearance of colonies on culture media) and biochemical tests as well as the Vitek® 2 System (BioMérieuX, Marcy l'Etoile, France) automated method, in order to ascertain the colonization and spread of these pathogens in occupationally exposed humans involved in the pig production in Yaoundé, Cameroon and KwaZulu-Natal, South Africa by original experimental research.

iv. To detect MRSA/ESBL-PE strains from nasal and hands swabs of exposed workers, using selective media, followed by delineating the bacterial characteristics (morphology after Gram staining, appearance of colonies on culture media) and biochemical tests as well as Vitek® 2 System (BioMérieuX, Marcy l'Etoile, France) automated method, in order to ascertain the colonization and spread of these pathogens in occupationally exposed humans involved in the pig production in Yaoundé, Cameroon and KwaZulu-Natal, South Africa by original experimental research.

v. To determine their antimicrobial resistance profiles through antibiotic susceptibility testing by minimum inhibitory concentration (MIC) determination using the broth microdilution with Vitek® 2 System (BioMérieuX, Marcy l'Etoile, France) automated methods by original experimental research.

vi. To compare the prevalence and resistance phenotypes of MRSA and ESBL-PE strains isolated from Cameroonian and South African slaughterhouses by original experimental research.

vii. To delineate risk factors associated with the colonization and dissemination of ESBL-PE and/or MRSA among exposed workers, including demographic data (age, sex, profession, education level, socioeconomic considerations, monthly income, etc.), clinical data (recent hospitalization, recent antibiotic use, diabetes mellitus, hypertension, asthma, tuberculosis, HIV/AIDS, skin and nasal problems, etc.), as well as occupational factors (animal gender, intensity of contacts with pigs, slaughterhouse practices, hygiene and safety precautions measures implemented), in order to establish the possible route of transmission between

human and animal and vice versa, using a structured questionnaire by original experimental research.

viii. To undertake molecular characterization of MRSA/ESBL-PE strains isolated from pigs and humans by whole genome sequencing (WGS) in order to provide information about the phylogenetic evolution, resistance and virulence genes and sequence type, as well as mobile genetic elements (MGEs) by original experimental research.

ix. To summarize the current state of knowledge and potential public health risks associated with the presence of MRSA or ESBL-E in pigs, to act as food-borne pathogens by way of original experimental research.

x. To suggest prevention and containment measures for antibiotic resistance in the food chain based on the original research conducted above and review papers.

3. Study design and methodology

Between March and October 2016, nasal and rectal swabs of pigs and nasal and hand swabs of occupationally exposed workers older than 21 years old were collected in five abattoirs in both Cameroon and South Africa. All samples were screened on selective media (MacConkey agar and Mannitol salt agar) supplemented with antibiotics (cefotaxime and cefoxitin) and identification was undertaken by colony morphology, catalase and oxidase tests, with confirmation by the Vitek® 2 System (BioMérieuX, Marcy l'Etoile, France). The cefoxitin screening and double disk synergy tests were used for methicillin resistance and extended-spectrum beta-lactamase production in staphylococci and *Enterobacteriaceae*, respectively. Repetitive-palindromic polymerase chain reaction (REP-PCR) and enterobacterial-repetitive-polymerase chain reaction (ERIC-PCR) were used to ascertain the clonal relatedness of MRSA and ESBL-PE isolates, respectively. Genomic DNA of representative isolates were further subjected to whole genome sequencing (WGS) using an Illumina MiSeq platform to delineate the molecular epidemiology, resistance genes, virulence factors, phage-related regions and circulating clonal lineages of these isolates. Generated reads were assembled with CLC Genomics Workbench and SPAdes, annotated with PGAP and RAST. ResFinder, VirulenceFinder, PlasmidFinder and PHAST were used to identify antibiotic resistance genes, virulence factors, plasmids and phage associated-regions. The multilocus sequence type (MLST) of the isolates was determined from the WGS data. Whole genome phylogenetic analysis was performed using the Rapid large-scale prokaryote pan genome analysis (Roary).

4. Thesis outline

The present study is outlined in the form of published journal articles and unpublished manuscripts, and is delineated by the following seven chapters:

- **Chapter 2. Article I:** Antibiotic Resistance in the Food Chain: A Developing Country-Perspective. *Front Microbiol. 7:1881. doi: 10.3389/fmicb.2016.01881.* This review evidences the danger of ABR in the food chain and particularly in developing countries as a serious global public health threat by highlighting the transmission routes of ABR along the food chain and comparing the status of ABR in food animals in developed vs. developing countries. Prevention and containment measures of ABR from farm-to-fork are also delineated. This paper addresses objectives One and Ten.
- **Chapter 3. Article II:** Antibiotic Resistance in Food Animals in Africa: A Systematic Review and Meta-Analysis. This systematic review analysed the published literature on the emergence/prevalence of ABR in food animals in Africa, in order to (1) describe the distribution of ABR-bacteria in food animals; (2) highlight the need to restrict the use of antibiotics in agriculture, and (3) provide evidence to follow the One Health approach to contain the emergence and spread of ABR on the continent. This manuscript addresses objectives Two and Ten. It has been submitted to Microbial Drug Resistance and is currently under review.
- **Chapter 4. Article III:** Emergence and Spread of Extended Spectrum Beta-Lactamase (ESBL)-Producing *Enterobacteriaceae* in Pig Slaughterhouses and Exposed Workers: A Multicentre Comparative Study Between Cameroon and South Africa. This original research article reports on and compares the prevalence, risk factors as well as clonal relatedness of ESBL-PE circulating in Cameroonian and South African slaughterhouses and addresses objectives Three, Four, Five, Six, Seven, Nine and Ten. It has been submitted to PloS One and currently under review.
- **Chapter 5. Article IV:** Mannitol-Fermenting Methicillin-Resistant Staphylococci (MRS) in Pigs and Abattoir Workers in Cameroon and South Africa: A Serious Food Safety Threat. It has been submitted to the International Journal of Food Microbiology and is currently under review. It reports on and compares the prevalence, risk factors as well as clonal relatedness of MRS circulating in Cameroonian and South African slaughterhouses and addresses objectives Three, Four, Five, Six, Seven, Nine and Ten.
- **Chapter 6. Article V:** Genome Analysis of Methicillin-Resistant *Staphylococcus aureus* Isolated from Pigs: Emergence of the Clonal Lineage ST398 in Cameroon and South Africa. This original research article has been submitted to Frontiers in Microbiology and

is currently under review. It reports on antibiotic resistance genes, virulence factors, mobile genetic elements and genetic lineages of circulating MRSA strains isolated from pigs in Cameroonian and South African abattoirs using whole genome sequencing (WGS) and addresses objectives Eight and Nine. This paper will reference "Mannitol-Fermenting Methicillin-Resistant Staphylococci (MRS) in Pigs and Abattoir Workers in Cameroon and South Africa: A Serious Food Safety Threat" which is expected to be published first.

- **Chapter 7. Article VI:** Whole Genome Sequencing of Circulating ESBL-producing *Klebsiella pneumoniae* Isolated from Pigs and Abattoir Workers in Cameroon. This original research article has been submitted to Frontiers in Microbiology and is currently under review. It reports on antibiotic resistance genes, virulence factors, mobile genetic elements, and genetic lineages of circulating ESBL-producing *K. pneumoniae* strains isolated in Cameroonian slaughterhouses using whole genome sequencing and addresses objectives Eight and Nine. This paper will reference the "Emergence and Spread of Extended Spectrum Beta-Lactamase (ESBL)-Producing *Enterobacteriaceae* in Pig Slaughterhouses and Exposed Workers: A Multicentre Comparative Study Between Cameroon and South Africa" paper, which is expected to be published first.
- **Chapter 8. Conclusion:** This chapter presents the extent to which the overarching aim was met by reviewing the key findings for each specific objective. It further delineates the significance, limitations and recommendations of the study for the field of food safety and antibiotic resistance.

CHAPTER 2

Article I. Antibiotic Resistance in the Food Chain: A Developing Country-Perspective

Antibiotic Resistance in the Food Chain: A Developing Country-Perspective

Author contributions

- Luria Leslie Founou, as the principal investigator, co-conceptualized the study, searched the literature, extracted and collated data, and drafted the manuscript.
- Raspail Carrel Founou extracted and collated data and contributed to the writing of the manuscript.
- Sabiha Yusuf Essack, as principal supervisor, co-conceptualized the study and undertook substantial critical revision of the manuscript.

Objectives met: This paper meets objective One (to evidence the danger of antibiotic resistance in the food chain and particularly in developing countries as a serious global public health threat) and Ten (to describe prevention and containment measures for antibiotic resistance in the food chain).

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REVIEW
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Antibiotic Resistance in the Food Chain: A Developing Country-Perspective

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Antibiotics are now "endangered species" facing extinction due to the worldwide emergence of antibiotic resistance (ABR). Food animals are considered as key reservoirs of antibiotic-resistant bacteria with the use of antibiotics in the food production industry having contributed to the actual global challenge of ABR. There are no geographic boundaries to impede the worldwide spread of ABR. If preventive and containment measures are not applied locally, nationally and regionally, the limited interventions in one country, continent and for instance, in the developing world, could compromise the efficacy and endanger ABR containment policies implemented in other parts of the world, the best-managed high-resource countries included. Multifaceted, comprehensive, and integrated measures complying with the One Health approach are imperative to ensure food safety and security, effectively combat infectious diseases, curb the emergence and spread of ABR, and preserve the efficacy of antibiotics for future generations. Countries should follow the World Health Organization, World Organization for Animal Health, and the Food and Agriculture Organization of the United Nations recommendations to implement national action plans encompassing human, (food) animal, and environmental sectors to improve policies, interventions and activities that address the prevention and containment of ABR from farm-to-fork. This review covers (i) the origin of antibiotic resistance, (ii) pathways by which bacteria spread to humans from farm-to-fork, (iii) differences in levels of antibiotic resistance between developed and developing countries, and (iv) prevention and containment measures of antibiotic resistance in the food chain.

Keywords: antibiotic resistance, zoonotic transmission, food chain, developing country, one health approach, global action plan, prevention, containment measures

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INTRODUCTION

Antibiotics are natural, synthetic, or semi-synthetic substances which interfere that the growth of or kill microorganisms, specifically bacteria, and are used to treat or prevent infections in humans and animals (O'Neill, 2015a; WHO, 2015a). Their development is one of the greatest accomplishments of modern medicine, their availability being vital to medical practices in communities and hospitals globally (O'Neill, 2015a; WHO, 2015a). Antibiotics are now an "endangered species" facing extinction due to the worldwide emergence of antibiotic resistance (ABR) and the void in the development of new therapeutic substances (O'Neill, 2015a; WHO, 2015a). An increasing number of treatment failures have been reported in patients with infections caused by multi-, extensive-, and pan-drug

resistant bacteria. Once antibiotics normally used against bacteria are no longer effective, it becomes necessary to use others, so-called "reserve" or "last resort" options that are often more expensive and/or toxic preparations (O'Neill, 2015a).

Antibiotic use is the key factor in the selection of resistant bacteria, with community and hospital settings forming the principal ecological niches of emergence in human health (O'Neill, 2015a; WHO, 2015a). However, it is the use of antibiotics in animals that has contributed to the magnitude of the global challenge of ABR (FAO, 2015). In the intensification of food animal production and aquaculture, antibiotics are administered not only as therapy, but also as metaphylactics, where the identification of disease symptoms in one animal prompts the treatment for the whole flock or herd, and as prophylactics where, sub-therapeutic doses are administered to counteract the adverse effect of stress responses that generally lead to infectious diseases (FAO, 2015). In addition, the extensive use of antibiotics as growth promoters for the rapid growth of food animals and fish exacerbates the emergence and spread of ABR (FAO, 2015). The emergence of ABR in the food chain is considered a cross-sectoral problem, as (i) antibiotics are widely used in aquaculture, livestock production, and crop culture (Acar and Moulin, 2006; FAO, 2015), (ii) antibiotic-resistant bacteria and antibiotic-resistant genes (ARGs) can easily spread at each stage of the food production chain (da Costa et al., 2013; FAO, 2015), and (iii) can cause infections in humans (Chang et al., 2015; WHO, 2015a). The emergence of ABR along the food chain is thus a major global public health issue, with several studies having reported food animals and products being colonized and/or infected and contaminated by antibiotic-resistant strains, such as methicillin-resistant *Staphylococcus aureus* (MRSA) (Price et al., 2012), antibiotic-resistant *Campylobacter* spp. (Ewnetu and Mihret, 2010), and extended spectrum-beta-lactamase (ESBL) producing-*Enterobacteriaceae* (viz. *Salmonella* spp., *Shigella* spp., *Escherichia coli*, *Klebsiella* spp., etc.; Fischer et al., 2012; Al Bayssari et al., 2015). The situation has been recently worsened with the emergence of antibiotic-resistant bacteria having significant pandemic potential, such as carbapenem-resistant *Enterobacteriaceae* (CRE) (harboring a VIM-1 carbapenemase resistant to the beta-lactam antibiotics family plus additional co-resistance; Fischer et al., 2012) and colistin-resistant *E. coli* (harboring mcr-1 gene and co-resistance genes; Liu et al., 2016), as well as emerging livestock associated-methicillin resistant *S. aureus* (LA-MRSA; Price et al., 2012), in German, Chinese, and Dutch pigs, respectively. In fact, these resistant strains recognized as having an animal origin, are associated with multiple hosts adaptability (Ewers et al., 2012; Price et al., 2012; Fernandes et al., 2016; Liu et al., 2016), virulence mechanisms (Ewers et al., 2012; Price et al., 2012) and high genetic exchanges (Price et al., 2012; Liu et al., 2016), are serious threats to the world as they could lead to the emergence of new and more resistant, virulent and mobile strains, unknown from the human immune system (Wulf and Voss, 2008). This phenomenon termed "superburg" is one of the main concern feared by the international scientific community as it could result in pandemic situations caused by as resistant and virulent bacteria (Wulf and Voss, 2008; Ewers et al., 2012).

Antibiotic-resistant bacteria may reach humans (i) indirectly along the food chain through consumption of contaminated food or food derived products, and (ii) following direct contact with colonized/infected animals or biological substances such as blood, urine, feces, saliva, and semen among others (Chang et al., 2015). Given the direct interaction of humans with the animal-ecosystem interface, it is essential to prevent the zoonotic transmission of antibiotic-resistant bacteria and ARGs from food animals-associated reservoirs to humans. The Food and Agriculture Organization of the United Nations (FAO), the World Organization for Animal Health (OIE), and the World Health Organization (WHO) endorsed the One Health approach, affirming that healthy animals contribute to healthy people and environments (FAO, 2015; WHO, 2015a). In keeping with this, supranational programs and systems for monitoring antimicrobial resistance in animals and foodborne (viz. originating from food or food products) pathogens, namely, the Global Foodborne Infections Network, the WHO's Advisory Group on Integrated Surveillance of Antimicrobial Resistance and the Codex Alimentarius Commission, have been established. The WHO's Global Action Plan (WHO, 2015a) and FAO's Action Plan on Antimicrobial Resistance (FAO, 2016) were recently published to address this worldwide threat.

Several programs monitoring antibiotic use and ABR in food animals, foodstuff, and humans, have been successfully implemented in high resource settings such as the European Union (EU), Denmark, Netherlands, Sweden, Japan and United States (US) (The Japanese Veterinary Antimicrobial Resistance Monitoring System in the Field of Animal Hygiene (JVARM), 2013; DANMAP, 2014; FDA, 2014; NethMap-MARAN, 2015; SWEDRES-SVARM, 2015; EFSA and ECDC, 2016). A number of low- and middle-income countries (LMICs) have also initiated efforts to contain ABR, albeit with a focus on human health. India introduced and has begun to implement national guidelines for antibiotic use in 2013. China launched a National Antibiotic Restraining Policy to reduce antibiotic consumption in the country, Thailand implemented a national strategy for emerging diseases, including ABR, and a policy for rational drug use in 2011, as well as an ABR Containment Program for 2012–2016. South Africa developed and implemented a National Framework of Antimicrobial Resistance for 2014–2024.

The majority of LMICs are however still far behind high-resource settings in terms of curbing the spread of ABR generally, and via the food chain specifically. This despite that substantial risk factors for communicable diseases and extensive animal-occupational exposure exist in LMICs. The true burden of ABR in food animals is only partially documented and its threat via the food chain is under-estimated in low resource settings. Given the expansion of the human population, globalization of trade in animals and food products, international travels and host movements, ABR can easily spread globally via the food chain (Holmes et al., 2016). If preventive and containment measures are not applied locally, nationally, regionally and internationally, the limited interventions in one country or continent, for instance, in the developing world, can compromise the efficacy and endanger

the policies of containment of ABR implemented in other parts of the world, the best-managed high-resource countries included. ABR is therefore a global problem, requiring integrated, multi-sectoral, and global solutions, as there are no geographic boundaries to its worldwide spread.

This review evidences the danger of ABR in the food chain and particularly in developing countries as a serious global public health threat. First, it assesses and summarizes background information on the emergence of ABR. Second it highlights transmission routes of ABR along the food chain; third, it compares the current status of ABR in food animals in developed vs. developing countries and finally, it delineates prevention and containment measures of ABR from farm-to-fork.

EMERGENCE OF ANTIBIOTIC RESISTANCE

Antibiotic use has always been associated with the development of resistance. Indeed, whenever an antibiotic is consumed, it eliminates susceptible bacterial cells, leaving behind or selecting those unusual strains that continue to grow in its presence through a Darwinian selection process. Those resistant variants then multiply, becoming the predominant bacterial population, and transmit their genetic resistance characteristics to offspring (Apata, 2009; Holmes et al., 2016). Such phenomenon can occur in saprophytic, commensal and pathogenic bacteria in humans, animals. and the environment.

Food animals, fish, and vegetables are considered large reservoirs of antibiotic-resistant bacteria, as the food production chain is an ecosystem composed of different ecological niches, where large quantities of antibiotics are used and numerous bacteria co-exist (Acar and Moulin, 2006). There are two principal biological pathways involved in the evolution and development of ABR. First, resistance can be mediated by a pre-existing phenotype in natural bacterial populations. During the evolutionary process, bacterial cells accumulate genetic errors in existing genes (in the chromosome or plasmid) and transfer the resistant genes to progeny cells via vertical gene transfer (VGT), leading to an *innate* or *intrinsic* or *natural* resistance (**Figure 1**). The second scenario, called *acquired* resistance, involves genetic exchanges within and between bacterial species (Apata, 2009; Holmes et al., 2016). It implies horizontal gene transfer (HGT) and the acquisition of new resistant genes harbored on mobile genetic elements, such as plasmids, integrons, transposons, insertion sequences, and phage-related elements. Such genetic materials are transferred through conjugation (transfer of DNA from donor to recipient bacterial through cell-to-cell contact and aided by a fertility-factor called pili), transformation (naked-DNA present in the environment is taken-up by the recipient cell), and transduction (a bacteriophage acts as vector and inserts DNA into recipient cell; Apata, 2009; Holmes et al., 2016; **Figure 1**).

HGT leads to new bacterial populations carrying a combination of new resistant genes and mechanisms, resulting in different resistance profiles (Acar and Moulin, 2006). This genetic process has been identified as the main driving force

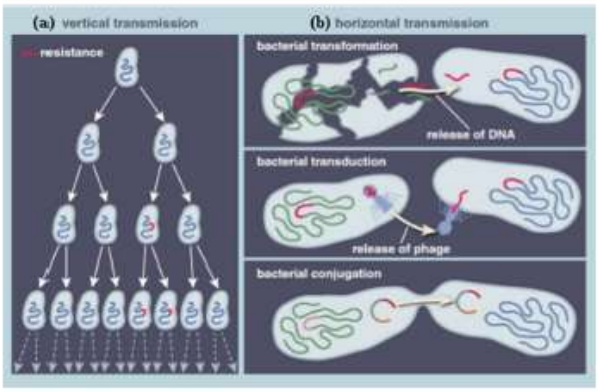

FIGURE 1 | Principal biological pathways involved in the evolution and development of antibiotic resistance. (A) Vertical transmission **(B)** Horizontal transmission. Reprinted by permission from American Scientist (Dantas and Sommer, 2014), Copyright (2014).

behind the spread of ABR, as it allows interspecies transmission, which is especially effective in the bacterial microbiome (gastro-intestinal tract, naso-pharyngeal mucosa, and skin) of animals and humans (Apata, 2009; Holmes et al., 2016). It is further important to note that VGT and HGT may occur concomitantly in a bacterial population (Acar and Moulin, 2006). These two types of mechanisms create a resistant population where selective pressure exerted by antibiotic use will substantially increase their number and risk of spread (Apata, 2009; Holmes et al., 2016). Antibiotic-resistant bacteria may act as reservoir of ARGs for other bacteria, which might be conserved within the bacterial population, even without the related antibiotics (Apata, 2009; Holmes et al., 2016).

TRANSMISSION ROUTES OF ANTIBIOTIC RESISTANCE ALONG THE FOOD CHAIN

The spread of ABR is possible along the food chain through direct or indirect contact. Direct contact occurs following immediate exposure of humans with animals and biological substances (such as blood, urine, feces, milk, saliva, and semen), and enhances the rapid and easy dissemination of resistant bacteria from host-to-host. Occupationally exposed workers such as veterinarians, farmers, abattoir workers and food handlers, as well as those directly in contact with them, are at high risk of being colonized or infected with antibiotic-resistant bacteria (Marshall and Levy, 2011). Although this transmission did not initially appear as a threat at population-health level, it is now acknowledged that exposed workers and their families provide a likely route for the entry of antibiotic-resistant bacteria and ARGs into the community and health care settings, where subsequent exchanges and the acquisition of resistance mechanisms are evident (Marshall and Levy, 2011).

In addition, the human population may be exposed indirectly to antibiotic-resistant bacteria and ARGs via contact with or consumption of contaminated food products (e.g., meat, eggs,

milk, and dairy products). This indirect transmission through the food chain is a far-reaching and more complex pathway (**Figure 2**). Recently, numerous reports have described the presence of large quantities of antibiotic-resistant bacteria and ARGs in various food products (ready-to-eat meat, cooked meat, and bulk milk) from various animal sources, such as cattle, poultry, swine, goat, and sheep, and from different stages of food production (Price et al., 2012; Coetzee et al., 2016; Liu et al., 2016). Several studies have further identified similar or clonally related antibiotic-resistant bacteria and ARGs of animal origin in human populations without occupational exposure, providing likely evidence for transfer following the consumption and/or handling of food (Acar and Moulin, 2006; Marshall and Levy, 2011). Farmers, abattoir workers and food handlers as well as consumers are thus the large number of people directly at risk of acquiring antibiotic-resistant bacteria via the food chain. In the developing world, where biosecurity and food safety measures are limited along the farm-to-fork continuum and where humans interact intimately with animals and the environment, the public health risk is likely to be associated with both the direct and indirect transmission of antibiotic-resistant bacteria and ARGs (Padungtod et al., 2008). In contrast, in developed countries, the indirect contamination seems to be more prevalent as antibiotic-resistant bacteria and ARGs emerging on-farms, are maintained throughout the food production and contaminated food products reach the end consumers to create foodborne infections (EFSA and ECDC, 2015).

A large proportion of antibiotics are not transformed into inactive compounds, and retain their activities after renal or biliary excretion (Thanner et al., 2016). The dissemination of active antibiotics, metabolites or degradation products of those antibiotics, termed antibiotic residues, as well as ARGs and antibiotic-resistant bacteria excreted via food animals waste, have established the environment as another important reservoir of ABR (da Costa et al., 2013; Zhu et al., 2013; Woolhouse et al., 2015; Thanner et al., 2016; **Figure 2**). Farm soils, manure, and wastewater have been identified as "hot spots" of ABR pollution, with antibiotic residues being detected across the world (Zhu et al., 2013; Wu et al., 2014; Thanner et al., 2016). Antibiotic residues lead to various adverse effects for human health, such as allergic hypersensitivity reactions, toxic effects, hepatotoxicity, nephropathy, mutagenicity, carcinogenicity, and ABR (Mensah et al., 2014). Although the concern associated with the presence of antibiotic residues in food animals has been mitigated in high resource economies, with <1% of these substances detected in food products in the European Union (EU), it remains a major public health threat in LMICs, with prevalence ranging from 4 to 90% (Mensah et al., 2014). The high prevalence of these residues in various ecological niches in the farm-to-fork continuum exacerbates the issue of ABR in the developing world, as it enhances the pool of antibiotic-resistant bacteria and ARGs in the ecosystem as a result of exposure to sub-inhibitory concentrations of antibiotics and their residues.

HGT has further been documented in environmental niches, such as manure, water and soil, leading to microbial communities containing various levels of antibiotic-resistant bacteria and ARGs (Zhu et al., 2013; Thanner et al., 2016). Several LMICs use animal and human waste, without appropriate treatment, as fertilizer in crop lands and for feeding of fish and shellfish in aquaculture (Zhu et al., 2013). The environmental pollution may therefore lead to the emergence and spread of new antibiotic-resistant bacteria and ARGs in food products, fish,

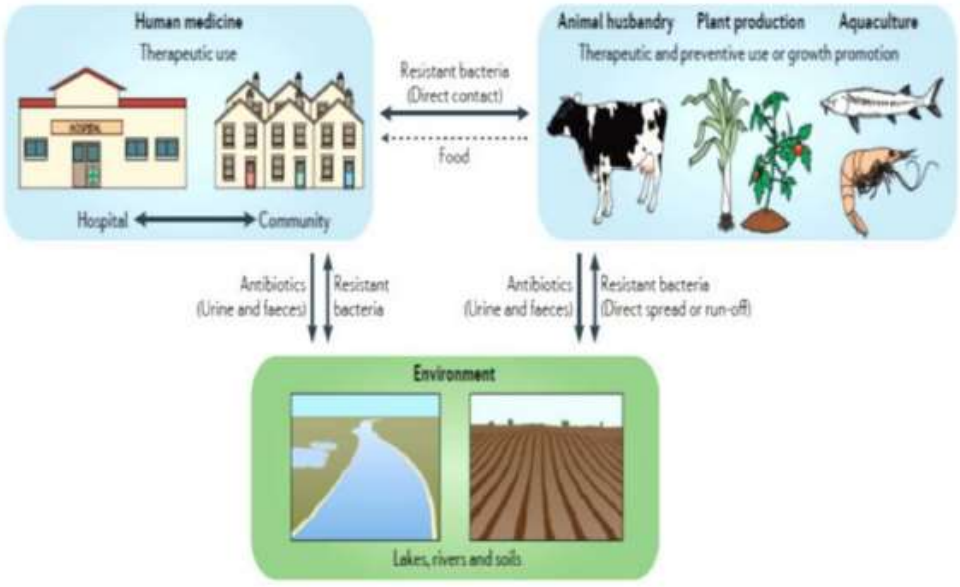

FIGURE 2 | Antibiotic use and different transmission routes of antibiotic resistance in the food chain. Adapted by permission from MacMillan Publishers Ltd: [Nature Reviews Microbiology], (Andersson and Hughes, 2014), Copyright (2014).

shellfish, vegetables, feed and water, which may in turn colonize or infect animals and humans, thereby enhancing the public health risks associated with the presence of ABR in the food chain (Acar and Moulin, 2006; Thanner et al., 2016). The key issues regarding these reservoirs are (i) identifying the different ecological niches and pathways associated with the presence of ABR in the food chain, and, (ii) understanding the public health implications for the human population when the transfer of these antibiotic-resistant bacteria and ARGs from these intermingled reservoirs occurs. It further reveals knowledge gaps regarding the magnitude and dynamic nature of the spread of antibiotic-resistant bacteria and/or ARGs within and between different ecological niches in the farm-to-fork continuum, which deserves to be considered when assessing the transmission of ABR along the food chain (Acar and Moulin, 2006; Woolhouse et al., 2015). Substantial metagenomics studies ascertaining the bacterial resistome, the HGT between antibiotic-resistant bacteria present in the above niches, as well as providing phylogenetic evidence and transmission dynamics within and between populations, are vital to determine the evolutionary origins of bacterial lineages, and may provide new perspectives on understanding the implications of ABR in the food chain (Woolhouse et al., 2015).

CURRENT STATUS OF ANTIBIOTIC RESISTANCE IN THE FOOD CHAIN

Different types of antibiotic-resistant bacteria have increasingly been identified in food animals, products, and feeds as well as in humans (exposed or not). Such antibiotic-resistant bacteria are either (i) indicator or commensal, (ii) foodborne, or (iii) emergent organisms. ARGs emerging in commensal bacteria are likely spread to foodborne ones and vice versa, while the numerous genetic exchanges occurring between different ecological niches lead to new resistant bacteria or genes or combination thereof. Antibiotic-resistant bacteria and ARGs of animal origin are globally reported, with the prevalence differing greatly between geographic locations and differentially resourced settings. MRSA and ESBL-producing *Enterobacteriaceae* (e.g., *Salmonella* spp., *E. coli*, *Shigella* spp., *K. pneumoniae*, *Enterobacter* spp., etc.) are among the best-known examples.

Developed Countries

In high-resource developed countries, an assortment of systems and programs to monitor antibiotic use, as well as ABR in food animals, food products, and humans have been implemented (**Table 1**). Such initiatives have led to the substantial decrease of antibiotic consumption and rates of resistance in these settings. In Norway for instance, The NORM/NORM-VET reported a very low annual antibiotic use of 5.927 tons in terrestrial animals in 2014, representing a total decrease of 38% from 1995 to 2014 (NORM/NORM-VET, 2015). The 2014's DANMAP report revealed a decrease of 2% compare to 2013, with 114 tons of active compounds consumed in livestock (DANMAP, 2014). Similarly, in the Netherlands, 207 tons of antibiotics were consumed in 2014, showing a reduction of 4.4% from 2013, and an overall decrease of 58.1% over the period 2009–2014 (NethMap-MARAN, 2015). In the European Union (EU), antibiotics were banned for growth-promotion in 2006, while the United States (US) implemented legislation based on a voluntary cessation of antibiotic use for growth-promotion as well as re-labeling of antibiotics.

Transmission of antibiotic-resistant bacteria via direct contact from animals to exposed workers, and indirectly following consumption of contaminated food products (broiler meat, beef, pork, milk, etc.), is well-documented in these countries (**Table 2**; DANMAP, 2014; NORM/NORM-VET, 2015; EFSA and ECDC, 2016). In the EU, a new legislation on monitoring of ABR in animals and food was implemented to ensure the comparison of similar and high-quality data in 2013 (EFSA and ECDC, 2016). As a result, harmonized data from the 28 EU member states were collated by the EFSA and ECDC to provide continent-specific estimates of ABR in food animals and products. The 2014's EFSA/ECDC EU Summary report on ABR in zoonotic bacteria revealed increased prevalence of multi-drug resistant (MDR) *S. Infantis* (more than 70% in broiler meats), MDR-*E. coli* (55% in broiler meats) and MRSA (26.5% in all food animals) in the EU (EFSA and ECDC, 2016). These bacteria are recognized as a serious public health threat due to their resistance to several antibiotics, often critically important for human health, thereby limiting the choice of effective antibiotic agents available for treatment.

In a country-specific context, the NORM/NORM-VET detected high levels of ESBL-producing *E. coli* from broilers (36%) and broiler meat samples (30%) in Norway, whereas rates of vancomycin resistant *Enterococcus* spp. were quite low (7%) in 2014 (NORM/NORM-VET, 2015). Meanwhile, monitoring of ABR in food animals in the Netherlands by MARAN revealed a 12% prevalence of ESBL-producing *Salmonella* spp. and 43% of fluoroquinolone-resistant *Salmonella* spp. in poultry. It also showed diverse levels of ESBL/AmpC- producing *E. coli* in food animals and products, these being 67% in broilers, 18% in pigs, 9% in dairy cows, 51% in turkey meat, and 67% in poultry meat (NethMap-MARAN, 2015). The DANMAP reported low rates of MDR-*Salmonella* spp. (7%) in pigs and ESBL-producing *E. coli* (9%) in broiler meat in Denmark, confirming the country as an example in terms of containing ABR (DANMAP, 2014). Transmission of antibiotic-resistant bacteria along the food chain has nevertheless been demonstrated in the country, with a 28% prevalence of ESBL-producing *E. coli* from imported broiler meat samples (DANMAP, 2014). This evidence strongly suggests that there are no geographic borders to curtail the spread of antibiotic-resistant bacteria, and that their emergence in the food chain threatens the world equally. In US, the epidemiology of ABR in food animals appears to be notably different than in European countries, where the substantial ABR research has been carried out and antibiotic use for growth promotion has been prohibited for more than a decade. Antibiotic use in food animals is estimated to account 70% of the country's annual antibiotic consumption (FDA, 2014). The report from the NARMS, over the period 2012–2013, identified MDR-*E. coli* in turkey (62%), chicken (62%), and swine (22%) as well as MDR-non-typhoidal

TABLE 1 | Examples of programs for surveillance and containment of antibiotic resistance.

Monitoring programs	Country	Target population	Target bacteria	References
Danish Integrated Antimicrobial Resistance Monitoring and Research Programme (DANMAP)	Denmark	Humans, animals, and food products	*Salmonella* spp. *Campylobacter* spp. *Enterococcus* spp. *Escherichia coli* *Klebsiella pneumonia* *P. aeruginosa* *Streptococcus* spp. *Staphylococcus aureus*	DANMAP, 2014
Norwegian Surveillance System for Antimicrobial Drug Resistance (NORM/NORM-VET)	Norway	Humans, animals, and food products	*Escherichia coli* *Enterococcus* spp. *Salmonella* spp. *Campylobacter* spp. *Yersinia enterocolitica* *Shigella* spp.	NORM/NORM-VET, 2015
Swedish Veterinary Antimicrobial Resistance Monitoring (SVARM)	Sweden	Animals and food products	*Salmonella* spp. *Campylobacter* spp. *Staphylococcus aureus* *Staphylococcus pseudointermidius* *Enterobacteriaceae* *Enterococcus* spp.	SWEDRES-SVARM, 2015
European Antimicrobial Resistance Surveillance Network (EARS-Net)	Multinational[a]	Humans	*Streptococcus pneumoniae* *Staphylococcus aureus* *Enterococcus* spp. *Escherichia coli* *Klebsiella pneumoniae* *P. aeruginosa*	ECDC, 2015
European Surveillance of Antimicrobial Consumption Network (ESAC-Net)	Multinational[b]	Humans	*Streptococcus pneumoniae* *Staphylococcus aureus* *Enterococcus* spp. *Escherichia coli* *Klebsiella pneumoniae* *P. aeruginosa*	ECDC, 2014
Monitoring and analysis of food-borne diseases in Europe (EFSA)	Multinational[c]	Humans, animals and food products	*Salmonella* spp. *Campylobacter* spp. *Escherichia coli* *Staphylococcus aureus*	EFSA and ECDC, 2016
Monitoring of Antimicrobial Resistance and Antibiotic Usage in Animals in the Netherlands (MARAN)	Netherlands	Animals and food products	*Salmonella* spp. *Campylobacter* spp. *Escherichia coli* *Enterococcus* spp.	NethMap-MARAN, 2015
National Antimicrobial Resistance Monitoring System (NARMS)	United States	Humans, animals and food products	*Salmonella* spp. *Campylobacter* spp. *Enterococcus* spp. *Shigella spp.* *Escherichia coli* *Vibrio spp.* other than *V. cholera*	FDA, 2014

(Continued)

TABLE 1 | Continued

Monitoring programs	Country	Target population	Target bacteria	References
Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS)	Canada	Humans, animals and food products	*Salmonella* spp. *Campylobacter* spp. *Enterococcus* spp. *Escherichia coli*	Government of Canada, 2014
L'Observatoire National de l'Epidémiologie de la Résistance Bactérienne aux Antibiotiques (ONERBA)	France	Humans and Animals	*Enterobacteriaceae and non-fermenters* *Staphylococcus aureus* *Streptococcus* spp. *Enterococcus* spp.	ONERBA, 2015
The Japanese Veterinary Antimicrobial Resistance Monitoring System in the Field of Animal Hygiene (JVARM)	Japan	Animals	*Salmonella* spp. *Campylobacter* spp. *Enterococcus* spp. *Escherichia coli*	The Japanese Veterinary Antimicrobial Resistance Monitoring System in the Field of Animal Hygiene (JVARM), 2013
Japanese Nosocomial Infections Surveillance (JANIS)	Japan	Humans	*Enterobacteriaceae and non-fermenters* *Staphylococcus* spp. *Streptococcus* spp. *Enterococcus* spp.	JANIS, 2016
The Finnish Veterinary Antimicrobial Resistance Monitoring and Consumption of Antimicrobial Agents report (FINRES-VET)	Finland	Animals and food products	*Escherichia coli* *Enterococcus* spp. *Salmonella* spp. *Campylobacter* spp. *Staphylococcus aureus* *Staphylococcus pseudointermidius*	FINRES-Vet, 2007-2009
Pilot Surveillance Program for Antimicrobial Resistance in Bacteria of Animal Origin	Australia	Animals	*Campylobacter* spp. *Escherichia coli* *Enterococcus* spp.	The department, 2007
Colombian Integrated Program for Antimicrobial Resistance Surveillance (COIPARS)	Colombia	Humans, Food animals, and products	*Salmonella* spp. *Campylobacter* spp. *Escherichia coli*	COIPARS, 2011
Pilot Integrated Food Chain Surveillance System	Mexico	food animals and products	*Salmonella* spp. *Campylobacter* spp.	Zaidi et al., 2008, 2012

[a] *33 European countries.*
[b] *34 European countries.*
[c] *28 European countries.*

Salmonella in turkey (34%) and ground beef (20%). Several studies have further reported human colonization and outbreak situations associated with antibiotic-resistant bacteria and ARGs of food animal origins (FDA, 2014).

Notwithstanding the difference in rates of ABR demonstrated in the food chain in developed countries, it is important to mention that most have national programs to monitor antibiotic use and ABR from humans, animals, and food products (**Table 1**). In response to the data generated from these programs, these countries have adopted a set of policies and control measures to contain ABR while preserving their productivity. Denmark has taken major initiatives to address the challenge of ABR over the last 20 years, and actions taken by the Danish government have been acknowledged as a worldwide model (O'Neill, 2015b). The DANMAP, a system for monitoring the impact of withdrawal of antibiotic growth-promoters on the development of ABR in food animals and humans was established in the country to measure the effect of interventions and inform future policies (DANMAP, 2014). From the ban of avoparcin in 1995 up to 2008, poultry production has increased slightly and overall antibiotic consumption decreased by 90%, from 5 to 0.5 tons (used exclusively for therapy)

TABLE 2 | Prevalence of antibiotic-resistant bacteria isolated from food animals and products in developed and developing countries.

Country	Isolation year	Origin/type of specimens	Antibiotic-resistant bacteria	Prevalence (%)	References
DEVELOPED COUNTRIES					
European Union	2014	Broiler meat	MDR-*Salmonella Infantis*	>70	EFSA and ECDC, 2016
		Broiler meat	MDR-*E. coli*	55	
Norway	2014	Broiler	ESBL-producing *E. coli*	36	NORM/NORM-VET, 2015
		Broiler meat	ESBL-producing *E. coli*	30	
		Broiler	Vancomycin resistant *Enterococcus* spp.	7	
Netherlands	2014	Poultry	ESBL-producing *Salmonella* spp.	12	NethMap-MARAN, 2015
		Poultry	Fluoroquinolone-resistant *Salmonella* spp.	43	
		Pig	ESBL/AmpC-*E.coli*	18	
		Dairy cow	ESBL/AmpC-*E.coli*	9	
		Turkey meat	ESBL/AmpC-*E.coli*	51	
		Poultry meat	ESBL/AmpC-*E.coli*	67	
Denmark	2014	Pigs	MDR-*Salmonella* spp.	7	DANMAP, 2014
		Broiler meat	ESBL-producing *E. coli*	9	
United States	2012–2013	Turkey	MDR-*E. coli*	62	FDA, 2014
		Turkey	MDR-non-typhoidal *Salmonella*	34	
		Chicken	MDR-*E. coli*	62	
		Pig	MDR-*E. coli*	22	
		Ground beef	MDR-non-typhoidal *Salmonella*	20	
DEVELOPING COUNTRIES					
China	2015	Pig	Plasmid mediated colistin resistant *E. coli*	21	Liu et al., 2016
		Raw meat	Plasmid mediated colistin resistant *E. coli*	15	
Thailand	2007	Poultry	ESBL-producing *S. Typhimurium*	77.3	Padungtod et al., 2008
		Pig	ESBL-producing *S. Typhimurium*	40.4	
	2012–2013	Pig	ESBL-producing *E. coli*	77	Boonyasiri et al., 2014
		Pork	ESBL-producing *E. coli*	61	
		Pork	MDR-*A. baumannii* and *P. aeruginosa*	40	
		Poultry	ESBL-producing *E. coli*	40	
		Poultry meat	ESBL-producing *E. coli*	50	
Vietnam	2007	Poultry	MDR-*E. coli*	91.5	Usui et al., 2014
Indonesia	2007	Poultry	MDR-*Enterococcus faecalis*	84.5	
Brazil	2000–2016	Pigs	Plasmid mediated colistin resistant *E. coli*	1.8	Fernandes et al., 2016
		Chicken	Plasmid mediated colistin resistant *E. coli*	5	
Egypt	2010	Meat and dairy products	MDR-Shiga-toxin-producing *E. coli* O157:H7	57.4	Ahmed and Shimamoto, 2015a
	2010	Meat and dairy products	MDR-*Shigella spp.*	89	Ahmed and Shimamoto, 2015b
Algeria	2005–2006	Broilers	MDR- *Salmonella spp.*	51	Elgroud et al., 2009
Tunisia	2010–2011	Raw meat	MDR-*Enterococcus spp.*	24.5	Klibi et al., 2013
		Raw meat	LA-MRSA ST398	0.6	Chairat et al., 2015
South Africa	2014	Cattle	MDR-*E. coli*	100	Iweriebor et al., 2015
	2015	Poultry	Plasmid mediated colistin-resistant *E. coli*	79	Coetzee et al., 2016

MDR, Multi-drug resistant.

in 2008 (DANMAP, 2014). The prevalence of vancomycin resistant *Enterococcus faecium* substantially declined in pig and poultry production between 1996 and 2008 (DANMAP, 2014). Several developed countries have also recently responded to and followed the recommendations of the WHO, OIE, and FAO tripartite alliance to strengthen the containment of ABR, with the development and implementation of their national action plans.

Developing Countries

In contrast with developed countries, the majority of LMICs have minimal or no programs or systems to monitor antibiotic use or ABR in food animals, food products, and humans, its true burden being only partially documented, and reliant on point-prevalence rather than long-term studies. Moreover, antibiotics are extensively used in agriculture for growth-promotion, and the danger of ABR in the food chain is further neglected and under-estimated (Eagar et al., 2012; Van Boeckel et al., 2014). For example, two-third of the 1500 tons of antibiotics sold for animal use over a 3 years' period (2002–2004) in South Africa were for growth-promotion purposes and included WHO-banned substances (Eagar et al., 2012). A recent modeling study suggests that up to a third of the global increase (67%) in antibiotic consumption in food animals, over the period 2010-2030, will be attributable to LMICs (Van Boeckel et al., 2015). For Brazil, Russia, India, China, and South Africa, this increase is projected to reach 99%, up to seven times the estimated population growth of these countries (Van Boeckel et al., 2015). By 2030, antibiotic use in food animals is estimated to be 51,851 tons in Asia alone, representing more than 80% of the global antibiotic consumption in food animals in 2010 (Van Boeckel et al., 2015). In 2007, China was the largest antibiotic producer and consumer, with 210,000 tons produced, with 46% being used in livestock production. The country was also the world's pig and poultry producer in 2014, with 56.7 and 17.5 million tons, respectively (Liu et al., 2016). The increase in agriculture and food production in LMICs parallels what developed countries experienced over the last 100 years when income rose (Collignon and Voss, 2015). The damaging consequences of this intensification in food production has resulted in several sub-optimal practices, such as over-crowding of animals, large quantities of antibiotics consumed, and often poor hygienic measures and farming practices (Collignon and Voss, 2015). The incidence of ABR is thus undoubtedly worse in developing countries, where humans interact intimately with animals and the environment, infectious diseases rates are higher, regulations on antibiotic use, and the development, implementation, and monitoring of ABR prevention and containment measures are rare and frequently non-existent.

Several studies have documented new resistance mechanisms and elevated rates of antibiotic-resistant bacteria in Asian and African developing nations (**Table 2**). A recent Chinese routine surveillance study revealed a highly mobile and transferable plasmid-mediated colistin-resistance gene, called mcr-1, in commensal *E. coli* isolated from pigs (21%) and raw meat (15%; (Liu et al., 2016)). The study also revealed that 1% of clinical isolates from hospitalized Chinese patients harbored this new resistant gene (Liu et al., 2016). It is thought that this gene originated from animals and spread into humans via the food chain (Liu et al., 2016). Plasmid mediated mcr-1 colistin-resistant *E. coli* were also detected in chicken (14/515) and pigs (2/515) in Brazil (Fernandes et al., 2016). This finding is of great concern, as although not frequently used in human medicine, colistin remains a drug of last resort, with the global emergence of extensively-drug resistant Gram negative bacteria.

In Thailand, high levels of ESBL-producing *Salmonella Typhimurium* in poultry (77.3%) and pigs (40.4%) were reported, with an overall 86% prevalence of MDR (Padungtod et al., 2008). In the same country, Boonyasiri et al. (2014) identified elevated prevalence of ESBL-producing *E. coli* in the food chain during the years 2012–2013, these being 77% in pigs, 40% in poultry, 77% in farmers, 76% in food handlers, 61% in pork, and 50% in chicken. The same study further reported 40% of MDR-*Acinetobacter baumannii* and *Pseudomonas aeruginosa* in pork (Boonyasiri et al., 2014). MDR-*E. coli* strains were also detected in chicken fecal samples in Vietnam (91.5%) and Indonesia (62.8%) with isolates being concomitantly resistant to up to 10 antibiotics (Usui et al., 2014). In Africa, there is a paucity of research and consequently data on antibiotic-resistant bacteria and ARGs in food animals and food products. However, high level of MDR-bacteria isolated from various food animals have been documented in several African countries. Ahmed and Shimamoto (2015a,b) reported MDR-Shiga-toxin-producing *E. coli* O157:H7 (57.4%) and MDR-*Shigella* spp. (89%) from food products (meat and dairy products) in Egypt, while a maximum prevalence (100%) of MDR-*E. coli* has been identified in rectal samples of cattle in South Africa (Iweriebor et al., 2015). MDR-*Enterococcus* spp. (24.5%) (Klibi et al., 2013) and MDR-*Salmonella spp.* (51%) (Elgroud et al., 2009) were isolated from meat samples and broilers in Tunisia and Algeria, respectively. A recent nation-wide surveillance in poultry settings described substantial increases in colistin-resistance in *E. coli* strains in 2015 in South Africa. Accordingly, the emergence of mcr-1 was confirmed in 79% of colistin-resistant strains isolated (Coetzee et al., 2016). Clinical isolates of colistin-resistant *E. coli* from hospitalized ($n = 3$) and outpatients ($n = 6$) were further detected in the same study (Coetzee et al., 2016). Carbapenemase producing-*P. aeruginosa* and *A. baumannii* have further been recently identified in food animals in Lebanon, confirming the serious threat associated with the emergence of ABR in the food chain (Al Bayssari et al., 2015).

The majority of developing countries are substantive exporters of food animals and food products. Brazil is the world's largest exporter of chicken meat (Fernandes et al., 2016) and Thailand exports not <70% of its poultry production (Boonyasiri et al., 2014). This means that antibiotic-resistant bacteria and/or ARGs emerging in these countries and other LMICs will easily spread across the world via the food chain (Boonyasiri et al., 2014; Fernandes et al., 2016; Holmes et al., 2016). Grami et al. (2016) recently reported high prevalence of plasmid mediated mcr-1 *E. coli* in fecal samples of chicken imported from European countries in Tunisia. This is also of grave concern as antibiotic-resistant bacteria and ARGs emerging in food animals and products in developed nations could endanger safety, public health in already overburdened health care settings of developing

countries, where prevention and containment of ABR are limited and where high levels of ABR prevail (**Table 2**). This confirms that globalization of trade in food animals and products are major determinants of the worldwide dissemination of ABR from farm-to-fork.

Additionally, travelers visiting developing countries from developed nations may be colonized or infected by antibiotic-resistant bacteria and/or ARGs, and in turn become vectors of transmission in their home countries. Dutch travelers to North Africa (Tunisia), South America (Peru, Bolivia, and Colombia), and Asia (Thailand, China, Vietnam, Laos, and Cambodia) were recently reported to be colonized by colistin-resistant *E. coli* strains harboring mcr-1 gene (Coetzee et al., 2016). Likewise, the prevalence of ESBL-producing *Enterobacteriaceae*, colonization in Swedish, Dutch, and Australian tourists, increased from 2.4–8.6 to 30–49% following travel to India, Southeast Asia, and China (Boonyasiri et al., 2014). Travelers from developed nations seeking healthcare in LMICs, generally to avoid high costs, long delays or due to legal or cultural restrictions in their home countries, have also been involved in the spread of ABR (Chen and Wilson, 2013). A recent study confirmed that recent international travel was an independent risk factor for septicemia after trans-rectal prostate biopsy (Chen and Wilson, 2013). Several authors further documented the implication of travelers, including medical tourists, in the global spread of ABR (Coetzee et al., 2016; Fernandes et al., 2016; Holmes et al., 2016). The travelers likely acquired antibiotic-resistant bacteria and/or ARGs following the consumption of contaminated foods, water and/or contact with the environment in developing countries (Coetzee et al., 2016; Fernandes et al., 2016).

The variations observed between countries, with an important north-to-south gradient, reveal that the spread of antibiotic-resistant bacteria in the food chain is likely to be higher in LMICs than in developed countries (**Table 2**). There is clear evidence that ABR is a global public health problem, which appears to be worse in developing countries, although it threatens the world equally. Despite the fact that some governments, notably China, India, Thailand, Brazil, Malaysia, and South Africa have taken significantly actions and policies toward the rationalization of antibiotic use and containment of ABR in humans, such initiatives still lag far behind in food animals. Having good data on antibiotic consumption and trends of antibiotic-resistant bacteria and ARGs in food animals, food products and humans, as well as political commitment, are imperative to better understand and manage the concern of ABR via the food chain in developing countries.

PREVENTION AND CONTAINMENT MEASURES OF ANTIBIOTIC RESISTANCE FROM FARM-TO-FORK

Emergence and spread of antibiotic-resistant bacteria and ARGs in the food chain, has given rise to severe health and socio-economic repercussions globally. Resistant foodborne infections are amongst the main public health issues associated with the threat of ABR in the food chain. This global concern equally affects developed and developing countries, and may cause outbreaks and pandemic situations (Padungtod et al., 2008). The problem is more serious in the developing world, where resistant infections significantly increase morbidity and mortality rates, whereas in developed countries, these infections will enhance therapeutic costs (Harbarth et al., 2015). The majority of developing countries are not implementing adequate measures to prevent and curb the spread of ABR from farm-to-fork, thus, posing a significant threat for global public health. Combating ABR effectively at a global scale means tackling it in the developing world first, whether in the food chain or not. The One Health approach was endorsed to address the threat of ABR by supranational entities, following the coalition between the WHO, FAO, and OIE referred as the "Tripartite Alliance." The WHO, in collaboration with its Tripartite partners, published the Global Action Plan on Antimicrobial Resistance in 2015, an attempt to counteract effectively this worldwide concern (WHO, 2015a). The FAO similarly launched its Antimicrobial Resistance Strategy in September 2016 to support the implementation of the WHO's Global Action plan in the food and agricultural sectors (FAO, 2016). Noting that ABR in the food chain is a serious global threat which is considerably neglected/under-estimated in developing countries, this section consequently proposes solutions for an effective containment of ABR from farm-to-fork, albeit relatively applicable in both developed and developing nations. The five strategic objectives of the WHO's Global Action Plan, namely (i) heighten awareness and understanding on antibiotic use and antibiotic resistance, (ii) strengthen knowledge via surveillance and research, (iii) reduce infectious diseases, (iv) optimize rational antibiotic use, and (v) mobilize resources, research and development, have been used as framework to set out integrated prevention and containment measures of ABR in the food chain (WHO, 2015a, **Table 3**).

Heightening Awareness and Understanding of Antibiotic Resistance

Public Awareness Campaigns on Antibiotic Use and Antibiotic Resistance

Heightening national awareness campaigns on antibiotic use, ABR, and food safety are important to address this global challenge from farm-to-fork, as knowledge leads to prevention. Well-designed and simple key messages that target populations and engage all-stakeholders (viz. farm, and abattoir workers, food handlers, consumers, veterinarians, pharmacists, food production industries, healthcare workers) are essential for successful awareness campaigns (Harbarth et al., 2015). Strengthened awareness through mass and social media continuously repeating key messages may contribute to effectively lessen antibiotic consumption and ABR rates (Harbarth et al., 2015). The choice of indicators and targets are crucial in monitoring the impact of such campaigns (Harbarth et al., 2015).

Global awareness campaigns on antibiotic use and ABR have been increasingly advocated, with sustainable communication measures having been implemented at international level, e.g., the "European antibiotic awareness day" in EU, the "get smart:

TABLE 3 | Summary of prevention and containment measures of antibiotic resistance from farm-to-fork.

Measures	World Health Organization's Global Action Plan Strategic Objectives				
	Increase awareness	**Strengthen surveillance**	**Reduce infectious diseases**	**Optimize rational antibiotic use**	**Mobilize resources, strengthen research and development**
Basic	National awareness campaigns on antibiotic usage and ABR	Integrated food chain surveillance systems	Implement biosecurity measures	Prohibit the growth-promotion use of antibiotics	Political will
	Food safety awareness campaigns	Establish reference laboratory		Prohibit unrestricted access of antibiotics	
	Achieve effective "culture change"	Harmonized-laboratory methods	Institute massive immunization campaigns	Establish guidelines for veterinary use of antibiotics	Sustainable commitment involving all stakeholders
		Reinforced education and fostered excellence			Assess and manage food safety risk
Moderate	Cross-disciplinary research	Research on total bacterial resistome and mobilome	Organic farming practices	Develop methods to verify judicious antibiotic use	Institute sustainable collaboration (North-to-South, South-to-South, Private-to-Publi)
	Provide assistance, support and training to occupationally exposed workers		Well-controlled extensive farming practices	Institute veterinary oversight	
			Reinforce veterinary legislation and enforcement policies		Leverage resources
Advanced	Evaluate the impact of the educational programs	State-of-the-art methods	Predictive microbiology	Institute Incentives/disincentives	Pre-, pro-, and syn-biotics
			Diagnostic tool based-nanoscale materials	Implement legal regulatory framework	Phage-related therapies
					Genetically modified food animals
					Nano-antibiotics

know when antibiotics work" in US, and the first "World Antibiotic Awareness Week" held in November 2015. Although challenging, awareness or educational campaigns are relatively successful, with some good examples reported across the world (Harbarth et al., 2015). Farmers, abattoir workers, food handlers, and veterinarians are not the only ones affected by the threat of ABR in the food chain, but all categories of the general population must be involved and educated.

Food Safety Awareness Campaigns

Food safety is a scientific discipline aiming to ensure safe food and prevent foodborne diseases throughout all stages of the food production chain including handling, transport, storage, and preparation. Food safety awareness campaigns are essential to improved consumer's and food handlers' knowledge to prevent foodborne infectious diseases and hazards (including antibiotic residues, ARGs, and antibiotic-resistant bacteria). Some basic food safety measures, such as convenient hand-washing with water and soap several times during the day (especially before and after meal preparation, after usage of toilets), effective vegetable-washing, adequate cooking temperatures, and food storage are important to reduce the spread of antibiotic-resistant bacteria and the prevalence of ABR- foodborne infections. In this regard, the WHO has summarized these practices into a simple and strong tool called "five keys to safer food" to help ensure food safety from farm-to-fork (Mwamakamba et al., 2012). It is the responsibility of stakeholders involved along the food chain, including food production industries, agricultural practitioners, food handlers, and consumers to ensure safe food using the adequate food safety measures, as depicted by the WHO, albeit governments have the central role in providing an adequate framework to implement food safety-associated health promotion programs. Several countries in Africa and Asia have implemented food safety education programs using the WHO five keys to safer foods (Prabhakar et al., 2010; Mensah et al., 2012; Mwamakamba et al., 2012).

Behavior Change, Education, and Training

Behind these sensitization efforts, human behavior, whether through inter alia individualism, lack of education, culture or social beliefs, is an important factor that needs to be considered when educating/creating an awareness on antibiotic use and ABR, as new knowledge is not immediately translated into new practices. For a successful intervention, it is important to

implement cross-disciplinary research involving anthropologists, sociologists, psychologists, and ethnographers to investigate the target groups' perception and knowledge beforehand. This will enable the most efficient strategies to be developed to modify behaviors, and achieve and enact effective "culture change" (Prabhakar et al., 2010; Mensah et al., 2012; Mwamakamba et al., 2012; Harbarth et al., 2015).

Reinforced education and fostering excellence in both private and public veterinarians about conservative prescription of antibiotics, the emergence of ABR and the One Health approach have a crucial role to play in containing ABR from farm-to-fork. The OIE provides guidelines on initial veterinary education, highlighting the basis for an organized profession with high-quality professionals (OIE, 2016). It is also essential to provide assistance, support and training to occupationally exposed workers (viz. to farmers, abattoir and food workers), as well as guidance on rational antibiotic use and good agricultural practices, according to their education level. Such initiative must be supported at a global level, and adapted at the local level through leadership from relevant ministers, industries, agricultural practitioners, and veterinarians. Monitoring strategies must also be developed to evaluate the impact of the educational programs.

Strengthening Surveillance and Research

Integrated Food Chain Surveillance Systems

An essential step toward addressing the public health threat of ABR is to determine the resistance burden from different ecological niches in the entire food chain (farm, abattoir, market, etc.). This is more evident in developing countries, where data on ABR, antibiotic use and foodborne illnesses is scarce. Integrated food chain surveillance systems, conducting sustainable research (long-term epidemiological and molecular studies) from farm-to-fork, endorsing the One Health approach and the Codex Alimentarius Commission guidelines, and reporting a combination of passive, active, and outbreak data sources are needed to fill the data gaps, as well as to assess the impact of public awareness campaigns on rational antibiotic use and ABR (Prabhakar et al., 2010; FAO and WHO, 2011; Acar and Moulin, 2013). The generated information from such surveillance could inform decision-makers, evidence-based policies, and help to allocate appropriate resources for preventing and containing ABR via the food chain. These systems further need to establish harmonized laboratory methods and provide specifications for the target populations, samples, settings and bacteria, in keeping with the WHO's AGISAR, in order to compare results nationally and internationally.

The NARMS, DANMAP, and MARAN are well-known integrated food chain surveillance systems that have been established in developed countries to undertake research along the food chain, combine data from various sources and use strong multi-sectorial partnerships with other entities (DANMAP, 2014; FDA, 2014; NethMap-MARAN, 2015). Colombia is a notable developing country example, having successfully piloted an integrated surveillance system to monitor trends in antibiotic resistance on poultry farms, abattoirs and retail markets in order to develop adequate ABR prevention and containment measures (Donado-Godoy et al., 2015). The pilot Colombian Integrated Program for Antimicrobial Resistance Surveillance (COIPARS) was able to meet animal health and welfare requests of food producers, and address public health concerns associated with the antibiotic use in food animals and ensure food safety. The COIPARS is regarded as a model in Latin America by the International Molecular Subtyping Network for Foodborne Disease Surveillance (PulseNet International) and the WHO Global Foodborne Infections Network (Donado-Godoy et al., 2015). The WHO AGISAR has further used COIPARS as a guide to harmonize methods for monitoring of ABR and antibiotic use in food animals in the Americas (Donado-Godoy et al., 2015).

Similarly, Mexico implemented a pilot on integrated food chain surveillance system to prevent and contain *Salmonella* spp., *Campylobacter* spp., and *E. coli* from farm-to-fork in four regions and ascertained its cost-effectiveness. Samples were collected from sick and healthy humans, locally produced retail chicken, pork, beef, and their intestines at slaughterhouses, with a sample size representative of the regional consumption of each retail meat using standardized laboratory protocols (Zaidi et al., 2008, 2015; Donado-Godoy et al., 2015). Within a 3 years-period, and with a limited financial investment, the Mexican's integrated food chain surveillance system was able to yield meaningful epidemiological data on emerging public health threats, identified effectively strains of greatest public health threat, as well as the main food animal reservoirs, showing that these systems are technically and economically feasible in developing countries (Zaidi et al., 2008, 2012, 2015).

Reference Laboratory and Harmonized-Laboratory Methods

National reference laboratories are essential to provide harmonized, high quality, and precise information when assessing ABR bacteria (indicator, foodborne, and emerging bacteria) with the potential to threaten food safety. The majority of developed countries have well-designed reference laboratories that focus their activities on humans as well as animals, while few LMICs have established national reference laboratories, with those that do mainly focusing on humans. National reference laboratories in which bacteria are tested for antibiotic susceptibility exist in 6 out 47 and 6 out of 21 countries of the WHO African and Eastern Mediterranean Regions respectively (WHO, 2015b). Reorientation of the research strategy to include food animals, food derived products, and humans is vital to contain the spread of ABR from farm-to-fork in developing countries (de Balogh et al., 2013).

Research on the Total Bacterial Resistome

Given that food animals are key and persistent reservoirs of ABR, basic food chain surveillance programs focus primarily on foodborne or indicator bacteria. Effectively curbing the emergence and spread of ABR needs to consider the role of commensal flora in the emergence of resistance. Antibiotic use affects and selects largely resistant bacteria in commensal flora, which may subsequently transmit their ARGs to the pathogenic ones (Andremont, 2003). The commensal genetic pool is so huge that it involves several pathways of transferring resistance,

including mutations and complex resistance mechanisms (HGT) that are either expressed or silent within subdominant species, and are able to move freely between species (Andremont, 2003). Assessing the whole bacterial resistome and mobilome through food surveillance systems will enable the detection of emerging antibiotic-resistant bacteria and ARGs from farm-to-fork. This is important to inform issues that are often ignored by basic food chain surveillance programs, these being to detect early outbreaks and undertake timely correct actions. This is obviously more feasible in developed and well-resourced countries.

State-of-the-Art Methods

Detecting antibiotic-resistant bacteria and ARGs is based on culturing, antimicrobial susceptibility testing (AST), and polymerase chain reaction (PCR). Although AST and PCR-based methods are excellent for phenotyping resistance profiling and detecting ARGs respectively, both require bacterial culture and do not enable the discovery of distantly related or unknown elements that may be present, and which are particularly crucial in the farm-to-fork continuum. Whole genome sequencing enables the detection and exploration of complete bacterial genome, with a view to identify new genetic traits. Metagenomics also allows the analysis of the whole genome and identification of unknown genetic elements, regardless of the cultural characteristics of the bacteria (Allen, 2014; Thanner et al., 2016). Single nucleotide polymorphisms, novel resistance genes and unforeseen aspects of ARG ecology may be identified through the use of whole genome sequencing and functional metagenomics, respectively (Allen, 2014; Thanner et al., 2016). Plasmid sequence analysis and reconstruction may also be performed using whole genome sequencing and metagenomics. Microarray expression analysis provide useful insights into expressing profiles of bacteria under antibiotic pressure, while recent transcriptomics and meta-transcriptomics tools identifying functional genes are also an important advancement in detecting new genetic elements (Thanner et al., 2016). State-of-the-art tools may be very useful to understand transmission dynamics of resistance gene expression through the different ecological niches prevailing in the food chain (Thanner et al., 2016). Again, these are more feasible in developed and well-resourced countries.

Reducing the Burden of Infectious Diseases

Immunization and Biosecurity

Unprecedented global food demands result in farmers' reliance on antibiotics to produce large quantities of animal protein at low cost although. Evidence exits that improving animal welfare and health have the potential to diminish this over-dependence on antibiotics without affecting productivity and costs. The use of vaccines is recommended as method of preventing infections in animals and should significantly reduce antibiotic consumption while increasing the productivity. Although already available against some viral, parasitical, and bacterial infections (**Table 4**) in food animals, their routine use is generally limited due to few incentives promoting them and the absence of disincentives prohibiting antibiotic consumption in the food production industry (Woolhouse et al., 2015). Mass vaccination campaigns should be implemented as in humans to ensure animal health, as the greater the use of vaccines, the lesser the incidence of infections (Woolhouse et al., 2015). Vaccines can effectively protect animals against bacterial infections, but may require regular "updates" to ensure their continuous efficacy (O'Neill, 2015b).

Biosecurity measures in food and agriculture should further significantly reduce or eliminate ABR-bacteria from farm-to-fork, and thereby, lower the burden of infectious diseases. Biosecurity refers to a holistic concept used to define a set of ongoing measures taken to reduce the risk of the emergence/introduction and dissemination of diseases at herd-, region-, and country-levels (FAO, 2003; FAO and WHO, 2011). It uses "One Health" as a framework, and benefits from this integrated approach by encompassing various concepts, such as good agricultural practices, good hygiene practices, good veterinary practices, hazard analysis, and critical control points (HACCP)-based procedures and microbiological risk assessment and management (FAO, 2003; FAO and WHO, 2011). When animals are healthy, antibiotics are not needed to treat them. However, when ABR occurs, and effective barriers (e.g., good agricultural practices, good hygiene practices, and HACCP-based measures) are present, subsequent transmission will be impeded, with food safety, food security, and public health being ensured. Biosecurity should then play a fundamental role in any disease control program as it expands beyond agricultural production to human and environmental health (Nahar et al., 2014; Postma et al., 2016).

Farming Practices

Well-controlled extensive farming practices using small chemical substances and that favor animal health and welfare to limit the development of infectious diseases and reduce antibiotic use (e.g., all-in-all-out, extensive free-range systems), should be promoted and implemented rather than intensive farming practices. Organic farm systems have recently received considerable interest to meet the global food demand in an effort to reduce the extensive use of antibiotics (Prabhakar et al., 2010). In comparing the occurrence of ABR in food animals in conventional and organic farms, Österberg et al. (2016) reported a significant lower prevalence of ABR-*E. coli* isolated from organic pig farms than from conventional ones in four European countries (Österberg et al., 2016). This suggests that organic farms could be valuable agricultural alternatives to reduce the burden of ABR, although further research is needed to ascertain the consequences related to such practices and improve the productivity. Sweden, the first country that implemented a total ban of antibiotic growth-promoters in food production industry in 1986, is one of the world's references for containment of ABR in the food chain through well-implemented and adequate farming practices (O'Neill, 2015b). Following the 1986's ban, to facilitate the passage to new farming practices, Swedish authorities developed guidelines on management, hygiene, medication, and feed to improve animal health and prevent infectious diseases. Swedish efforts were considerably focused on problem-oriented research and provided extensive support for farmers (O'Neill, 2015b). This led to decrease of sales of antibiotics used in food animals

TABLE 4 | List of some available veterinary bacterial vaccines.

Target animal	Target pathogen	Brand name	Characteristics	References
Pig	*Lawsonia intracellularis*	Enterisol Ileitis	Live oral vaccine	Guedes and Gebhart, 2003
Fish	*Yersinia ruckeri*	AquaVac ERM	Killed oral vaccine	Meeusen et al., 2007
Fish	*Aeromonas salmonicida*	AquaVac Furuvac	Killed oral vaccine	
Fish	*Vibrio anguillarum*	AquaVac Vibrio	Killed oral vaccine	
Chicken	*Salmonella spp.*	Megan Vac1 MeganEgg	Live vaccine	Babu et al., 2004
Cattle	*Brucella abortus*	RB-51	Rifampin-resistant mutant	Moriyon et al., 2004
Pig	*Actinobacillus pleuropneumoniae*	PleuroStar APP	Recombinant proteins	Van Overbeke et al., 2001
Chicken	*Mycoplasma gallisepticum*	Vaxsafe MG	Live vaccine for eye drop administration	Barbour et al., 2000
Chicken	*Mycoplasma synoviae*	Vaxsafe MS	Live vaccine for eye drop administration	Meeusen et al., 2007
Turkeys	*Bordetella avium*	Art Vax	Live for spray inhalation or drinking water	
Sheep	*Chlamydophila abortus*	Ovilis Enzovax	Live vaccine for intramuscular or sub-cutaneous injection	
Pig	*Actinobacillus pleuropneumoniae*	Porcilis APP	Outer membrane proteins	

from 45 tons to around 15 tons of active substances by 2009 (O'Neill, 2015b). In contrast, up to a third of the projected 67% global increase in antibiotic consumption in food animals will be attributable to shifting agricultural practices in LMICs, where extensive farming systems will be substituted by large-scale intensive farming (Van Boeckel et al., 2015). This suggests that LMICs should follow the Swedish experience for sustainable agriculture independent of antibiotics although data relative to such initiatives was limited.

Rapid Infection Diagnostics

There are significant advantages of rapid diagnostic tools, such as culturing, PCR, microarray, and whole genome sequencing, being more readily available to quickly identify bacterial infections, resistance patterns, and determine appropriate treatment in animals and humans (Woolhouse et al., 2015). Chromogenic tests and Matrix-assisted laser desorption/ionization time-of-flight have been proposed, as they allow not only rapid bacterial identification and but also effective resistance profiling. These tools may be suitable for LMICs where diagnostic tool must be relatively economical, rapid, easy to use, and requires less logistic infrastructure (Holmes et al., 2016).

Several mathematical models, such as Predictive Microbial Modeling Lab (PMM-Lab), Pathogen Modeling Program (PMP), Geeraerd and Van Impe Inactivation Model Fitting Tool (GInaFiT), and Combined dataBase (ComBase) Predictor, that predict the bacterial behaviors in various stages of production (e.g., processing, storage, and distribution) have recently received significant interest in the food production industry (Plaza-Rodríguez et al., 2015). Predictive microbiology generates substantive quantitative data and useful estimates that can assist in decision-making during food production process, HACCP-based procedures and food safety risk analysis and management (Plaza-Rodríguez et al., 2015; Thanner et al., 2016). Predictive microbiology could be valuable to reduce antibiotic use and infectious diseases, and contain ABR. However, it must be optimized to encompass important factors, such as the total bacterial resistome, microbial loads, biological mechanisms, and cross-species transmission, with outcomes not only being lethality, but also incidence of diseases, level of pathogenicity and prevalence of antibiotic-resistant bacteria and ARGs (Plaza-Rodríguez et al., 2015; Thanner et al., 2016).

The Artificial Neural Network (ANN) is an advanced mathematical model capable of performing large and simultaneous computations for data processing and knowledge representation. It is able to combine data of different nature collected from various sources, populations and protocols, thereby forming an integrated diagnostic system. Several studies have demonstrated that ANNs could be built and trained to recognize bacterial resistance patterns, and to generate adequate conclusion in terms of ABR profiles (Budak and Übeyli, 2011; Lechowicz et al., 2013). Budak and Übeyli (2011) used ANN to predict the resistance profiles of *Salmonella spp.* to Ampicillin, Chloramphenicol, and Trimethoprim-sulfamethoxazole, and reported overall accuracies of more than 95% (Budak and Übeyli, 2011).

Modern methods, such as Fourier Transform Infrared Spectroscopy (FTIR) and Attenuated Total Reflection-based (ATR)-FTIR, that detect the presence and measure the infrared spectra of intact microbial cells have also been proposed for rapid bacterial strains detection in the food production chain (Rebuffo-Scheer et al., 2007; Lechowicz et al., 2013). The combination of infrared spectroscopy with ANN also allows the rapid identification of bacteria and determination of resistance patterns. Lechowicz et al. (2013) demonstrated that the Multi-Layer Perception network built using the ANN-based FTIR method was able to successfully detect uro-pathogenic *E. coli*, and to classify strains susceptible and resistant to cephalothin with more than 90% of accuracy. Although these tools require sufficient skills, they present an adequate level of accuracies to differentiate bacteria and determine resistance patterns, and could be used routinely for laboratory diagnostics (Rebuffo-Scheer et al., 2007; Lechowicz et al., 2013).

Nanoscale materials have also emerged as an innovative approach to enable rapid, sensitive and cost-effective diagnosis, and to adequately determine bacterial resistance profiles. Antibody-conjugated nanoparticles have reported to be able to identify highly pathogenic *E. coli* O157:H7 within 20 min. A rapid diagnostic tool, using gold nano-wire arrays in conjunction with a linker arm coated to specific *E. coli* antibodies, has recently

been developed to detect urinary tract infections (Huh and Kwon, 2011). On-going research using magnetic nanoparticles, such as dextrancoated super-magnetic iron oxide nanoparticles attached with con-A conjugated nano-sensors, is assessing bacterial metabolic activity and antibiotic susceptibility in opaque media, including blood and milk, without any sample preparation. The intention is to achieve fast and reliable diagnostic assays for bacterial infections adapted to the food production chain, although this is not as yet commercially available (Huh and Kwon, 2011).

Rapid diagnostics will allow agricultural practitioners to identify early infection in animals, to separate those that are infected from others, prevent the spread of infection to the whole herd or flock, and thereby reduce antibiotic use (FAO, 2015; O'Neill, 2015b). Technical and financial barriers, as well as resistance to the adoption of innovative ideas are slowing the implementation of such measures around the world, particularly in developing countries.

Optimizing Rational Antibiotic Use

Ban on Antibiotic Use

The burden of ABR has reached and surpassed the pace of development of new antibacterial agents entering clinical use, and has led to a radical re-evaluation of antibiotic use. Given the growing global concern about the spread of antibiotic-resistant bacteria from animals to humans, there is a significant interest in phasing out antibiotics in the food production industry. LMICs should determine their annual antibiotic consumption in food animals and prohibit the growth-promotion use of any antibiotic substances which the WHO considers to be "critically, highly and important antimicrobials in human medicine," as well as those not currently approved for veterinary use (WHO, 2011; OIE, 2015; O'Neill, 2015b). Avoparcin, was the first antibiotic growth-promoter banned in 1995 in Denmark, followed by virginiamycin in 1998 and a complete ban of antibiotics in 2000 (DANMAP, 2014). The effects of the ban were noticeable as the country experienced significant reduction in antibiotic use and ABR, without affecting the pig and poultry productions which continued to thrive albeit with a small drop in productivity at the inception of the ban and until biosecurity efforts were enhanced. Danish farmers increased their pig production by 47% over the period 1992–2008, preserving the standing of country as among the greatest exporters of pork worldwide, with 90% of their pig production being exported (O'Neill, 2015b). Denmark thus illustrated that very productive and sustainable food production is possible alongside low antibiotic use (O'Neill, 2015b). The majority of developing nations are still far behind a total ban of antibiotics for growth-promotion purposes. Considerable efforts are thus needed by way of an incremental process encompassing cultural, political, and socio-economic contexts while taking cognizance of the urgency and gravity of the ABR concern.

Prohibit Unrestricted Access of Antibiotics

As ARGs are generally carried on mobile genetic elements and all antibiotic families have the potential to select, to a certain degree, cross-resistance to other families. The unrestricted access of antibiotics to farmers should thus be prohibited unless under veterinary prescription or oversight. Meaningful veterinary oversight should further be mandatory when classes of antibiotics currently approved for dual use (both animal and human health) to treat or prevent infectious diseases are prescribed. It is also important to develop methods to verify judicious antibiotic use, establish goals for measuring progress and guidelines for consuming veterinary antibiotics (O'Neill, 2015b; OIE, 2016). In 2009, Dutch government implemented a registration process for veterinary prescription of antibiotics. As a result, between 2007 and 2012, Dutch antibiotic sales in food animals decreased by 56% without any loss in terms of productivity and profits. Dutch farmers have changed their farming practices from over-reliance on antibiotics to improved animal health and welfare (O'Neill, 2015b). To the best of our knowledge, such measures have not been significantly implemented in food animals and aquaculture in LMICs, where regulatory framework and legislation implementation are further limited.

Legislative Regulation and Enforcement Policies

The OIE recommended reinforcing veterinary legislation and enforcement policies to ensure compliance with the law and regulations promoting responsible and prudent use of Veterinary Critically Important Antimicrobial Agents (VCIA), Veterinary Highly Important Antimicrobial Agents (VHIA), and Veterinary Important Antimicrobial Agents (VIA) (OIE, 2015, 2016). When antibiotics are justifiably required, it is essential to follow the established principles and criteria for antibiotic use, according to the OIE "List of Antimicrobial Agents of Veterinary Importance." Achieving a balance between minimizing antibiotic use in food production industry, while meeting the unprecedented rise of worldwide food demands, is a fundamental food security/food safety challenge that must be considered when defining target goals for reducing antibiotic use. It is therefore important that every country involves stakeholders from various sectors (governments, industries, experts, practitioners, and international entities) to set an ambitious but realistic and achievable targets to reduce antibiotic consumption (O'Neill, 2016). Developing countries should examine and adapt the Danish, Dutch, and Swedish experiences as well as acquire scientific expertise from these countries in order to reduce their antibiotic consumption in food animals and aquaculture while preserving their productivity. It is further essential to promote and ensure sustainable agriculture not over-reliant on antibiotics in the developing world to reach the United Nations Sustainable Development Goals by 2030. Sustainable support from developed countries, funding agencies and international entities is essential to reduce antibiotic use and ABR in food animals and aquaculture in LMICs.

Sustainable Investment and Development of New Medicines

Political Will

The human and economic burden associated with containing ABR are trivial compared to the cost of complacency or

inaction. ABR will account for more than 10 million deaths by 2050, with a cumulative global economic loss of US $100 trillion over the 35 next years if not addressed substantively (O'Neill, 2015a). ABR should be considered a national priority to retain the attention, ensure sustainable investment and allocate resources to successfully contain it. Governments should translate surveillance estimates into policies leading to implementing long-term interventions and activities in animal husbandry systems and agriculture to ensure animal welfare and health, and to contain ABR at local, national, regional, and international levels. More public and private partnerships are also needed to assess the problem in its broader context and find lasting solutions. Active political will is thus indispensable to enact sustainable engagement, investment, research, and alternatives to antibiotics in the food production industry (O'Neill, 2015b; Woolhouse et al., 2015).

Sustainable Engagement

The need to address and fill the gaps in ABR knowledge have never been more imperative. Sustainable engagement of inter alia policy makers, agricultural practitioners, veterinarians, antibiotic providers, industries, food suppliers, the public, and others who may contribute to long-term solutions is an essential component to effectively contain ABR via the food chain. Multi-sectorial and coordinated efforts should cohesively identify and target research priorities as well as increase research funding to effectively overcome this global challenge (Woolhouse et al., 2015; O'Neill, 2016).

Leverage Resources

Addressing the issue of ABR globally requires greater efforts in developing countries, where interventions may be restricted as a result of inadequate resources. It is thus important to approach to donor funding agencies and supranational entities to improve prevention and containment of ABR via the food chain. An example is the Fleming Funds with its £265 million allocated by the UK government to help improve surveillance in LMICs (O'Neill, 2016). The World Bank's International Development Association (IDA) is another funding mechanism through which LMICs could request support to strengthen surveillance programs, built laboratory-capacity, and prevent and contain ABR. More international support to LMICs are however still needed.

Alternatives to Antibiotics for Food Animals

The global scientific community recognizes the urgent need to reinvigorate the therapeutic pipeline with new antibiotics. The community is further aware of the need for effective and adequate alternative solutions to antibiotics in food animals as an essential strategy to circumvent the challenge of ABR. The range of these potential alternatives is, for the most part, similar to those already documented in human medicine and may include prebiotics, probiotics, synbiotics (viz. mixing of prebiotics and probiotics), substances targeting bacterial communication (quorum sensing), phage enzymes, phage-related therapies and the use of genetically modified food animals (already resistant to infection; Woolhouse et al., 2015).

Synthetic biology (SB) provides a novel approach to discover new antibiotics, and to exploit and improve existing drugs. It uses the genomic data generated by state-of-art technology to identify biological machineries of various bacteria predicted to be able to produce antibiotics. Synthetic bacteria with beneficial functionalities are designed and new antibiotics bio-synthesized with novel antibacterial activity under artificial and engineered control systems (Takano and Breitling, 2014). SB offers an exciting prospect to expand the therapeutic arsenal, as it could generate libraries of novel, predicted and different drug variants with broader activities and better pharmacokinetics (Takano and Breitling, 2014).

Nano-antibiotics, either having antimicrobial activity by themselves or as novel nano-sized drug delivery platforms, are also part of the most interesting alternatives to antibiotics. Unlike other antibiotic substances and proposed strategies, nano-antibiotics exert their activity through several pathways, such as (i) a combination of numerous mechanisms to inhibit bacteria (e.g., nitric oxide-releasing nanoparticles, chitosan-, and metal-containing nanoparticles) (ii) encapsulating several antibiotics within the same nanoparticles, (iii) reduced uptake and increased drug efflux, and (iv) site specific action releasing high concentration of antibiotics at the site of infection (Huh and Kwon, 2011; Pelgrift and Friedman, 2013). Nano-antibiotics offer several advantages, not only in overcoming ABR and reducing acute adverse effects (although toxicity upon long-term exposure is yet uncertain) but they also remain stable on long-term storage and are cost-effective preparations compared with antibiotic synthesis (Huh and Kwon, 2011; Pelgrift and Friedman, 2013). Considerable investments in research are still needed before any of the above possibilities become commercially available on a global scale and can effectively replace antibiotics.

CONCLUSION

ABR is a global public health challenge with severe health and socio-economics repercussions that is significantly influenced by antibiotic use in food animals. Combating ABR effectively at a global scale means addressing it equally in the developed and developing world. Multifaceted, comprehensive and integrated strategies, as advocated by the WHO Global Action Plan and FAO Action Plan in line with the One Health approach, are urgently required to (i) prevent the transmission of ABR and infectious diseases from farm-to-fork, (ii) circumvent potential pandemic situations, and (iii) preserve the efficacy of antibiotics and assure food safety, food security, and global health. Countries should therefore follow WHO, OIE, and FAO recommendations to implement national action plans encompassing human, (food) animal, and environmental sectors to improve policies, interventions and activities that

address the prevention and containment of ABR from farm-to-fork.

AUTHOR CONTRIBUTIONS

LF co-conceptualized the study, searched the literature, extracted and collated data, and drafted the manuscript. RF extracted and collated data and contribute to the writing of the manuscript. SE co-conceptualized the study, provided substantial revision of the manuscript. All authors read and approved the final manuscript.

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The other authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

CHAPTER 3

Article II. Antibiotic Resistance in Food Animals in Africa: A Systematic Review and Meta-analysis

Antibiotic Resistance in Food Animals in Africa: A Systematic Review and Meta-analysis[2]

Author contributions

- Luria Leslie Founou, as the principal investigator, co-conceptualized the study, developed the protocol, searched the literature, screened titles abstract and full-texts, extracted and summarized the data, performed quality assessment, undertook statistical analysis and interpreted the results, prepared tables and figures, and drafted the manuscript**.**
- Daniel Amoako, participated in the screening of titles and abstracts and undertook critical review of the manuscript.
- Raspail Carrel Founou extracted and collated data, performed quality assessment and statistical analyses, interpreted the results, prepared tables and figures and contributed to the writing of the manuscript.
- Sabiha Yusuf Essack, as principal supervisor, co-conceptualized the study, screened titles, abstracts and full-texts, and undertook critical revision of the manuscript.

Objective met: This paper meets objective Two viz. to describe the distribution of ABR-bacteria in food animals in Africa, in order to highlight the need to restrict the use of antibiotics in agriculture, and provide evidence to follow the One Health approach to contain the emergence and spread of ABR on the continent.

[2] This paper has been submitted to Microbial Drug Resistance and is currently under review.

Antibiotic Resistance in Food Animals in Africa: A Systematic Review and Meta-analysis

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Abstract

Objectives: This study critically reviewed the published literature and performed a meta-analysis to determine the overall burden of antibiotic-resistant bacteria in food animals in Africa.

Methods: English and French published papers indexed in EBSCOhost, PubMed, Web of Science and African Journals Online were retrieved, with searches being conducted up to August, 2015. Data were pooled and meta-analysis performed using a random-effects model and the results are described as event rates.

Results: According to the predefined inclusion and exclusion criteria, 17 articles out of the 852 retrieved, were eligible for the qualitative and quantitative analysis. The studies included were mainly conducted in Nigeria, with *E. coli*, *Salmonella* spp., and *Campylobacter* spp. being the main bacteria. The pooled estimates showed high level of antibiotic resistance (86%; $p<0.001$) and multi-drug resistance (73%; $p=0.003$).

Conclusion: Our results suggest that antibiotic resistance is substantively prevalent, and poses a serious threat for food safety and security in Africa. These findings shed light on areas for future research concerning antibiotic resistant and multi-drug resistant bacteria in food animals as etiological agents of infectious diseases in humans. They further yielded some interesting findings on the burden of antibiotic resistance, that could be useful in developing measures to contain this threat in the farm-to-plate continuum in Africa.

3.1. Background

Antibiotic resistance (ABR) is a worldwide public health concern, with serious health, economic and societal repercussions (1). Its emergence is attributed to the selective pressure exerted by antibiotic use in the community, hospitals, veterinary health, agriculture, aquaculture, and the environment. Additionally aggravating the situation, is the fact that very few new antibiotics have recently been produced by pharmaceutical companies. It is widely acknowledged that food animals are key reservoirs of antibiotic-resistant bacteria, and that antibiotic usage in this population favours the emergence, selection and spread of resistance among animals and humans (2-4), both via zoonoses (infectious diseases transmitted between animals and humans) and the food chain (4-6).

Food animal production generally depends on the therapeutic and prophylactic use of antibiotics, and can be enhanced by the use of antibiotics for growth-promotion. Several antibiotic agents commonly used in food animals are either identical or linked to those administered in humans (7). This broad use of antibiotics in agriculture has increased the danger posed by the emergence and spread of ABR by selecting for new antibiotic-resistant (commensal and/or pathogenic) bacteria and infections caused by these bacteria (4, 6, 8, 9). Accordingly, the presence of ABR in food animals threatens food safety and by extension, global health. Given the sharing of bacteria between humans and animals, as well as the animal origin of 60% (10, 11) of emerging human pathogens, the Food and Animal Organization of the United Nations (FAO), World Organization for Animal Health (OIE) and World Health Organization (WHO) fully endorse the One Health approach as articulated in the WHO Global Action Plan on Antimicrobial Resistance (AMR) (12), the OIE Strategy on AMR and Prudent Use of Antimicrobials (13) and the FAO Action Plan on AMR (14).

Notwithstanding the situation evidenced by this global health challenge, the dearth of information concerning ABR in food animals in Africa leads to an under-estimation of the nature and extent of ABR, as well as the associated health and socio-economic impacts on

human, animal and environmental health regionally and globally. This systematic review analysed the published literature on the prevalence of ABR in food animals in Africa. By summarizing the available data, our objectives were to (1) describe the dissemination of antibiotic-resistant bacteria in food animals; (2) highlight the need to reduce, replace and refine the use of antibiotics in agriculture, and (3) provide evidence to follow the One Health approach to contain the emergence and spread of ABR on this continent.

3.2. Methods

The Preferred Reporting Items for Systematic Reviews and Meta-analyses [PRISMA, (15)] and Meta-analysis of Observational Studies in Epidemiology [MOOSE; (16)] statements were followed.

3.2.1. Outcomes of interest

The primary outcome of interest was to identify the prevalence of antibiotic-resistant bacteria isolated from apparently healthy, sick or dead food animals, products thereof and exposed workers at farms, abattoirs/markets or both. Resistance to beta-lactams, aminoglycosides, and fluoroquinolones, described by the WHO (17) and OIE (18) as critically important antibiotics in humans and animals as well as tetracyclines listed as critically important veterinary antimicrobial agents in animals (18), were used as the basis to ascertain multi-drug resistance (MDR) in our study. The secondary outcome of interest was the prevalence of multidrug-resistant bacteria, which, for the purpose of this review, is regarded as resistance to three or more classes of antibiotics.

3.2.2. Ethics approval

This systematic review and meta-analysis was based on an appraisal of published reports and was therefore exempted from ethical approval. Moreover, it did not involve any direct research on human participants and no informed consent was required.

3.2.3. Sources and literature search

A multifaceted search was conducted in four electronic databases, namely Medline via PubMed, Web of Science, EBSCOhost and African Journals Online up to August, 2015, using a combination of boolean operators (AND/OR), Medical Subject Heading (MeSH) and pre-defined keywords including 'antimicrobial resistan*', 'antibiotic resistan*', 'drug* resistan*', 'multi-drug resistan*', 'multi-drug resistan*', 'multiple-drug resistan*', 'multiple drug* resistan*', 'food animal*', 'farm animal*', 'domestic animal*', 'livestock animal*', 'poultry', 'pig', 'cattle', 'sheep', 'goat' and followed by refining terms: 'Africa*', 'East* Africa*', 'Western* Africa*', 'Southern* Africa*', 'Northern* Africa*', 'Central Africa*', 'Sub-saharan Africa*'. The truncation mark (*) specifies that diverse extensions were used during the search.

The reference lists of all included papers were further used to carry out a supplementary literature search. In addition, attempts were made to contact authors to obtain inaccessible abstracts and full-texts of included studies. Papers in English and French were retrieved and assessed for potentially relevant studies pertaining to antimicrobial resistance in food producing animals in Africa. The authors independently screened and evaluated the full texts of the papers following the first duplicated and blinded screening on the basis of titles and abstracts for relevance to the study objectives. Disagreements and inconsistencies among authors were resolved by consensus after discussion.

3.2.4. Exclusion and inclusion criteria

The authors individually assessed articles using pre-designed eligibility forms and according to pre-defined eligibility criteria (Table 1). Briefly, studies on parasites, viruses and fungi, as well as those dealing with ABR in aquatic, companion and wildlife animals, and the environment were excluded. Although, studies dealing with ABR in humans were excluded those reporting data of workers exposed to food animals and/or products thereof were included. Studies reporting data from outside Africa were further not selected, nor was grey literature

(foreign or domestic material usually inaccessible through relevant databases and indexes) and unpublished data.

The selection of French and English published papers was based on clearly defined populations involving living food animals at farms and/or processed/freshly slaughtered animals at abattoirs/markets. In order to be included, studies must have also performed antibiotic susceptibility testing with antibiotics belonging to beta-lactam/aminoglycoside, tetracycline, and fluoroquinolone classes of antibiotics via disk diffusion, agar dilution, broth micro-dilution or E-test methods, and results interpreted according to appropriate guidelines (Antibiogram Committee of the French Society of Microbiology: CA-SFM; European Committee on Antimicrobial Susceptibility: EUCAST; Clinical Laboratory Standards Institute: CLSI formerly known as National Committee on Clinical Laboratory Standards: NCCLS). Multi-site and intercontinental studies involving ABR in food animals in African countries were also considered.

3.2.5. Framework for literature screening and data extraction

EndNote (version X7, Thomson Reuters) was used for literature management and relevant data from included papers were extracted as outlined in Table 2. The data were abstracted and analysed using a framework onto an Excel® (Microsoft® Office Excel 2013) spreadsheet, including for each study, first author details, country of study, year of publication, aims, study population (e.g. pigs, poultry, cattle, sheep, goat, human), type of sample (e.g. nasal swabs, rectal swabs, faecal samples, meat products), sample size, clinical status (e.g. apparently healthy, sick and dead), study site (viz. slaughterhouse, farm, market), type of study (e.g. single, multisite, and international study), bacteria of interest (e.g. *S. aureus*, *Salmonella* spp., *Campylobacter* spp., *E. coli.*, *Enterococcus* spp.), antibiotics tested, antimicrobial susceptibility testing (AST) methods (disk diffusion, micro-broth dilution, agar dilution, E-test and automated methods), guidelines of interpretation of AST (e.g. CA-SFM, EUCAST, CLSI, NCCLS), ABR/MDR prevalence and results.

3.2.6. Quality assessment

Various types of observational studies addressing prevalence were considered in this systematic review. There are numerous reporting measures assessing the study quality in systematic reviews and meta-analyses, but these are generally limited to specific type of studies such as randomized-controlled trials, with no standard method for conducting quality assessment of prevalence data studies. Therefore, pre-existing scales could not be used to assess study quality. The modified critical appraisal tool (high-quality items rating scale) developed by Munn et al. (2014) was used to assess the quality of all included studies (19): (1) Was the basic data including study period, sample type, bacteria of interest and study site provided? (2) Were the study participants recruited in an appropriate way? (3) Was the sample size representative of the target population? (4) Were the study subjects and setting described in detail? (5) Was the data analysis conducted with sufficient coverage of the identified bacteria? (6) Were all important confounding factors/ subgroups/differences identified and accounted for? (7) Were objectives, standard criteria used to measure the condition? (8) Was the condition measured reliably?

Each item was answered with either a yes, no or unclear, and scored on a three-point scale, with 2 indicating high quality, 1 indicating moderate quality and 0 low quality. Summing up the scores of each item provided the overall score of the study, with a highest being 16. A total score ≥12 was regarded as high quality (low-risk of bias), between 6-12 as moderate quality (medium-risk of bias) and <6 as low quality (high-risk of bias). Only high-quality studies were included in the study. The quality assessment was undertaken individually by the authors.

3.2.7. Statistical analysis

Microsoft Excel® (2013 for Windows) was used to analyse the data following an initial extraction. Meta-analyses were performed for outcomes of which there were four or more studies that could be combined. Analyses were conducted across animal populations for the

two selected endpoints (resistance and multi-drug resistance). The rates of antibiotic-resistant and multi-drug resistant bacteria among included studies were calculated as follows:

$$Bacterial\ ABR\ rate\ (\%) = \frac{Number\ of\ strains\ confirmed\ resistant}{Number\ of\ strains\ isolated\ and\ screened\ for\ resistance}$$

$$Bacterial\ MDR\ rate\ (\%) = \frac{Number\ of\ strains\ confirmed\ Multi-drug\ resistant}{Number\ of\ strains\ confirmed\ resistant}$$

Meta-analyses of rates were undertaken to determine the overall prevalence of antibiotic-resistant and multi-drug resistant bacteria among food animals and exposed workers. Subgroup analyses were conducted for population-, sample-, settings-, organism-, and country-defined subgroups.

Forest plots of pooled event rates for the primary and secondary outcomes, with 95% confidence intervals, were generated using the Comprehensive Meta-analysis (CMA) software (Biostat, Inc., New Jersey, USA) version 3 for Windows. Studies were weighted in favor of those with more precise results (narrower confidence intervals) and results are presented as event rates. Data were pooled, and meta-analyses performed using the random-effects model to provide a more conservative estimate of resistance, allowing for any heterogeneity between studies. This method was used to assess the extent of bacterial resistance of the entire relevant population, not only the population in the included studies. The I^2 statistic with cut-off values of 25 % (low), 50% (moderate) and 75% (high) was used to assess heterogeneity between studies and the chi square test with p-value <0.05 was used to define a significant degree of heterogeneity within studies. Publication bias was assessed and visualized by a funnel plot and Egger's tests for small study effects.

3.3. Results

Figure 1 outlines the work-flow of the study selection process with reason of exclusion. The systematic search from the four electronic databases identified 852 articles. After duplicates

were removed, 463 papers were screened for potential inclusion based on their titles and abstracts, with 124 full-text articles being entirely assessed. Two articles were added following hand-searching and according to the predefined inclusion and exclusion criteria, yielding a final number of 68 studies being eligible for the quality assessment. Of these 68 studies, 17 were rated as good quality (low risk of bias), 47 were of moderate quality (medium risk of bias) and 4 were of poor quality (high risk of bias). Only good quality studies were finally included in the qualitative and quantitative synthesis.

3.3.1. Description and characteristics of included studies

Most of the data analysed were obtained from single centre studies conducted mainly in Nigeria (n=6) (Table 2 and Figure 2). The majority of studies (n=12) reported ABR only in food animals while two studies investigated ABR concomitantly in food animals, food products and exposed workers (32, 33). Similarly, three studies reported ABR conjointly in food animals and food products (34-37). *E. coli* (n=8), *Salmonella* spp. (n=6) and *Campylobacter* spp. (n=2) were the main antibiotic-resistant bacteria investigated and reported (Table 2 and Figure 2).

3.3.2. Assessment of antibiotic resistance of bacterial species

All papers (100%) included antibiotic susceptibility testing of the identified bacterial species. Overall, *E. coli* isolates were screened with 16 different antibiotics across all respective studies using disk diffusion (75%; 6/8) and broth micro-dilution (25%; 2/8). Similarly, 19 antibiotics were tested against *Salmonella* spp. isolates with disk diffusion (50%; 3/6) and broth microdilution (33.3%; 2/6) being the main AST methods (Table 3). The use of standardized guidelines was reported in all 17 studies. Susceptibility testing was performed most frequently to ampicillin (75%) followed by tetracycline, gentamicin, trimethoprim-sulfamethoxazole, streptomycin, ciprofloxacin, nalidixic acid, chloramphenicol and cefuroxime in *E. coli* (Table 3). Regarding *Salmonella* spp. the order was as follows, streptomycin, gentamicin, ciprofloxacin, tetracycline, chloramphenicol, ampicillin, nalidixic acid, sulphonamides,

trimethoprim-sulfamethoxazole. The overall estimated effects for *Campylobacter* spp., *S. aureus* and *Enterococcus* spp. were not calculated due to insufficient reports.

3.3.3. Primary analyses

Figures 3A and 4A represent forest plots of untransformed event rate estimates of ABR and MDR in selected studies. Pooled estimates generated 86% (95%CI, 76.3-92.20%, p=0.000) of ABR and 73% (95%CI; 58.3-83.9%, p=0.003) of MDR. Subgroup analyses were performed per population, bacteria, setting, sample and country to allow more specific results.

3.3.4. Subgroup analyses

a. Population

Figures 3B and 4B show forest plots of ABR and MDR per population with 95% CIs. The prevalence of ABR was very high in pigs with a prevalence of 93.6% (95%CIs; 77.7-98.4%; p<0.001). The prevalence of ABR was 78.2% (95%CIs; 44.1-94.3%; p=0.098) in cattle and 73.1% (95%CIs; 48.8-88.6%; p=0.062) in poultry. Despite the highest level of ABR being in pigs (93.6%; 95%CIs; 77.7-98.4%; p<0.001), MDR was assessed to be largely lower in this population [51.1% (95%CIs; 23.3-78.3%; p=0.942)] although it was not statistically significant. Conversely, overall prevalence of MDR was elevated in cattle and poultry, with 74.3% (95%CIs; 43.4-91.6%; p=0.117) and 84.3% (95%CIs; 56.0-95.8%; p=0.022) prevalence respectively. Pooled estimates for goats and sheep were not calculated due to insufficient data (only 2 reports). The I^2 values of the logit event estimates in cattle, poultry and pigs were 95.53%, 95.57% and 96.84% respectively (p=0.000).

b. Bacterial species

E. coli was the principal bacterium of interest (8 out of 17 studies) and was most frequently investigated individually with no other bacterial species in different populations. Significant levels of ABR [86.50% (95%CIs; 73.20-93.8%; p=0.000)] and MDR [77.50% (95%CIs; 58.90-89.2%; p=0.006)] were identified in *E. coli*. Similarly, rate of ABR was high in *Salmonella*

spp. [80.9% (95%CIs; 54-93.8%; p=0.028)], whereas MDR was estimated at 34.6% (95%CIs; 19.80-53.20%; p=0.102) (Figures 3C and 4C).

c. Setting

Pooled estimates were conducted for isolates collected from farms and abattoirs. Overall prevalence of ABR was higher in farms [88.6%, (95%CIs; 74.4-95.4%; p=0.000)] than abattoirs [79.3%, (95%CIs;52.4-93.0; p=0.032)]. Similarly, MDR prevalence was higher in farms [86.6% (95%CIs; 69.1-94.9%; p=0.001)] than in abattoirs [52.4% (95%CIs; 23.2-79.9%; p=0.886%)] (Supplementary Figures 1A and 2A).

d. Sample

Supplementary figures 1B and 2B depict forest plot of ABR and MDR analysed per sample. Faecal samples were the main isolation site with elevated rates of ABR (96.1%; 95%CIs, 89.2-98.6%, p=0.000) and MDR (69.5%; 95%CIs, 49.6-84%, p=0.054). Pooled estimates for carcasses were not calculated due to insufficient reports.

e. Country

Subgroup analyses per country provided a 95.9% (95%CIs; 78.1-99.3%; p=0.001) prevalence of ABR in Nigeria (Supplementary figure 1C), while the level of MDR was 61.9% (95%CIs; 35.4-82.80%; p=0.552) (Supplementary figure 2C). Prevalence in other countries could not be ascertained as only respectively one report was available for these countries.

3.4. Discussion

Antimicrobial resistance is one of the greatest public health challenges facing the world. The situation has become particularly worrying as a result of the escalating global emergence of multi-drug resistant bacteria in the food chain, (6, 26). This systematic review and meta-analysis was undertaken to analyse the published literature reporting prevalence of ABR in food animals in Africa. Out of the 852 records found through databases searching, 20 records describing 17 different studies were included in the qualitative and quantitative analysis. The study proved that antibiotic-resistant foodborne pathogens are under-investigated on this

continent with reports from only 12 of the 54 African countries. The overall prevalence of ABR and MDR was 86% and 73%, respectively. These results could be attributed to agricultural practices being over-reliant on antibiotic use in Africa (4, 5, 27). This is consistent with a recent modelling study which suggested that a shift on agricultural practices from small to industrial scale in developing countries will lead to up to a third of the global increase in antibiotic consumption in food animals by 2030 (28).

At the animal species level, pigs and poultry were the leading population colonized or infected by antibiotic-resistant bacteria and multi-drug resistant bacteria in our study. The high prevalence of multi-drug resistant bacteria observed among poultry isolates, reflects the relatively large consumption of various antibiotics for their breeding, whereas the high rate of single resistance in pigs suggests that few classes of antibiotics are used to treat or prevent infections. Our findings are in accordance with that reported elsewhere in other developing countries such as Thailand and Vietnam (29-31). In Denmark, the first country to have implemented a surveillance program of ABR, as well as in the rest of the European Union (EU), the prevalence of antibiotic-resistant and multi-drug resistant bacteria in food animals was relatively lower (range: 4-65%) than in our study (26, 32-35). Differences in the level of resistance could be associated with long-term surveillance programs, infection prevention and control and biosecurity measures, antibiotic use monitoring and a ban on antimicrobials as growth-promoters for many years in food animals in these high-income European countries. It is probable that such measures and policies would also be appropriate to contain the emergence and spread of ABR in food animals in Africa.

A sound analysis and interpretation of our findings raised some fundamental questions: (i) As antibiotic-resistant and multi-drug resistant bacteria have been isolated from healthy and sick animals across the continent, what are the genetic elements (resistance and virulence genes) and clonal relatedness of these bacteria within and between both populations as well as within

and between countries? (ii) Are healthy animals becoming clinically ill following the asymptomatic carriage of antibiotic-resistant bacteria? (iii) What are the global health, societal and economic implications if these animal-originating strains succeed in spreading and undergoing host-adaptive micro-evolutionary changes that could lead to the emergence of new and more resistant/virulent strains in the human population? There was unfortunately limited data to answer these questions, thereby highlighting areas for future research.

Subgroup analysis per bacteria displayed high prevalence of ABR and MDR in *Salmonella* spp. A meta-analytical study carried out in Ethiopia, revealed a diverse prevalence of *Salmonella* spp., these being 7.07% in cattle, 8.41% in sheep, 9.01% in goats and 43.81% in pigs with antimicrobial susceptibility testing data not reported (36). Our results are also higher than that described in the Netherlands where 12 and 43% of ESBL-producing and fluoroquinolone resistant *Salmonella* spp. were observed in poultry respectively (37). This finding could be correlated to poor farming/slaughterhouse practices and sub-optimal hygiene measures.

The high prevalence of ABR and MDR in *E. coli* reported in our study is of further great concern as the involved antibiotic resistance genes (ARGs) may be carried on mobile genetic elements (MGEs). ABR and MDR in *E. coli* could be responsible of serious infections in humans on this continent, and serve as reservoirs of ARGs that could potentially be disseminated to other commensal and pathogenic bacteria such as *Salmonella* spp. which, in turn, may spread via the food chain (2, 4, 6). This therefore confirms that monitoring ABR in indicator bacteria such as *E. coli* in food animals and products thereof, is imperative to understand the evolution and transmission dynamics of antibiotic-resistant bacteria and ARGs in the food chain (26, 35, 38). Despite the fact that the nature and extent of antibiotic use in food animals were not ascertained, the high prevalence of ABR and MDR observed among *E. coli* and *Salmonella* spp. isolates is indicative of widespread use of antibiotics in farming practices both for prevention and treatment of infectious diseases in food animals in Africa.

Antibiotic-resistant and multi-drug resistant bacteria were highly prevalent in food animals at farms and abattoirs. Multi-drug resistant bacteria detected in food animals at farms (86.6%; 95%CIs, 69.1-94.9%, p=0.001) were directly representative of the antimicrobial use in these settings, whereas those detected at abattoirs (52.4%; 95%CIs, 23.2-79.9%, p=0.886) reflected bacteria surviving the processing stage, and therefore able to reach the consumer. This is a grave public health threat, as given the globalization of trade in food animals and food products as well as international travels, there are no geographic borders to contain the global dissemination of antibiotic-resistant and multi-drug resistant bacteria emerging in Africa.

A 95.9% and 61.9% prevalence of ABR and MDR were respectively described in Nigeria. However, this data was not comparable with other African countries due to insufficient reports. These findings should in no way implicate Nigeria as a country with a high prevalence of ABR, but rather that ABR in the food chain has been recognized as serious public health concern in this country. Our results suggest that more high-quality studies are needed on this continent, that a minimum package of criteria for monitoring systems needs to be established and implemented, and collaboration of various sectors and disciplines has to be reinforced as advocated by the WHO's Advisory Group on Integrated Surveillance of Antimicrobial Resistance (38).

Our study should be interpreted considering certain limitations. Information about antibiotic consumption in food animals in included African countries was not available due to the scarcity of data in these nations. The resistance to specific antimicrobials, particularly those regarded as 'critically important' in animal and human health and correlation with resistance genes and virulence factors could not be ascertained in this study, reflecting the limited laboratory capacity in Africa. In addition, it is probable that there is publication bias due to the poor quality of studies and lack of reporting with only 17 published reports from 12 out of 54 countries meeting our strict inclusion criteria, with those not included failing to report on ABR (Figure

5). A high level of heterogeneity associated with a number of factors including origin of animals, farming and slaughterhouse practices, study design and exposure to environmental aspects such as stress, was also observed. Whilst the inclusion criteria and subgroup analyses used in this study helped in reducing heterogeneity, we could not confidently assume that studies were fully comparable. It is further important to note that effects of all presumptive factors such as *Salmonella* and *Campylobacter* resistance epidemiology per serotype and species, could not be analysed due to scarcity of data and limited number of studies in some subgroups.

Conclusion

To the best of our knowledge, this is the first systematic review and meta-analysis of ABR in food animals in Africa. Given the findings of the review, it seems clear that ABR is substantively prevalent and poses a serious threat for food safety and security on this continent. Areas for future research concerning antibiotic-resistant and multi-drug resistant pathogens in food animals as etiological agents of infectious diseases in humans were identified. Data generated here yielded some interesting findings on the burden of ABR, that could be useful in developing measures to contain this threat from farm-to-plate in Africa. We therefore strongly recommend that the One Health approach and recommendations advocated by the WHO, OIE and FAO, be followed to restrict the use of antibiotics and thus ABR in animal and human health. In addition, sound sampling and laboratory analyses schemes, cooperation and good communication between sectors (agriculture, veterinary and public health sectors), qualitative and quantitative risk assessment for emerging and potential hazards, and sustainable political will and financial support across the food chain are required.

Declarations

Availability of data and materials

All data generated or analyzed during this study are included in this published article and its supplementary information files.

Competing interest

Professor Essack is a member of the Global Respiratory Infection Partnership and Global Analgesic Steering Committee sponsored by an unrestricted educational grant from Reckitt and Benckiser. All other authors declare that there is no competing financial interest.

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Author contributions

LF co-conceptualized the study, developed the protocol, searched the literature, screened titles abstracts and full-texts, extracted and summarized the data, performed quality assessment, statistical analysis and interpreted the results, prepared tables and figures, and drafted the manuscript. **DA** contributed to titles and abstracts screening, and undertook critical review of the manuscript. **RF** screened full-texts, performed quality assessment, statistical analysis, interpreted the results and prepared tables and figures. **SE** co-conceptualized the study, developed the protocol, screened titles and abstracts, and undertook critical review of the manuscript. All authors read and approved the final manuscript.

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Table 1. Characteristics of eligible studies

Country	Study population (N)	Clinical status	Type of sample (N)	Study site (N)	Bacteria (N)	R	MDR	Detection of genes		References
								ABR	Virulence	
STUDIES CONDUCTED ONLY IN FOOD ANIMALS										
Algeria	Poultry (300)	NR	Dropping (100), caeca (100), neck skins (100)	Farm (6) Abattoir (4)	*Campylobacter spp.* (263)	+	+	N/A	N/A	(51)
Cameroon	Poultry (150)	NR	Carcasses (150)	Abattoir (8)	*Salmonella spp.* (103)	+	+	N/A	N/A	(52)
Kenya	Cattle (80) Pig (105) Poultry (50)	NR	Carcasses (90), feces (95), cloacal swab (48) pharyngeal swab (12)	Abattoir (2)	*E. coli* (235)	+	+	+	N/A	(53, 54)
Nigeria	Poultry (100) Pig (100)	Healthy	Fecal samples (200)	Abattoirs (NR)	*E. coli* (162)	+	+	+	N/A	(55)
Nigeria	Poultry (400)	Healthy and sick	Cloacal swabs (201), tracheal swabs (196), internal organs (903)	Farms (100)	*E. coli (805)* *S. aureus (660)*	+	NR	+	N/A	(56)
Nigeria	Poultry (525)	NR	Internal organs (235), feces (140), cloacal swabs (150)	Abattoirs (3)	*Salmonella spp.* (41)	+	+	N/A	N/A	(57)
Nigeria	Pig (306)	NR	Fecal samples (306)	Farms (31)	*Salmonella spp.* (229)	+	+	+	N/A	(58)
South Africa	Pig (400)	NR	Fecal samples (400)	Farms (2)	*Enterococcus spp.* (320)	+	+	N/A	+	(59)
Tunisia	Poultry (136)	Healthy	Fecal samples (136)	Farms (36)	*E. coli* (67)	+	+	+	N/A	(60)
Uganda	Pig (465)	Sick and healthy	Fecal samples (465)	Farms (93)	*Salmonella spp.* (53)	+	+	N/A	N/A	(61)
Zambia	Cattle (376)	NR	Fecal samples (376)	Farms (104)	*E. coli* (371)	+	+	N/A	N/A	(62)
Zimbabwe	Poultry (14 165)	NR	Cloacal swabs (2 833)	Farms (NR)	*Salmonella spp.* (206)	+	+	N/A	N/A	(63)

N: Sample size; NR: Not reported; N/A: Not applicable; +: positive result; -: negative result; R: resistance; MDR: multidrug resistance; ABR: antibiotic resistance

Table 1. Characteristics of eligible studies (Continued)

Country	Study population (N)	Clinical status	Type of sample (N)	Study site (N)	Bacteria (N)	R	MDR	Detection of genes		References
								ABR	Virulence	
STUDIES CONDUCTED IN FOOD ANIMALS, FOOD PRODUCTS AND EXPOSED WORKERS										
Ethiopia	Cow (195) Food products (195) Human (22)	NR	Fecal (195) Milk samples (195) Human stool (22)	Farms (23)	*Salmonella spp.* (24)	+	+	N/A	N/A	(32)
Ghana	Goat (51) Sheep (44) Pig (12) Poultry (103) Human (395)	NR	Animal feces (210) Human stool (58)	Farms (NR)	*E. coli* (178)	+	+	N/A	N/A	(33)
STUDIES CONDUCTED IN FOOD ANIMALS AND FOOD PRODUCTS										
Nigeria	Cattle (407) Sheep (168) Goat (281) Pig (409) Meats (868)	NR	Fecal samples (1 265) Pork (200) Mutton (450) Beef (448) Goat-meat (175)	Farms (NR) Abattoirs (NR) Meat markets (NR)	*E. coli* (154)	+	+	N/A	+	(37)
	Cattle (800)	NR	Carcasses (800) Meats (250)	Abattoirs (4)	*E. coli* (227)	+	+	N/A	N/A	(64)
Senegal	Poultry (250)	NR	Carcasses (250)	Abattoirs (80)	*Campylobacter spp.* (205)	+	NR	+	N/A	(34-36)

N: Sample size; NR: Not reported; N/A: Not applicable; +: positive result; -: negative result; R: resistance; MDR: multidrug resistance; ABR: antibiotic resistance

Table 2. Summary of antibiotic resistance profiles across included studies

Study	Guidelines	Bacterial species	AST methods	No. of strains	Resistant isolates (%)	MDR Isolates (%)	Antibiotic resistance profiles of bacterial species isolated (%)																					
							Beta-lactams beta-lactams inhibitors							Aminoglycosides					Macrolides	Fluoroquinolones			Tetracyclines	Phenicols	Furazolidone	Sulphonamides	
							Ampicillin	Amoxicillin	Amoxicillin-clavulanate	Cefuroxime	Cefoxitin	Cefotaxime	Ceftriaxone	Gentamicin	Streptomycin	Kanamycin	Amikacin	Neomycin	Erythromycin	Nalidixic acid	Ciprofloxacin	Enrofloxacin	Tetracycline	Chloramphenicol	Nitrofurantoin	Sulphonamide	Trimethoprim-sulfamethoxazole
Messad et al. 2014 (51)	CA-SFM	*Campylobacter jejuni and coli*	Disk diffusion	263	100	100	75.3		46.8					0					21.7	100	83.7		83.7	0			
Wouafo et al. 2010 (52)	CA-SFM	*Salmonella enterica*	Disk diffusion and agar dilution	103	89.3	39.8		13.6	1		0	0		0	44.7					34	0		84.5	1		19.4	11.6
Addis et al. 2011 (32)	NCCLS	*Salmonella enterica*	Disk diffusion	24	100	83.3	100						0	15.5	55.6	31.1					0		36.6	7.8	35.6		0
Donkor et al. 2012 (33)	NCCLS	*E. coli*	Disk diffusion	187	NR	91.6	90.35			49.5		0		84.5			56						91	82.5			62
Kikuvi et al. 2006 (53)	CLSI	*E. coli*	Disk diffusion	235	65.5	37.9	40.6							1	36	11.6							28.6	4			22
Fortini et al. 2011 (55)	EUCAST	*E. coli*	Disk diffusion	162	100	NR	100							NR	NR	NR				55	55		NR	NR		NR	
Ojo et al. 2010 (37)	CLSI	*E. coli*	Broth microdilution	154	96.2	69.5	82.5								50.3			24		37.7	22.1	25.3	75.3	42.9			

Olatoye 2010 (64)	NCCLS	*E. coli*	Disk diffusion	116	100	100	72.9			65.7				11.4						35.7			91.4	72.9	72.9		44.3
Mamza et al. 2010 (56)	NCCLS	*E. coli*	Broth microdilution	805	11		13.5	13.5						13.5							13.5		14				
		S. aureus		660	8.8	100	24.3												24.3		14		24				
Raufu et al. 2009 (58)	EUCAST CLSI	*Salmonella enterica*	Broth microdilution	41	58	NR	0	0						0	4.8			0	4.6	56	56			2.4		0	0
Fashae et al. 2014 (57)	CLSI	*Salmonella enterica*	Disk diffusion	224	NR	22.8								0.9	18.3					15.2	0.9		22.8	11.6		12.9	
Cardinale et al.2003 (34)	NR	*Campylobacter jejuni and coli*	E-test method	205	34	NA															73.5						
Mnif et al. 2012 (60)	EUCAST	*E. coli*	Disk diffusion	67	100	100								6			6			89.5			94				73.1
Ikwap et al. 2014 (61)	CLSI	*Salmonella spp.*	Broth microdilution	53	36	57.9	3.7					0		0	15	1.8				0	0		3.7	5.6		43.4	
Mainda et al. 2015 (62)	EUCAST	*E. coli*	Disk diffusion	371	16.4	NR	6.5							2							1.5		11.2				4
Makaya et al.2015 (63)	CLSI	*Salmonella spp.*	Disk diffusion	206	26	12.1	0	6.8							3.9			0	2.4			3.4	9.2				0
Iweriebor et al. 2015 (59)	CLSI	*Enterococcus spp.*	Disk diffusion	320	100	93.8		39.1							100		77.3	92	98.7		69						

***AST**: Antimicrobial Susceptibility Testing; **NR:** Not Reported; **NA**: Not Applicable; **CA-SFM:** Antibiogram Committee of the French Society of Microbiology; **EUCAST:** European Committee on Antimicrobial Susceptibility; **CLSI:** Clinical and Laboratory Standards Institute; **NCCLS**: National Committee on Clinical Laboratory Standards*

Antibiotic resistance rates (%):

0; >0-10; >10-20; >20-30; >30-40; >40-50; >50-60; >60-70; >70-80; > 80-90; >90-100

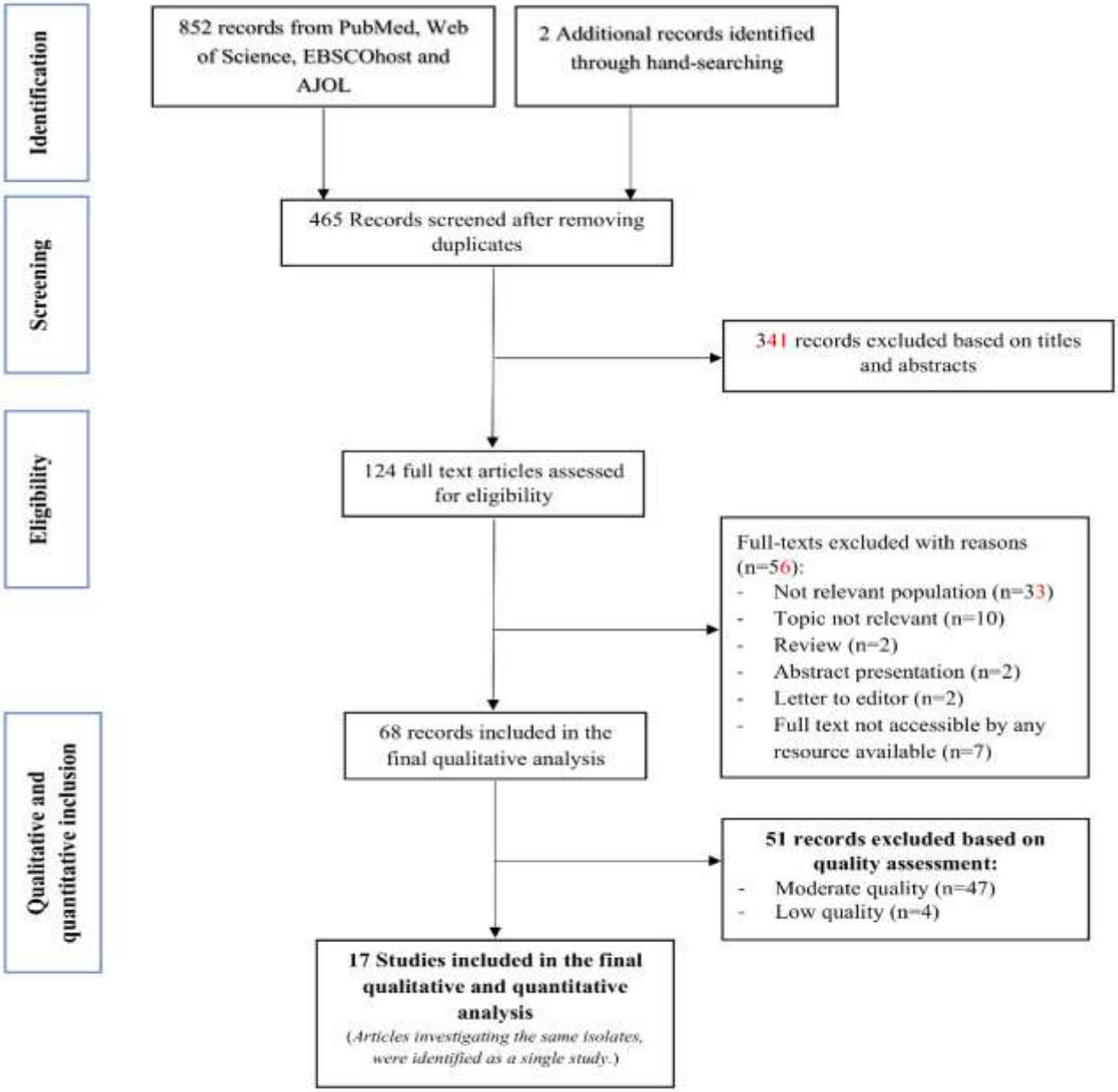


Figure 1. Flow-chart demonstrating the identification and inclusion process for articles included in this systematic review and meta-analysis

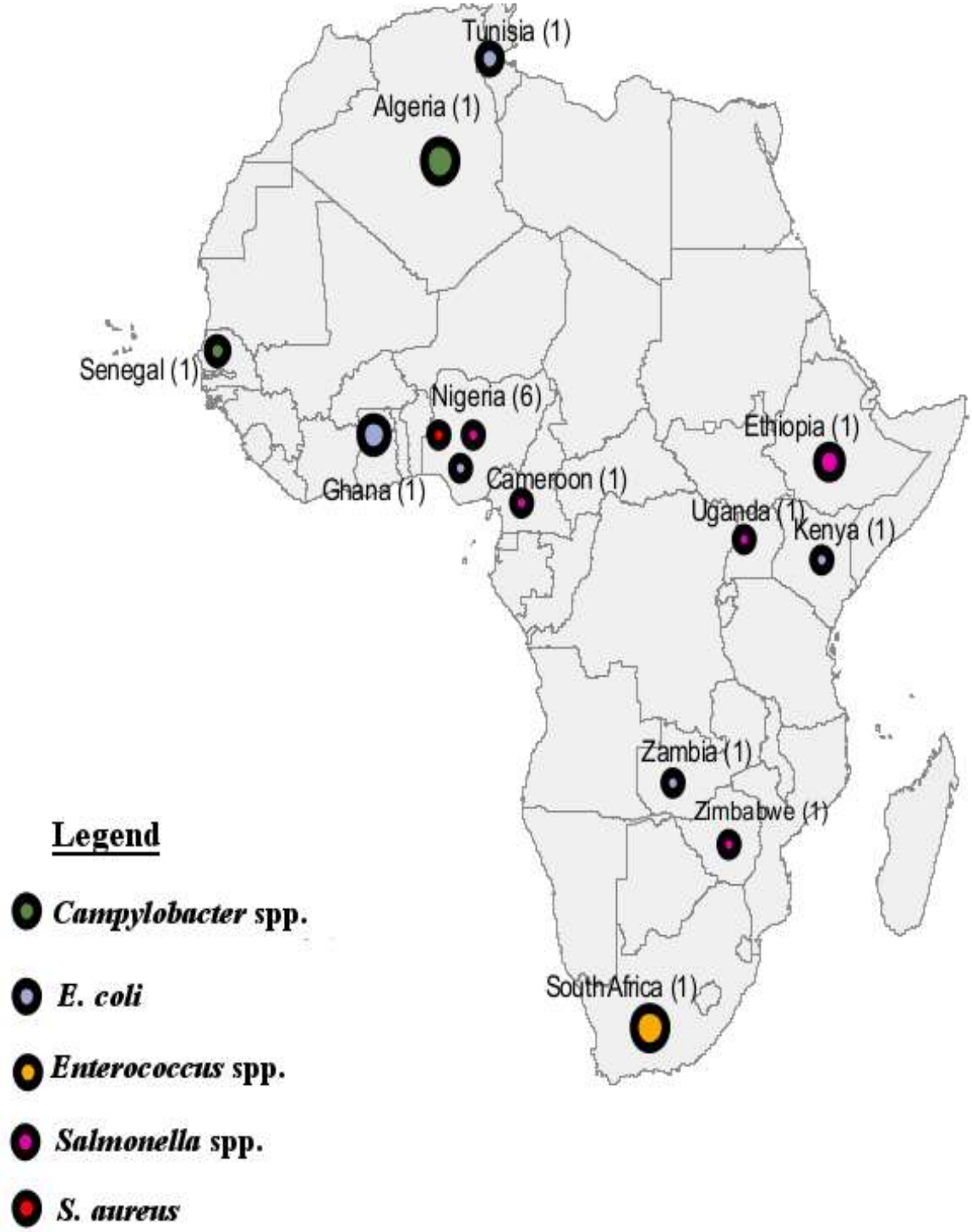


Figure 2. Graphical representation of antibiotic resistant bacteria reported in food animals in Africa. Each bacterium is annotated with a coloured circle. The number of studies carried out in each country is also indicated. **Map was created using ArcGIS® and** ArcMap ™ **software version 10.3 (Esri, CA, USA).**

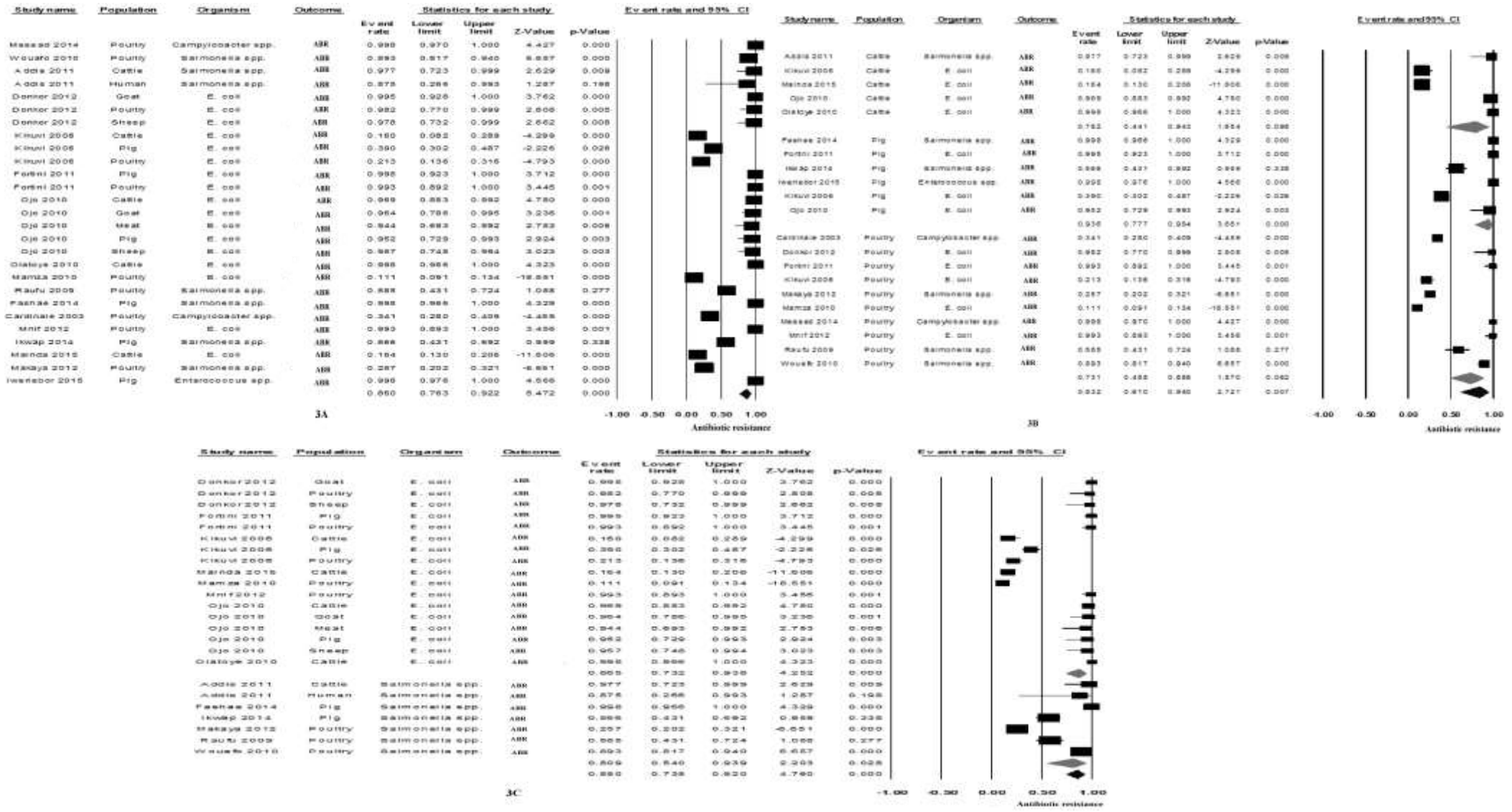
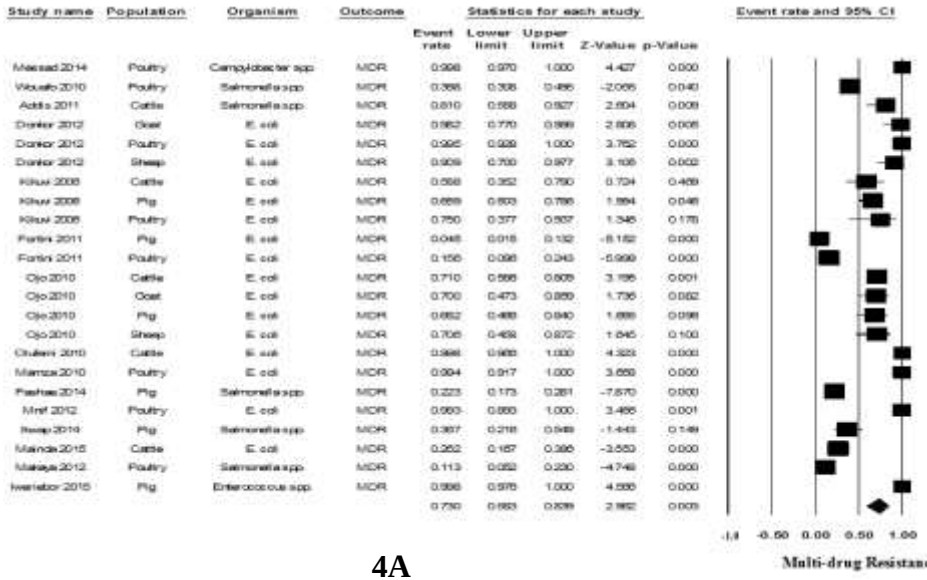
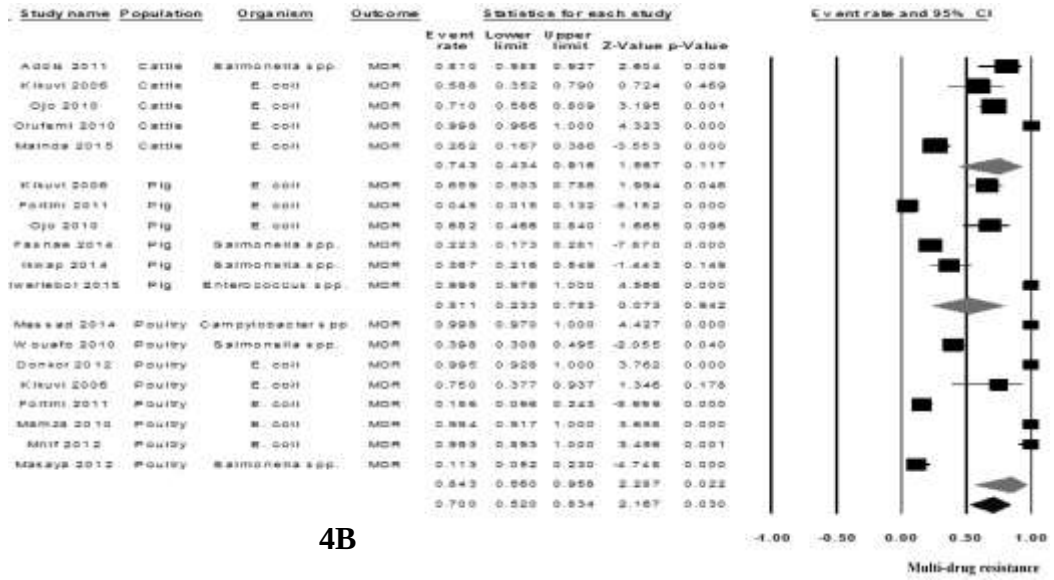


Figure 3. Meta-analyses of overall rate and sub-group analyses of bacterial resistance. Each box represents the value of each included study while the diamond represents the overall and summary effect for each sub-group. The line in the middle is the line of null effect, the right-hand side of the line is in favour of resistance whereas the left is in favour of susceptibility. **3A** Pooled random-effects estimate of overall (95% CI) bacterial resistance in selected studies; **3B** Sub-group analysis per population; **3C** Sub-group analysis per bacteria.



4A



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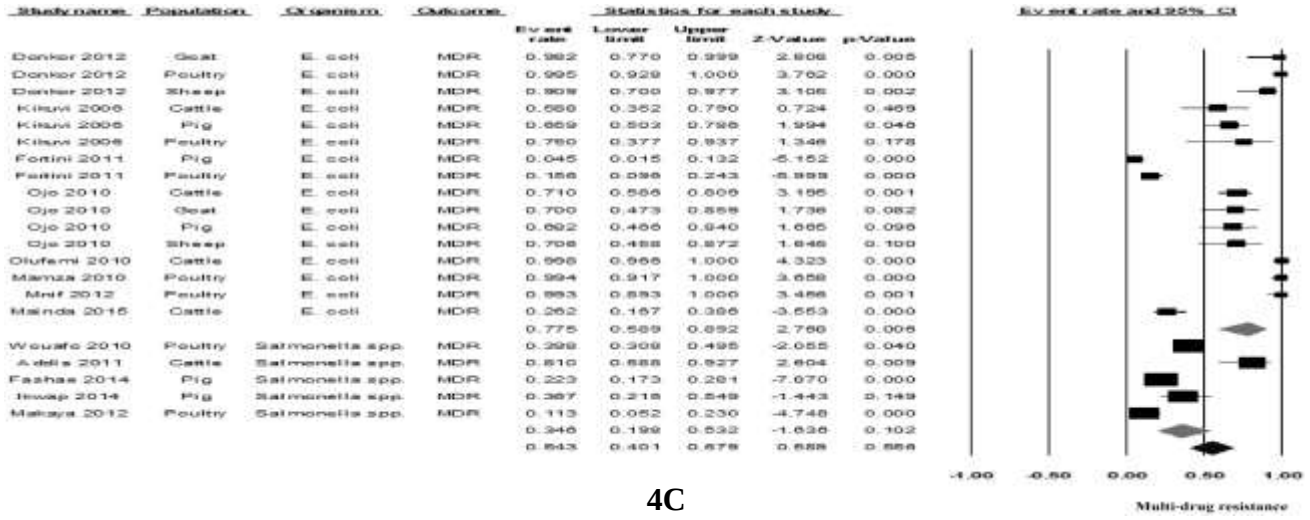


4C

Figure 4. Meta-analyses of overall rate and sub-group analyses of bacterial multi-drug resistance. Each box represents the value of each included study while the diamond represents the overall and summary effect for each sub-group. The line in the middle is the line of null effect, the right-hand side of the line is in favour of resistance whereas the left is in favour of susceptibility. **4A** Pooled random-effects estimate of overall (95% CI) bacterial resistance in selected studies; **4B** Sub-group analysis per population; **4C** Sub-group analysis per bacteria.

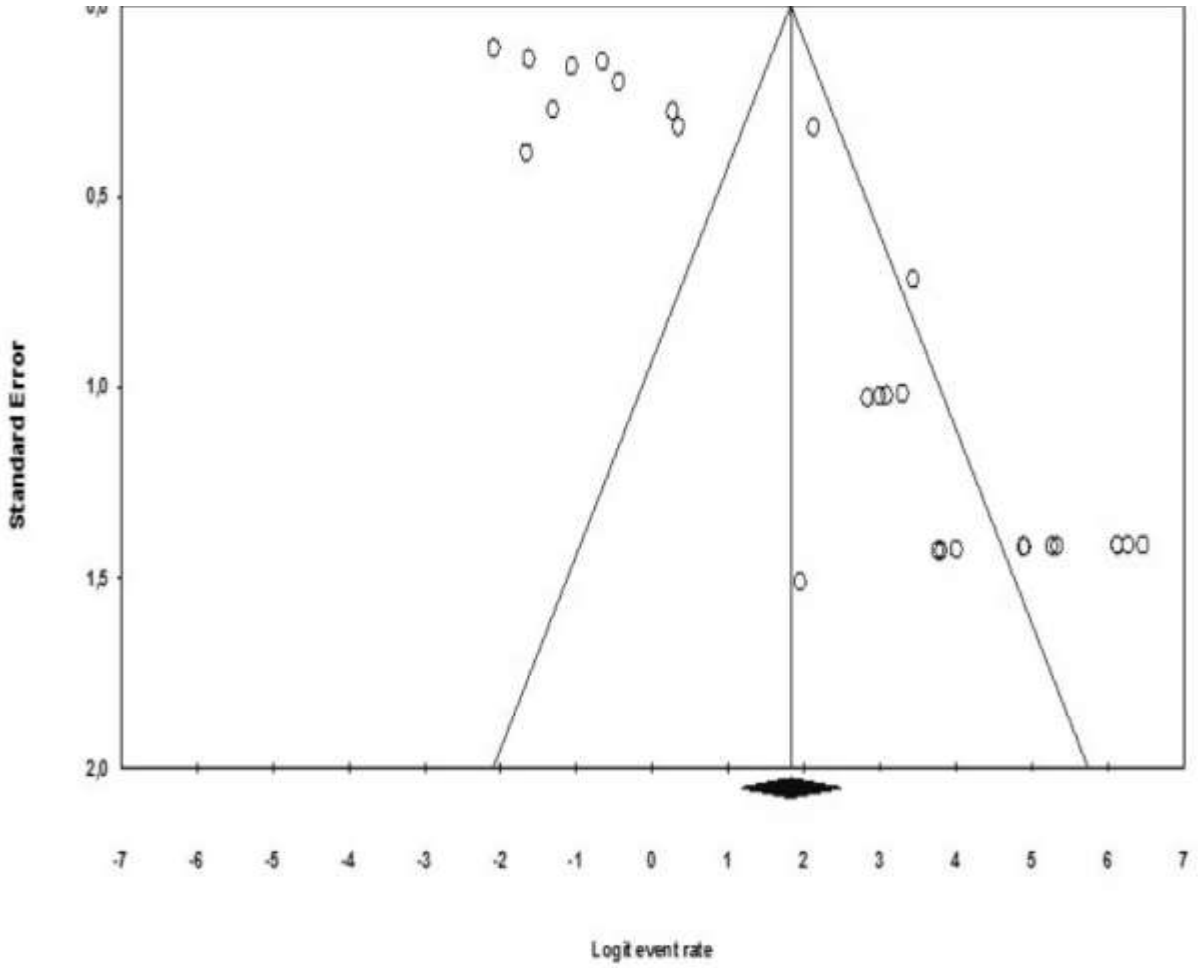


Figure 5. Bias assessment (Funnel) plot with 95% confidence limits for studies included in the analysis

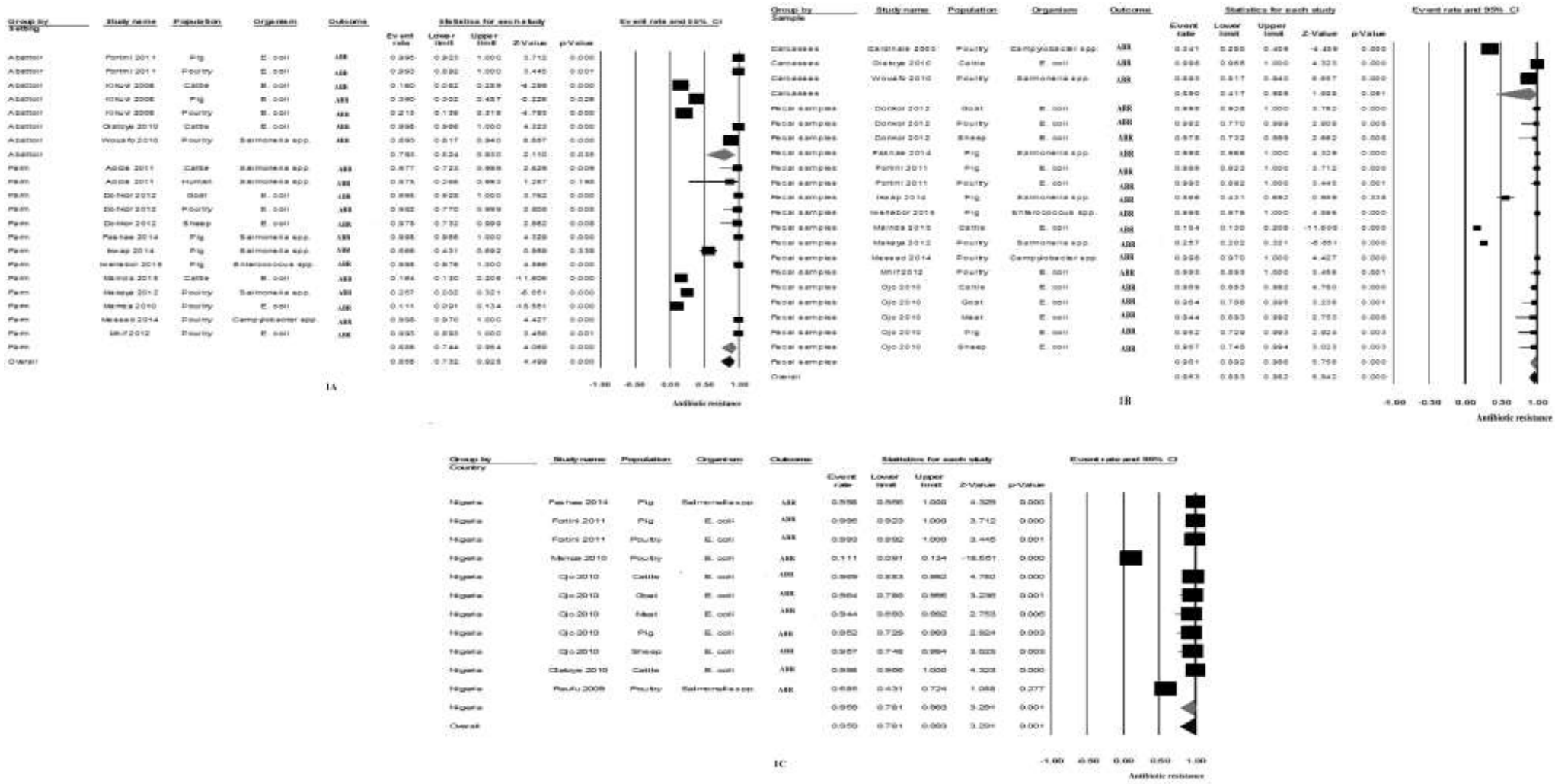
Supplementary material

Supplementary Table 1. PRISMA Checklist

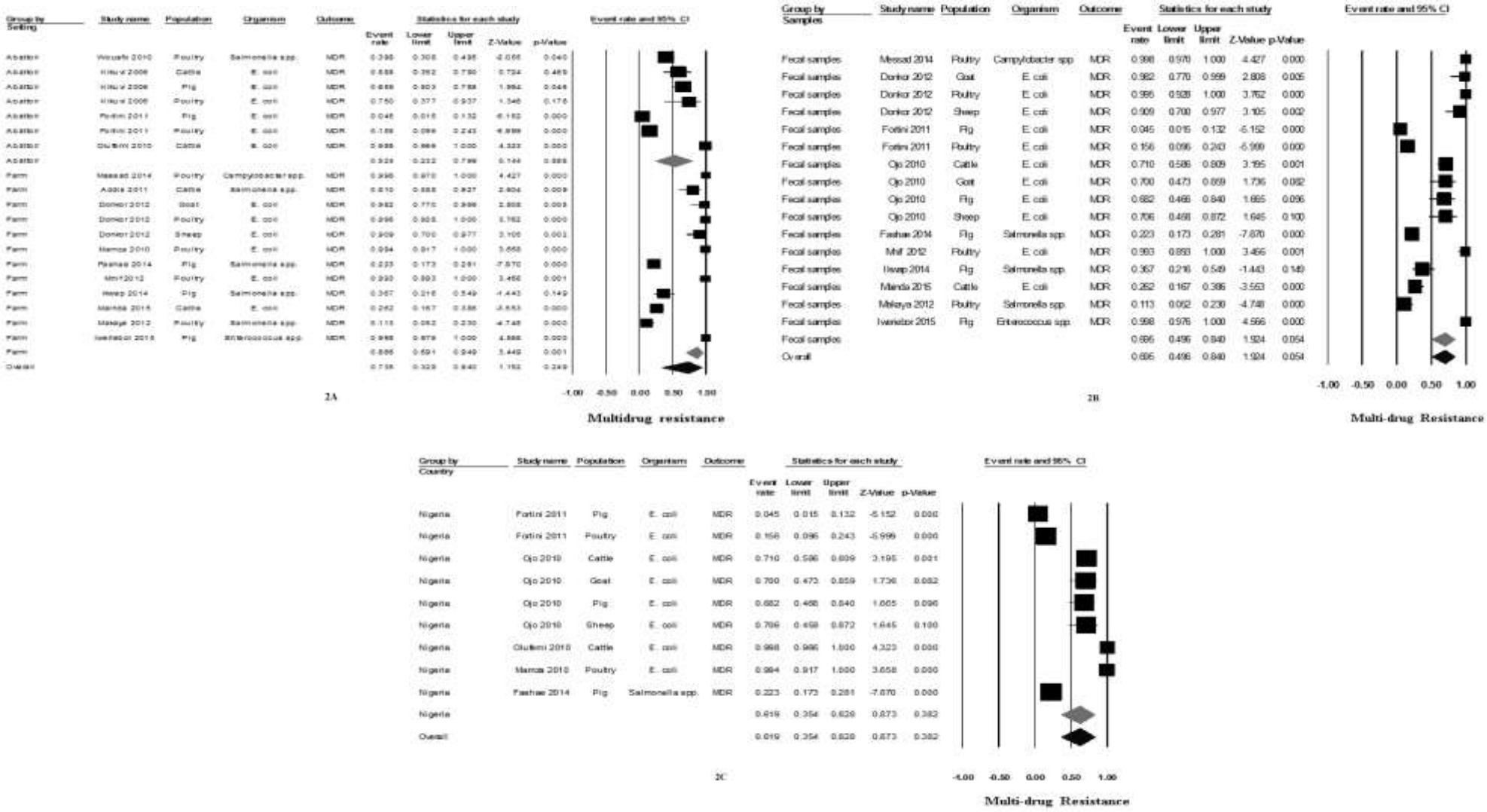
Section/topic	#	Checklist item	Reported on page #
TITLE			
Title	1	Identify the report as a systematic review, meta-analysis, or both.	1
ABSTRACT			
Structured summary	2	Provide a structured summary including, as applicable: background; objectives; data sources; study eligibility criteria, participants, and interventions; study appraisal and synthesis methods; results; limitations; conclusions and implications of key findings; systematic review registration number.	2
INTRODUCTION			
Rationale	3	Describe the rationale for the review in the context of what is already known.	3-5
Objectives	4	Provide an explicit statement of questions being addressed with reference to participants, interventions, comparisons, outcomes, and study design (PICOS).	5
METHODS			
Protocol and registration	5	Indicate if a review protocol exists, if and where it can be accessed (e.g., Web address), and, if available, provide registration information including registration number.	NA
Eligibility criteria	6	Specify study characteristics (e.g., PICOS, length of follow-up) and report characteristics (e.g., years considered, language, publication status) used as criteria for eligibility, giving rationale.	7, Suppl Table 3
Information sources	7	Describe all information sources (e.g., databases with dates of coverage, contact with study authors to identify additional studies) in the search and date last searched.	7
Search	8	Present full electronic search strategy for at least one database, including any limits used, such that it could be repeated.	6, Suppl Table 2
Study selection	9	State the process for selecting studies (i.e., screening, eligibility, included in systematic review, and, if applicable, included in the meta-analysis).	6-8, Figure 1.
Data collection process	10	Describe method of data extraction from reports (e.g., piloted forms, independently, in duplicate) and any processes for obtaining and confirming data from investigators.	6-8

Data items	11	List and define all variables for which data were sought (e.g., PICOS, funding sources) and any assumptions and simplifications made.	5-8, Suppl Table 2
Risk of bias in individual studies	12	Describe methods used for assessing risk of bias of individual studies (including specification of whether this was done at the study or outcome level), and how this information is to be used in any data synthesis.	8-9
Summary measures	13	State the principal summary measures (e.g., risk ratio, difference in means).	9-10
Synthesis of results	14	Describe the methods of handling data and combining results of studies, if done, including measures of consistency (e.g., I^2) for each meta-analysis.	9-10
Risk of bias across studies	15	Specify any assessment of risk of bias that may affect the cumulative evidence (e.g., publication bias, selective reporting within studies).	Figure. 5
Additional analyses	16	Describe methods of additional analyses (e.g., sensitivity or subgroup analyses, meta-regression), if done, indicating which were pre-specified.	9-10
RESULTS			
Study selection	17	Give numbers of studies screened, assessed for eligibility, and included in the review, with reasons for exclusions at each stage, ideally with a flow diagram.	10-11, Fig 1.
Study characteristics	18	For each study, present characteristics for which data were extracted (e.g., study size, PICOS, follow-up period) and provide the citations.	10-11, Table 1
Risk of bias within studies	19	Present data on risk of bias of each study and, if available, any outcome level assessment (see item 12).	NA
Results of individual studies	20	For all outcomes considered (benefits or harms), present, for each study: (a) simple summary data for each intervention group (b) effect estimates and confidence intervals, ideally with a forest plot.	11-13, Figures 3-4;
Synthesis of results	21	Present results of each meta-analysis done, including confidence intervals and measures of consistency.	11-13; Figures 3-4
Risk of bias across studies	22	Present results of any assessment of risk of bias across studies (see Item 15).	Figure 5
Additional analysis	23	Give results of additional analyses, if done (e.g., sensitivity or subgroup analyses, meta-regression [see Item 16]).	11-13; Figures 3-4
DISCUSSION			

Summary of evidence	24	Summarize the main findings including the strength of evidence for each main outcome; consider their relevance to key groups (e.g., healthcare providers, users, and policy makers).	13-19
Limitations	25	Discuss limitations at study and outcome level (e.g., risk of bias), and at review-level (e.g., incomplete retrieval of identified research, reporting bias).	18-19
Conclusions	26	Provide a general interpretation of the results in the context of other evidence, and implications for future research.	19-20
FUNDING			
Funding	27	Describe sources of funding for the systematic review and other support (e.g., supply of data); role of funders for the systematic review.	20-21



Supplementary figure 1. Subgroup analyses of bacterial antibiotic resistance. 1A Subgroup analysis per settings; **1B** Subgroup analysis per sample; **1C** Subgroup analysis per country.



Supplementary figure 2. Subgroup analyses of bacterial multi-drug resistance. 2A Subgroup analysis per settings; **2B** Subgroup analysis per sample; **2C** Subgroup analysis per country.

CHAPTER 4

Article III. Emergence and Spread of Extended Spectrum Beta-Lactamase (ESBL)-Producing *Enterobacteriaceae* in Pigs and Exposed Workers: A Multicentre Comparative Study Between Cameroon and South Africa

Emergence and Spread of Extended Spectrum Beta-Lactamase (ESBL)-Producing *Enterobacteriaceae* in Pigs and Exposed Workers: A Multicentre Comparative Study Between Cameroon and South Africa[3]

Author contributions

- Luria Leslie Founou, as the principal investigator, co-conceptualized the study, undertook sample collection, laboratory and statistical analyses, prepared tables and figures, and drafted the manuscript.
- Raspail Carrel Founou undertook sample collection, laboratory analyses, contributed to data analysis and vetting of the results, and reviewed the manuscript.
- Noyise Ntshobeni participated in the genomic extraction and fingerprinting analysis.
- Usha Govinden contributed materials and reagents for sample collection and primary laboratory analyses, and took part in the design of the study.
- Linda Antoinette Bester contributed materials and reagents for sample collection and primary laboratory analyses, and took part in the design of the study.
- Hafizah Yousuf Chenia contributed materials and reagents for and vetting of genomic fingerprinting results.
- Cyrille Finyom Djoko contributed materials, equipment and reagents, took part in the design of the study, coordinated the field implementation in Cameroon and reviewed the manuscript.
- Sabiha Yusuf Essack, as principal supervisor, co-conceptualized the study, contributed to vetting of the results and undertook critical revision of the manuscript.

Objectives met: This original research article reports and compare the prevalence, risk factors as well as clonal relatedness of ESBL-PE circulating in Cameroonian and South African slaughterhouses and meets objectives Three, Four, Five, Six, Seven, Nine and Ten.

[3] This paper has been submitted to PloS One and is currently under review.

Emergence and Spread of Extended Spectrum Beta-Lactamase - Producing *Enterobacteriaceae* (ESBL-PE) in Pigs and Exposed Workers: A Multicentre Comparative Study Between Cameroon and South Africa

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Running title: Emergence and spread of ESBL-producing *Enterobacteriaceae* in the food chain

Keywords: Antibiotic resistance, *Enterobacteriaceae*, ESBL, food chain, One Health approach

Abstract

Background: Extended spectrum beta-lactamase-producing *Enterobacteriaceae* (ESBL-PE) represent a significant public health concern globally and are recognized by the World Health Organization as pathogens of critical priority. However, the prevalence of ESBL-PE in food animals and humans across the farm-to-plate continuum is yet to be elucidated in Sub-Saharan countries including Cameroon and South Africa. This study determined the risk factors, carriage, antimicrobial resistance profiles and genetic relatedness of extended spectrum beta-lactamase producing *Enterobacteriaceae* (ESBL-PE) among pigs and exposed workers in Cameroon and South Africa.

Methods: The study was carried out from March to October 2016. ESBL-PE from pooled samples of 432 pigs and nasal and hand swabs of 82 humans were confirmed with VITEK 2 system. Genomic fingerprinting was performed by ERIC-PCR. Univariate and multivariate logistic regression analyses were performed to identify risk factors for ESBL-PE carriage in humans from a questionnaire survey amongst slaughterhouse workers.

Results: ESBL-PE prevalence in animal samples from Cameroon were higher than for South Africa and ESBL-PE carriage was observed in Cameroonian workers only. Nasal ESBL-PE colonization was statistically significantly associated with hand ESBL-PE (21.95% vs 91.67%; p=0.000; OR=39.11; 95% CI 2.02-755.72; p=0.015). Low level of education, insufficient monthly income, previous hospitalization, recent antibiotic use, inadequate handwashing, lack of training and contact with poultry were the risk factors identified.

Conclusion: The study highlights the threat posed by ESBL-PE in the food chain and recommends the implementation of effective strategies for antibiotic resistance containment in both countries.

4.1.Introduction

Antibiotic resistance (ABR) is a global public health concern, with grave health and socio-economic implications (1, 2). It is a natural evolutionary process emerging wherever antibiotics are used including in hospitals, communities, veterinary health, agriculture, aquaculture, and the environment. ABR is exacerbated by the fact that very few new antibiotics have lately been introduced by pharmaceutical industries. ABR can occur in saprophytic, commensal and pathogenic bacteria present in (food) animals, humans, and the environment (1, 2). Food animals are considered important reservoirs of antibiotic-resistant bacteria (ARB) that spread directly and indirectly in the farm-to-plate continuum (1, 2, 3). Direct contact occurs due to exposure of humans to animals and their biological substances such as urine, blood, feces, saliva, milk, and semen, and this enhances the rapid and easy dissemination of ARB from person-to-person (1, 2, 3). Whilst this transmission mode did not originally appear as a danger at population-health level, it is now widely recognized that exposed workers and their families provide a likely route for the entry of ARB and antibiotic resistance genes (ARGs) into the community and health care settings, where potential for subsequent genetic exchanges and emergence of resistance mechanisms is high (3, 4). Humans might also be indirectly exposed to ARB and ARGs by contact with or consumption of contaminated food products such as meat, eggs, milk and other dairy products. Farmers, abattoir workers, veterinarians and food handlers as well as consumers are thus at great risk of acquiring ARB and ARGs if these emerge at any step in the farm-to-plate continuum (1, 3).

The family *Enterobacteriaceae* is a heterogeneous group of rod-shaped, Gram-negative bacteria, fermenting glucose, usually motile and facultative anaerobes, with the majority of genera being natural residents of gastrointestinal tract of animals, humans and some of these bacteria can be found in the environment (1, 5, 6). They are causative agents of several clinical diseases, ranging from intestinal infections such as typhoid fever and dysentery, to extra-intestinal infections such as urinary tract infections, pneumonia, peritonitis, septicaemia, wound infections and meningitis, thereby constituting amongst the most clinically important group of bacteria in human and animal health (1, 7). The extensive use of beta-lactam antibiotics, especially third and fourth generation cephalosporins, has led to the emergence of extended spectrum beta-lactamase-producing *Enterobacteriaceae* (ESBL-PE), that are able to inactivate beta-lactam antibiotics by hydrolysis and opening of the beta-lactam ring. ESBL-PE are resistant to the majority of beta-lactam antibiotics with a few exceptions of carbapenems, cephamycins and beta-lactamase inhibitors that are hydrolysed by other enzymes such as Amp-

C enzymes, carbapenemases and inhibitor-resistant enzymes, respectively. They can also exhibit additional resistance to several classes of antibiotics, including aminoglycosides, fluoroquinolones, potentiated sulfonamides, cyclins and phenicols, which lead to multi-drug resistance (MDR) and limit effective therapeutic options for treatment of infectious diseases (8).

Several studies have detected ESBL-PE in food animals, especially pigs, poultry and cattle, and food products throughout the world and their transmission from livestock to humans in the farm-to-plate continuum has been evidenced (5, 6, 8-10). ESBL-PE can easily contaminate humans, food animals and products during an assortment of procedures occurring in the farm-to-plate continuum, including farming (directly via person-to-person contact and indirectly via food, water, soil, air), slaughtering processes (stunning, cutting, scalding, evisceration), storage, food transportation and consumption, with increased transmission risks to the global population, as there are no geographic boundaries to contain their worldwide dissemination (3, 8, 9). ESBL-PE thus represent a significant public health concern globally and have recently been classified by the World Health Organization as pathogens of critical priority (11).

However, the prevalence of ESBL-PE in food animals and humans across the farm to fork continuum is yet to be elucidated in Sub-Saharan countries including Cameroon and South Africa. It is therefore imperative to understand the epidemiology and determine the burden of ESBL-PE in food animals in order to highlight the threat posed by these resistant bacteria and provide evidence for decision-makers to implement effective prevention and containment measures of ABR in Cameroon and South Africa. The objectives of this study were therefore to determine the carriage, antimicrobial resistance profiles and genetic relatedness of ESBL-PE among pigs and exposed workers, and delineate risk factors of ESBL-PE carriage in humans in Cameroon and South Africa.

4.2.Materials and methods

4.2.1. Study design and study sites

A cross-sectional, multicentre study was conducted between March and October, 2016 in three slaughterhouses/markets in Cameroon and two abattoirs in South Africa, that were encoded for ethical reasons as SH001, SH002, SH003 and SH004 and SH005, respectively. All slaughterhouses were visited at different time points to allow better representativeness of the pig population.

In Cameroon, samples were randomly taken at the two biggest pig abattoirs (SH001 and SH002) of Yaoundé, where more than 80% of pigs are slaughtered and with annual productivity scale ranging from 25 000 to 45 000 pigs (12). Samples from an auxiliary abattoir (SH003)

were additionally collected to ensure a geographically representative distribution since animals originating from surrounding farms of Yaoundé are typically underrepresented in these slaughterhouses. These slaughterhouse/markets were located in three geographically distinct districts of Yaoundé. In South Africa, samples were taken at two of the biggest and most productive abattoirs of the province of KwaZulu-Natal (SH004 and SH005), with annual productivity ranging from 120 000 to 150 000 pigs.

4.2.2. Ethical considerations

Ethical approvals from the Biomedical Research Ethics Committee (**Ref. BE365/15**) and Animal Research Ethics Committee (**Ref. AREC/091/015D**) of the University of KwaZulu-Natal as well as from the National Ethics Committee for Research in Human Health of Cameroon (**Ref. 2016/01/684/CE/CNERSH/SP**) were obtained prior the implementation of the study. Ministerial approvals from the Cameroonian Ministry of Livestock, Fisheries and Animal Industries (**Ref. 061/L/MINEPIA/SG/DREPIA/CE**) and Ministry of Scientific Research and Innovation (**Ref. 015/MINRESI/B00/C00/C10/C14**) were also obtained.

4.2.3. Sampling procedure and questionnaire

a. Procedures for animal data

A randomized sampling method of apparently healthy and freshly slaughtered/stunned pigs was undertaken in both countries. Nasal (inner cavity of both anterior nares) and rectal swabs of pigs were collected using Amies swabs without charcoal (Copan Italia Spa, Brescia, Italia). Overall, 864 swabs (432 nasal and 432 rectal) were collected from 432 pigs in Cameroon (n=216) and South Africa (n=216), with the number of specimens from each slaughterhouse (SH001, n=129; SH002, n=57; SH003, n=30; SH004, n=120; SH005, n=96) being proportionally calculated to be representative of the number of pigs slaughtered annually per site.

b. Procedures for human subjects

All abattoir workers older than 21 years old and willing to participate were included in the study after oral and written informed consent. Upon this step, participants were asked to complete a questionnaire addressing socio-demographic and clinical information, as well as other potential risk factors associated with ESBL-PE colonization and dissemination. Both anterior nares and hands (between fingers for each right and left hand) were collected with Amies media and all samples were processed within 4 h after collection.

4.2.4. Laboratory analysis

For microbiological analysis, three individual pig samples were pooled per slaughterhouse and according to the gender, specimen type and area of breeding to yield 144 nasal and 144 rectal pools representing 432 original nasal and rectal samples respectively collected from 432 pigs. A total of 288 swabs from the 144 nasal and 144 rectal pools constituted the final pig sample. Pooled samples of pigs and human swabs were streaked onto an in-house screening MacConkey agar supplemented with 2 mg/L cefotaxime (MCA+CTX) and incubated for 18-24 h at 37°C for ESBL-PE screening. Presumptive ESBL-PE were phenotypically confirmed with Vitek® 2 System (BioMérieux, Marcy l'Etoile, France).

a. ESBL detection, species identification and antimicrobial susceptibility testing

Each colony growing on MCA+CTX and with a unique morphotype was screened for the production of ESBLs through the standard double disk synergy test (DDST), using cefotaxime and ceftazidime, alone and in association with clavulanic acid as recommended by the Clinical Laboratory and Standards Institute (CLSI). An increase in size of the inhibition zone of more than 5 mm in the presence of clavulanic acid was regarded as positive for ESBL production (13). Upon this two-step screening, a representative subset of isolates, underwent phenotypic identification via Vitek® 2 System (BioMérieuX, Marcy l'Etoile, France).

Using an 18-24 h fresh culture, 1 to 2 colonies were mixed with 3.70 ml of sterile saline solution, resulting into a 0.5 turbidity on the McFarland scale. The minimum inhibitory concentrations (MIC) of ampicillin, amoxicillin + clavulanic acid, cefuroxime, cefuroxime acetyl, cefoxitin, cefotaxime, ceftazidime, cefepime, ertapenem, imipenem, meropenem, gentamicin, amikacin, ciprofloxacin, tigecycline, nitrofurantoin, piperacillin/tazobactam, colistin and trimethoprim-sulfamethoxazole, were determined by broth microdilution method using Vitek® 2 System (BioMérieuX, Marcy l'Etoile, France) and Vitek® 2 Gram Negative Susceptibility card (AST-N255) (BioMérieux, Marcy l'Etoile, France). The results were interpreted according to the CLSI guidelines (13) with the exception of colistin, amoxicillin + clavulanic acid, piperacillin/tazobactam and amikacin that were based on EUCAST breakpoints (14) with *E. coli* ATCC 25922 being used as the control.

b. Genomic Extraction

Genomic DNA was extracted from a subset of ESBL-PE strains selected on the basis of their antimicrobial resistance profiles. Samples were cultured in 3 ml of Tryptone soya broth with moderate shaking for 18 h at 37ºC in normal atmosphere. After incubation 1.5 ml of broth was centrifuged at 12,000 g for 10 min and the pellet was prepared for genetic analysis using the Thermo Scientific® GeneJet Genomic DNA purification kit (Thermo Fisher Scientific, South

Africa) according to the manufacturer's instructions. DNA templates were stored at -20°C until used.

c. Genotypic relatedness determination of ESBL-producing *Escherichia coli* strains

Genomic DNA was extracted using the Thermo Scientific® GeneJet Genomic DNA purification kit (Thermo Fisher Scientific, South Africa) according to the manufacturer's instructions. ERIC-PCR was performed with primers ERIC 1 5'-ATG TAA GCT CCT GGG GAT TCA C-3' and ERIC2 5'-AAG TAA GTG ACT GGG GTG AGC G-3' (8). Reactions were carried out in a 10 µl final solution containing 0.1 µl of each primer (100 µM), 5 µl Dream*Taq* Green Polymerase Master Mix 2× (Thermo Fisher Scientific, South Africa), 2.8 µl nuclease free water and 2 µl DNA template and run in an Applied Biosystems 2720 programmable thermal cycler (Thermo Fisher Scientific, South Africa) with the following protocol: initial denaturation at 94°C for 3 min, 30 cycles consisting of a denaturation step at 94°C for 30 s, annealing at 50°C for 1 min, extension at 65°C for 8 min, a final extension step at 65°C for 16 min and final storage at 4°C. ERIC profiles were digitized for analysis using Bionumerics software (version 7.6, Applied Maths, TX, USA). The similarity between each strain was determined from the homology matrix using Dice coefficient and dendrograms constructed using the algorithm Unweighted Pair-Group Method (UPGMA).

d. Data analysis

Data was coded and entered into Excel spreadsheet (Microsoft Office 2016) and Epi Info (version 7.2, CDC, Atlanta, GA, USA), and analysed STATA (version 14.0, STATA Corporation, TX, USA). A data set was created for individual human results and, aggregated animal and abattoir data. Abattoirs were classified as ESBL-positive if an ESBL-PE was detected from at least one pooled sample (nasal or rectal). Likewise, each human was categorized as carrier or non-carrier, with carrier being defined as having ESBL-PE in at least one site (nose or hand).

4.3.Results

4.3.1. Demographic characteristics

Altogether, 114 people were contacted in the five selected abattoirs and 83 (73%) workers agreed to participate in the study. Seventy-six filled out the questionnaire and provided both nasal and hand swabs, six provided questionnaire and hand swabs only, while one withdrew before the sampling step. The response rate was higher in Cameroon (71%) than in South Africa (59%), with the most common reason for non-participation being lack of time or interest and fear of the sampling procedure and of the results.

Table 4.1. describes nasal and hand ESBL-PE carriage of exposed workers in relation to individual, clinical and abattoir-related characteristics. Unlike in South Africa where no human was colonized by ESBL-PE, the prevalence of ESBL-PE carriage in Cameroonian workers was significantly high (67.92%; p=0.000). People with a lower educational level (p=0.048), monthly income (p=0.007) and not having received training (p=0.000) were more prone to be colonized by ESBL-PE.

Table 4.1. Nasal and hand ESBL-E carriage of exposed workers in relation to individual, clinical and abattoir-related characteristics[4].

Variables	Nasal sample			Hand sample		
	Frequency n (%)	**Prevalence ESBL-E (%)**	**Overall p-value**	**Frequency n (%)**	**Prevalence ESBL-E (%)**	**Overall p-value**
Individual characteristics						
Country						
Cameroon	53 (69)	67.92	**0.000**	53 (64)	79	**0.000**
South Africa	24 (31)	0		30 (36)	0	
Gender						
Female	9 (12)	44.44	0.883	12 (12)	41.67	0.503
Male	68 (88)	47.06		71 (88)	52.11	
Age						
[21-30]	31 (40)	41.94	0.084	32 (39)	46.88	0.063
[31-40]	26 (34)	50		28 (34)	42.86	
[41-50]	13 (17)	38.46		14 (17)	71.43	
[51-60]	5 (6)	100		6 (7)	83.33	
Above 60	2 (3)	0		3 (3)	0	
Educational level						
Never been to school	4 (5)	50	**0.048**	5 (6)	40	**0.032**
Primary school not completed	6 (8)	50		7 (8)	42.86	
Primary school	34 (44)	64.71		35 (42)	71.43	
Secondary school	27 (35)	25.93		27 (33)	37.04	
High school/university	6 (8)	33.33		8 (10)	25	
Average monthly income (US $)						
Below 55	8 (10)	62.50	**0.007**	8 (14)	62.5	**0.004**
55-110	14 (19)	78.57		14 (29)	85.71	
110-165	12 (16)	66.67		12 (17)	58.33	
165-220	10 (13)	40		10 (17)	70	
220-275	20 (27)	20		24 (10)	25	
Above 275	11 (15)	27.27		13 (12)	30.77	
Relative working at hospital or with animals						
Yes	42 (55)	64.29	**0.001**	44 (53)	68.18	**0.001**
No	35 (45)	25.71		39 (47)	30.77	
Clinical factors						
Previous hospitalization (within the year of sampling)						
Yes	21 (27)	39.29	**0.032**	21 (25)	71.43	**0.027**

[4]Six out of the 84 enrolled workers refused the nasal sampling, one withdrew prior to the sample collection, for a total of 77 nasal and 83 hand samples collected. Few questions were not answered by workers while other could not recall the exact information leading to missing information that were not considered in the analysis.

No	56 (73)	66.67		62 (75)	43.55	
Nasal problem						
Yes	11 (14)	36.36	0.456	11 (13)	45.45	0.714
No	66 (86)	48.48		72 (87)	51.39	
Skin problem						
Yes	14 (18)	28.57	0.132	14 (17)	35.71	0.222
No	63 (82)	50.76		69 (83)	53.62	
Recent antibiotic use (month prior the sampling)						
Yes	38 (49)	55.26	0.140	38 (64)	71.05	**0.001**
No	39 (51)	38.46		45 (36)	33.33	
Abattoir-related factors						
Proximity of abattoir with house						
Yes	32 (42)	40.63	0.363	14 (33)	17	0.184
No	45 (58)	51.11		28 (67)	34	
Abattoir						
SH001	21 (27)	76.19	**0.000**	21 (25)	85.71	**0.000**
SH002	19 (25)	36.84		19 (23)	63.16	
SH003	13 (17)	100		13 (16)	92.31	
SH004	4 (5)	0		10 (12)	0	
SH005	20 (26)	0		20 (24)	0	
Principal activity or working area						
Slaughterer	34 (44)	58.82	**0.012**	34 (41)	58.82	**0.000**
Transport of pig/pork	5 (7)	80		5 (6)	80	
Wholesaler	7 (9)	28.57		7 (8)	85.71	
Butcher	5 (7)	80		5 (6)	80	
Retailer of viscera*	7 (9)	71.43		7 (8)	85.71	
Retailer of grilled pork#	1 (1)	0		1 (1)	100	
Scalding of pigs	3 (4)	0		3 (4)	0	
Evisceration	8 (10)	0		14 (17)	0	
Transport of viscera/blood	1 (1)	0		1 (1)	0	
Veterinarian	5 (7)	20		5 (6)	20	
Meat inspector	1 (1)	0		1 (1)	0	
Training to practice profession						
Yes	28 (36)	3.57	**0.000**	34 (41)	2.94	**0.000**
No	49 (64)	71.43		49 (59)	83.67	
Year in profession						
[0-4]	31 (43)	35.48	0.356	31 (39)	38.71	0.357
[5-9]	6 (8)	66.67		8 (10)	50	
[10-14]	22 (30)	50		24 (30)	58.33	
Above 15	14 (19)	57.14		16 (20)	62.50	
Intensity of pig's contact						
Always	35 (45)	51.43	0.348	35 (42)	57.14	0.136
Almost always	32 (42)	37.50		38 (46)	39.47	
Sometimes	10 (13)	60		10 (12)	70	
Contact with other animals						
Yes	38 (50)	60.53	**0.046**	39 (48)	69.23	**0.004**
No	38 (50)	34.21		42 (52)	35.71	
Intensity of contact with other animals						
Always	8 (21)	87.50	**0.025**	8 (20)	100	**0.006**
Almost always	9 (24)	22.22		10 (26)	30	
Sometimes	17 (45)	58.82		17 (44)	70.59	
Rarely	4 (10)	100		4 (10)	100	

***retailer of viscera*:** street-vendor purchasing pig's viscera from the abattoir, undertaking manual cleaning and sells ready-to-eat meal; ***#retailer of grilled pork*:** street-vendor purchasing pork at the abattoir and sells ready-to-eat grilled pork.

4.3.2. Risk factors of ESBL-PE carriage in humans

Table 4.2 (univariate and multivariate logistic regression) shows the association between ESBL-PE carriage in humans and the main potential risk factors. Previous hospitalization, recent antibiotic use, inadequate handwashing, occupation of relatives and year in the employment, were identified through univariate logistic regression as the main risk factors for nasal and hand ESBL-PE carriage. In contrast, the level of education, average monthly income, training, convenient handwashing and wearing of protective working clothes significantly reduced the odds of being colonized by ESBL-PE for both nares and hands (Table 4.2).

Table 4.2. Predictors of nasal and hand ESBL-PE carriage among exposed workers. Univariate and multivariate analysis (logistic regression)

Variables	Univariate Analysis				Multivariate Analysis			
	Nasal carriage of ESBL-PE		Hand carriage of ESBL-PE		Nasal carriage of ESBL-PE		Hand carriage of ESBL-PE	
	OR (95% CI)	*P*	OR (95% CI)	*P*	OR (95% CI)	*P*	OR (95% CI)	*P*
Abattoir	0.43 (0.22-0.85)	**0.014**	0.28 (0.15-0.55)	**0.000**	2.54 (0.47-17.75)	0.254	1.75 (0.19-15.54)	**0.615**
Hand ESBL-PE carriage (Yes or No)	39.11 (2.02-755.73)	**0.015**	…		…		…	
Nasal ESBL-PE carriage (Yes or No)	…		39.11 (2.02-755.72)	**0.015**	…		…	
Gender	1.11 (0.10-12.39)	0.932	1.52 (0.20-11.38)	0.682	8.74 (1.03-74.16)	**0.047**	27.94 (1.68-463.05)	**0.020**
Educational level	0.61 (0.39-0.94)	**0.024**	0.72 (0.56-0.91)	**0.006**	…		…	
Monthly Income	0.57 (0.36-0.89)	**0.014**	0.60 (0.36-0.99)	**0.045**	0.58 (0.35-0.97)	**0.039**	0.76 (0.44-1.31)	0.324
Training	0.01 (0.0003-0.79)	**0.038**	0.006 (0.0005-0.0658)	**0.000**	0.004 (0.00009-0.22)	**0.006**	0.0008 (0.000008-0.09)	0.003
Principal Activities	0.63 (0.50-0.78)	**0.000**	0.63 (0.46-0.87)	**0.004**	…		…	
Occupation of relative [a]	5.2 (1.46-18.56)	**0.011**	4.82 (0.64-36.56)	0.128	5.62 (1.02-30.82)	**0.047**	3.58 (0.57-22.43)	0.172
Year in Profession	1.40 (0.66-2.97)	0.387	1.35 (0.68-2.69)	0.398	…		…	
Age	1.09 (0.80-1.48)	0.595	1.07 (0.61-1.89)	0.817	…		…	
Recent hospitalization [b]	3.09 (1.26-7.59)	**0.014**	3.24 (1.18-8.86)	**0.022**	1.28 (0.24-6.87)	0.769	0.57 (0.08-4.12)	0.576
Recent antibiotic use [c]	1.97 (0.40-9.73)	0.402	4.91 (1.20-20.03)	**0.027**	…		…	
Skin problem	0.39 (0.17-0.89)	**0.025**	0.48 (0.21-1.08)	0.076	…		…	
Nasal problem	0.61 (0.29-1.28)	0.192	0.79 (0.34-1.82)	0.578	…		…	
Proximity of abattoir with house	0.65 (0.20-2.15)	0.485	0.53 (0.25-1.12)	0.097	…		…	
Protective working clothes	0.04 (0.002-0.812)	**0.036**	0.022 (0.002-0.258)	**0.002**	…		…	
Inadequate Handwashing	4.71 (2.28-9.70)	**0.000**	3.9 (1.01-15.01)	**0.048**	…		…	
Convenient handwashing	0.08 (0.017-0.41)	**0.002**	0.04 (0.013-0.145)	**0.000**	…		…	
Intensity of contact with pigs	0.97 (0.50-1.87)	0.920	0.96 (0.40-2.31)	0.934	…		…	
Contact with other animals	2.95 (0.87-10.04)	0.084	4.05 (1.42-11.53)	**0.009**	…		…	
Intensity of contact with other animals	1.09 (0.42-2.83)	0.854	1.03 (0.52-2.06)	0.927	…		…	
Contact with poultry	5.83 (1.58-21.48)	**0.008**	8.41 (2.27-31.11)	**0.001**	9.93 (1.37-71.63)	**0.023**	24.22 (1.28-457.35)	**0.034**
Pig contamination Nasal ESBL (yes or No)	1.04 (0.93-1.16)	**0.509**	1.06 (0.95-1.17)	**0.313**	1.36 (0.84-2.21)	**0.216**	1.22 (0.58-2.56)	0.597
Pig contamination Rectal ESBL (yes or No)	1.03 (0.93-1.15)	**0.585**	1.05 (0.96-1.16)	**0.273**	0.82 (0.53-1.25)	**0.353**	0.91 (0.46-1.79)	0.778
Sow	1.04 (0.87-1.24)	0.697	1.05 (0.88-1.25)	0.565	…		…	
Boar	0.84 (0.66-1.07)	0.151	0.80 (0.66-0.96)	**0.019**	…		…	

a: Family members working at hospital, with food animals or crop production, b: Exposure within 12 months prior the date of sampling; c: Within one month prior the date of sampling

Nasal ESBL-PE colonization was highly statistically associated with hands contamination of ESBL-PE (21.95% vs 91.67%; p=0.000) with an odds ratio (OR) of 39.11 (95% CI 2.02-755.72; p=0.015). The univariate analysis further reveals that nasal and hand ESBL-PE carriage were however not associated with rectal (OR=1.03, 95% CI 0.93-1.15; p=0.585; vs. OR=1.05, 95% CI 0.96-1.16; p=0.273) and nasal ESBL-PE contamination (OR=1.04, 95% CI 0.93-1.16; p=0.509 vs. OR=1.05, 95% CI 0.95-1.17; p=0.313) in pigs, although without statistical significance. In contrast, nasal and hand ESBL-PE carriage in humans were associated with contact with other animals, especially poultry with high statistical significance for both sample types (OR=5.83, 95% CI 1.58-21.48, p=0.008; vs OR=8.41, 95% CI 2.27-31.11, p=0.001; Table 4.2).

4.3.3. ESBL-PE status in humans

Out of the 53 workers sampled in Cameroon 42 (79%) and 36 (68%) were colonized by hand and nasal ESBL-PE, respectively. Multiple colonies (up to eight) were isolated from majority of workers, with a total of 109 and 83 presumptive ESBL-PE being detected from hand and nasal swabs, respectively. Due to the large number of isolates a selection based on statistical analysis allowed the selection of a subset of 82 non-duplicate (hand=48, nasal=34) ESBL-PE isolates representative of the main population for phenotypic analysis. All of these 82 (100%) presumptive human ESBL-PE isolates selected were confirmed via Vitek® 2 System (BioMérieuX, Marcy l'Etoile, France) as *Enterobacteriaceae* and antimicrobial susceptibility testing (with double disc synergy and VITEK 2 system) revealed that 78/82 (95%) were ESBL-PE. The main species identified were *E. coli*, *K. pneumoniae* and *Enterobacter spp.* (S1 Table). In contrast, in South Africa, *Enterobacteriaceae* was not isolated from slaughterhouse workers (S1 Table).

4.3.4. Epidemiological background of ESBL-PE in pigs

From the five abattoirs, 144 pooled nasal samples (three nasal swabs each, proportionally to abattoir productivity, 72 per country) and 144 pooled rectal samples (three rectal swabs each, proportionally to abattoir productivity, 72 per country) taken from 432 pigs were analysed. Overall, ESBL-PE were isolated from 75% (108/144, p=0.000) and 71% (102/144, p=0.000) of the pooled nasal and rectal samples respectively (Table 3), with multiple colonies (up to ten) isolated from the majority of ESBL-positive pooled samples in both countries. Nasal ESBL-PE colonization in pigs was highly statistically associated with rectal ESBL-PE positivity (OR=15.95; 95%CI 6.06-36.85; p=0.000). When comparing the results at country-level, a maximum level of ESBL-PE (100%) was detected in both type of pooled samples in Cameroon,

whereas 42% (30/72; p=0.011) and 50 % (36/72; p=0.284) ESBL-PE were respectively isolated in rectal and nasal pooled samples in South Africa (Table 3).

Table 4.3. Extended-spectrum β-lactamase-producing *Enterobacteriaceae* (ESBL-PE) in pooled nasal and rectal samples

Characteristics	Nasal samples			Rectal samples		
	Frequency Pooled samples, n (%)	**Nasal ESBL, n (%)**	**Overall p-value**	**Frequency Pooled samples, n (%)**	**Rectal ESBL, n (%)**	**Overall p-value**
Country						
Cameroon	72 (50)	72 (100)	**0.000**	72 (50)	72 (100)	**0.000**
South Africa	72 (50)	36 (50)		72 (50)	30 (41.67)	
Abattoir						
SH001	43 (30)	43 (100)	**0.000**	43 (30)	43 (100)	**0.000**
SH002	19 (13)	19 (100)		19 (13)	19 (100)	
SH003	10 (7)	10 (100)		10 (7)	10 (100)	
SH004	40 (28)	19 (47.50)		40 (28)	9 (22.50)	
SH005	32 (22)	17 (53.13)		32 (22)	21 (65.63)	
Gender						
Sow	79 (55)	64 (81.01)	0.066	79 (55)	59 (74.68)	0.262
Boar	65 (45)	44 (67.69)		65 (45)	43 (66.15)	
Time point						
First	42 (29)	31 (73.81)	0.149	42 (29)	34 (80.95)	**0.050**
Second	54 (38)	45 (83.33)		54 (38)	40 (74.07)	
Third	48 (33)	32 (66.67)		48 (33)	28 (58.33)	

4.3.5. Prevalence of ESBL-PE based on the time point

Figs 1A and 1B illustrate the rectal and nasal ESBL-PE colonization in pigs per abattoir and at each time point, respectively. Overall, in South Africa, the prevalence of rectal ESBL-PE decreased from 65 to 23% from the first to the third time point with high statistical significance (p=0.011). At abattoir level, maximum prevalence of 100 and 90% rectal ESBL-PE were recorded in SH005 during the first and second time point (p=0.000), respectively; whereas less than 40% prevalence was detected in SH004 at all time points (p=0.051) (Fig 1). ESBL-PE positivity associated with nasal colonization was more diverse along time points in the country, with the highest prevalence being 61% at the second point of collection. In contrast with South Africa, 100% ESBL-PE carriage was detected at all time-points and for both type of pooled samples in Cameroon.

Fig 1. Overall prevalence of ESBL-PE positivity in pigs per abattoir and time point. A. Prevalence of nasal ESBL-PE positivity; **B.** Prevalence of rectal ESBL-PE positivity

4.3.6. ESBL-PE status in pooled samples

In total, 262 and 258 ESBL-PE were detected from nasal and rectal pooled samples respectively in Cameroon. Due to the large number of isolates, post-stratification allowed the selection of a subset of 162 non-duplicate (nasal=79 and rectal=83) ESBL-PE strains representative of the main population and slaughterhouse for phenotypic analyses. Out of these, 138 (85%) were identified as *Enterobacteriaceae* with 137 (99%) being phenotypically confirmed as ESBL-PE. The main species identified were *E. coli* (61%), *Klebsiella pneumoniae* (25%), *Enterobacter spp.* (6%), *Shigella sonnei* (3%) and *Citrobacter freundii* (3%) (S2 Table). In South Africa, a total of 159 presumptive ESBL-PE (81 nasal and 78 rectal) strains underwent phenotypic characterization, with *E. coli* (24%) being the only *Enterobacteriaceae* species isolated in both types of pooled samples in South Africa. *Bordetella bronchoseptica* (23%), *Acinetobacter baummanii* (3%), *Aeromonas salmonicida* (3%), *Pseudomonas aeruginosa* (3%) and *Myroides spp*. (1%), were the other bacterial species identified (S2 Table).

4.3.7. Antimicrobial resistance patterns in humans

Overall, ESBL-PE were not detected in humans in South Africa, but strains isolated from those in Cameroonian abattoirs, expressed high resistance to ampicillin (77%), trimethoprim-sulfamethoxazole (50%), cefuroxime (43%), cefuroxime-acetyl (43%), cefotaxime (32%), ceftazidime (18%) and amoxicillin-clavulanic acid (18%) (Table 4.4). One strain isolated from hand was resistant to colistin (4 µg/ml) although not being multi-drug resistant. No resistance to imipenem, ertapenem, meropenem and tigecycline was observed in either country (Table 4.4).

Table 4.4. **Resistance to antibiotics of extended-spectrum beta-lactamase-producing *Enterobacteriaceae* (ESBL-PE) isolated from pigs and humans**

Antibiotics	Cameroon				South Africa	
	Pig		Human		Pig	
	MIC (µg/ml) Range	No. (%) resistant isolates	MIC (µg/ml) Range	No. (%) resistant isolates	MIC (µg/ml) Range	No. (%) resistant isolates
Ampicillin	≥32	126 (95)	≤2−≥32	32(73)	≥32	38 (100)
Amoxicillin-clavulanate	4−≥32	54(40)	≤2−≥32	8(18)	8−16	2(5)
Piperacillin-tazobactam	≤ 4−≥128	24(18)	≤4−64	2(5)	≤4	0
Cefuroxime	4−≥64	124(93)	≤1−≥64	19(43)	≥64	38(100)
Cefuroxime-acetyl	4−≥64	125(93)	≤1−≥64	19(43)	≥64	38(100)
Cefoxitin	≤4−≥64	10(7)	≤4−≥64	3(7)	≤4	0
Cefotaxime	≤1−≥64	118(88)	≤1−≥64	14(32)	4−≥64	38(100)
Ceftazidime	≤1−≥64	93(69)	≤1−≥64	8(18)	≤1−4	1(3)
Cefepime	≤0.5−≥64	6(4)	≤1−≥64	2(5)	≤1−4	1(3)
Meropenem	≤0.25	0	≤0.25	0	≤0.25	0
Imipenem	≤0.25	0	≤0.25	0	≤0.25	0
Ertapenem	≤0.5	0	≤0.5	0	≤0.5	0
Amikacin	≤2−16	11(8)	≤2−16	1(2)	≤2−16	1(3)
Gentamicin	≤1−≥16	43(32)	≤1−≥16	3(7)	≤1−≥16	7(18)
Ciprofloxacin	≤0.25−≥4	33(25)	≤0.25−≥4	2(5)	≤0.25	0
Tigecycline	≤0.5−2	0	≤0.5−1	0	≤0.5−1	0
Nitrofurantoin	≤16−64	0	≤16−128	1(2)	≤16−64	0
Colistin	≤0.5	0	≤0.5−4	1(2)	≤0.5−8	1(3)
Trimethoprim-sulfamethoxazole	≤20−≥320	119 (89)	≤20−≥320	22(50)	≤20−≥320	36(95)

Table 4.5. presents the resistance patterns detected in ESBL-PE in humans. AMP.TMP/SXT.CXM.CXM-A.CTX (34%) and AMP.AMC.TZP.CXM.CXM-A.CTX.CAZ.TMP/SXT (7%) were the main resistance patterns in hand and nasal ESBL-*E. coli* respectively, in humans in Cameroon. Seven percent of hand ESBL-*K. pneumoniae* expressed the AMP.AMC.CXM.CXM-A.CTX.TMP/SXT.GM.FT, AMP.AMC.CXM.CXM-A.CTX.CAZ.AN.GM.CIP and AMP.TMP/SXT.CXM.CXM-A.CTX.CAZ profiles, that were resistant to four, three and two classes of antibiotics respectively (Table 4.5). The same analysis could not be performed with South African samples as there were no ESBL-PE detected in humans in this country.

Table 4.5. Antimicrobial resistance profiles of extended-spectrum beta-lactamase-producing *Enterobacteriaceae* (ESBL-PE) strains isolated from humans

Bacteria	Resistance patterns	Cameroon	
		Nasal, n (%)	Hand, n (%)
E. coli	AMP.AMC.TZP.CXM.CXM-A.CTX.CAZ.TMP/SXT	1 (50)	0
	AMP.CXM.CXM-A.CAZ.CS	0	1(8)
	AMP.TMP/SXT.CXM.CXM-A.CTX	0	4(31)
	AMP.TMP/SXT.CXM.CXM-A.CTX.CAZ.AMC.GM.CIP.FEP	0	1(8)
	AMP.TMP/SXT.CXM.CXM-A.CTX.CAZ.AMC.TZP	0	1(8)
	AMP.TMP/SXT.CXM.CXM-A.CTX	0	1(8)
	AMP.CXM.CXM-A.CTX.FEP.TMP/SXT	0	1(8)
E. dissolvens	AMP.AMC.CXM.CXM-A.FOX.CTX.TMP/SXT	1 (50)	0
S. sonnei	AMP.TMP/SXT.CXM.CXM-A.CTX.CAZ	0	1(8)
K. pneumoniae	AMP.AMC.CXM.CXM-A.CTX.TMP/SXT.GM.FT	0	1(8)
	AMP.AMC.CXM.CXM-A.CTX.CAZ.AN.GM.CIP	0	1(8)
	AMP.TMP/SXT.CXM.CXM-A.CTX.CAZ	0	1(8)
Grand Total		2 (100)	13 (100)

AMP: Ampicillin; AMC: Amoxicillin-clavulanate; TZP: Piperacillin-tazobactam; CXM: Cefuroxime; CXM-A: Cefuroxime-Acetyl; CTX: Cefotaxime; CAZ: Ceftazidime; TMP/SXT: Trimethoprim-Sulfamethoxazole; FOX: Cefoxitin; GN: Gentamicin; CIP: Ciprofloxacin; FEP: Cefepime; CS: Colistin

4.3.8. Antimicrobial resistance patterns in pigs

Table 4.6. presents the antimicrobial resistance profiles in ESBL-PE isolated from animals in both Cameroon and South Africa. ESBL-PE isolated from pigs in Cameroon, exhibited high resistance to ampicillin (95%), cefuroxime (93%), cefuroxime-acetyl (93%), cefotaxime (88%), ceftazidime (69%) and trimethoprim-sulfamethoxazole (89%) (Table 4.4). No ESBL-PE showed resistance to ertapenem, meropenem, imipenem and only few isolates expressed resistance to cefoxitin, cefepime, gentamicin, amikacin and ciprofloxacin (Table 4.4). Similarly, 100% resistance to ampicillin, cefuroxime, cefuroxime-acetyl, and cefotaxime, and 95% resistance to trimethoprim-sulfamethoxazole were observed in ESBL-PE isolated from

pigs in South Africa. One strain expressed high resistance to colistin (8 µg/ml) and to nine other antibiotics, whereas none was resistant to imipenem, ertapenem, meropenem and tigecycline in the country (Table 4.6).

Table 4.6. Antimicrobial resistance profiles of extended-spectrum beta-lactamase-producing *Enterobacteriaceae* (ESBL-PE) isolated from pigs

Bacteria	Resistance patterns	No. Antibiotics	No. Classes	Cameroon		South Africa	
				Nasal, n (%)	Rectal, n (%)	Nasal, n (%)	Rectal, n (%)
E. coli	AMP.TMP/SXT.CXM.CXM-A.CTX	5	2	3(5)	2(4)	0	29(94)
	AMP.TMP/SXT.CXM.CXM-A.CTX.CAZ	6	2	7(12)	1(20)	0	0
	AMP.TMP/SXT.CXM.CXM-A.CTX.CAZ.FEP	7	2	2(3)	2(4)	0	0
	AMP.TMP/SXT.CXM.CXM-A.CTX.CAZ.GM.CIP	8	4	1(2)	2(4)	0	0
	AMP.TMP/SXT.CXM.CXM-A.CTX.CAZ.CIP	7	3	1(2)	0	0	0
	AMP.TMP/SXT.CXM.CXM-A.CTX.CAZ.AMC	8	3	1(2)	3(5)	0	0
	AMP.TMP/SXT.CXM.CXM-A.CTX.CAZ.AMC.GM.CIP	9	4	2(3)	3(5)	0	0
	AMP.TMP/SXT.CXM.CXM-A.CTX.CAZ.AMC.TZP	8	2	5(8)	5(9)	0	0
	AMP.TMP/SXT.CXM.CXM-A.CTX.CAZ.AMC.TZP.FOX.FEP.GM.CIP	12	4	1(2)	0	0	0
	AMP.CXM.CXM-A.CTX.CAZ	5	1	0	2(4)	0	0
	AMP.TMP/SXT.CXM.CXM-A.CTX.CAZ.FEP.GM.	8	3	0	1(2)	0	0
	AMP.TMP/SXT.CXM.CXM-A.CTX.CIP	6	3	0	1(2)	0	0
	AMP.TMP/SXT.CXM.CXM-A.CTX.CAZ.CIP.AMC	8	3	0	5(9)	0	0
	AMP.TMP/SXT.CXM.CXM-A.CTX.GM	6	3	0	1(2)	0	0
	AMP.TMP/SXT.CXM.CXM-A.CTX.CAZ.CIP.AMC.TZP	9	3	0	1(2)	0	0
	AMP.TMP/SXT.CXM.CXM-A.CTX.CAZ.CIP.GM.TZP.FEP	10	4	0	1(2)	0	0
	AMP.CXM.CXM-A.CTX	4	1	0	0	0	2(6)
	AMP.CXM.CXM-A.CTX.TMP/SXT.CAZ.FEP.AK.GM.CS	10	4	0	0	1(14)	0
	AMP.CXM.CXM-A.CTX.TMP/SXT.GM	6	3	0	0	1(14)	0
	AMP.CXM.CXM-A.CTX.TMP/SXT.GM.AMC	7	3	0	0	5(71)	0
	AMP.TMP/SXT.CXM.CXM-A.CTX.CAZ.CIP.GM.AMC.TZP	10	4	0	2(4)	0	0
K. pneumoniae	AMP.TMP/SXT.CXM.CXM-A.CTX.GM	6	3	2(3)	1(2)	0	0
	AMP.TMP/SXT.CXM.CXM-A.CTX.CAZ.AK.GM.CIP.AMC	10	4	0	6(11)	0	0
	AMP.TMP/SXT.CXM.CXM-A.CAZ.AMC.TZP	7	2	0	1(2)	0	0
	AMP.TMP/SXT.CXM.CXM-A.CTX.CAZ.GM.AMC.TZP	9	3	4(7)	2(4)	0	0
	AMP.TMP/SXT.CXM.CXM-A.CTX.CAZ.AMC.TZP.AK.GM.CIP	11	4	0	3(5)	0	0
	AMP.TMP/SXT.CXM.CXM-A.CTX	5	2	1(2)	0	0	0
	AMP.TMP/SXT.CXM.CXM-A.CTX.CAZ	6	2	4(7)	0	0	0

	AMP.TMP/SXT.CXM.CXM-A.CTX.CAZ.GM	7	3	8(14)	0	0	0
	AMP.TMP/SXT.CXM.CXM-A.CTX.CAZ.GM.AMC.TZP	9	3	4(7)	0	0	0
K. ozanae	AMP.TMP/SXT.CXM.CXM-A.CTX.CAZ.AK.GM.CIP.AMC	10	4	1(2)	0	0	0
Enterobacter cloacae	AMP.AMC.CXM.CXM-A.FOX.CTX.TMP/SXT	7	2	4(7)	0	0	0
C. freundii	AMP.AMC.CXM.CXM-A.FOX.CTX.TMP/SXT	7	2	3(5)	0	0	0
S. sonnei	AMP.CTX.CAZ.TMP/SXT	4	2	1(2)	0	0	0
	AMP.TMP/SXT.CXM.CXM-A.CTX.CAZ	6	2	4(7)	0	0	0

AMP: Ampicillin; AMC: Amoxicillin-clavulanate; AK: Amikacin; CXM: Cefuroxime; CXM-A: Cefuroxime-acetyl; CTX: Cefotaxime; CAZ: Ceftazidime; CS: Colistin; CIP: Ciprofloxacin; FEP: Cefepime; FOX: Cefoxitin; GM: Gentamicin; TMP/SXT: Trimethoprim-sulfamethoxazole; TZP: Piperacillin-tazobactam

The majority of rectal ESBL-producing *E. coli* isolated from pigs in South Africa showed the resistance profile AMP.TMP/SXT.CXM.CXM-A.CTX (94%) while the pattern AMP.CXM.CXM-A.CTX.TMP/SXT.GM.AMC (71%) was more prevalent in nasal ESBL-producing *E. coli* with resistance to two and five classes of antibiotics, respectively. In Cameroonian pigs, AMP.TMP/SXT.CXM.CXM-A.CTX.CAZ (20%), AMP.TMP/SXT.CXM.CXM-A.CTX.CAZ.AMC.TZP (9%) and AMP.TMP/SXT.CXM.CXM-A.CTX.CAZ.CIP.AMC (9%) were the main resistance profiles detected in rectal ESBL-producing *E. coli*, while AMP.TMP/SXT.CXM.CXM-A.CTX.CAZ (12%) and AMP.TMP/SXT.CXM.CXM-A.CTX.CAZ.AMC.TZP (8%) were most prevalent in nasal ESBL-*E. coli* (Table 4.6). ESBL-producing *K. pneumoniae* were not detected in South Africa but in Cameroon, the main resistance profiles were AMP.TMP/SXT.CXM.CXM-A.CTX.CAZ.AK.GM.CIP.AMC (11%) and AMP.TMP/SXT.CXM.CXM-A.CTX.CAZ.GM (14%) in rectal and nasal pooled samples, respectively (Table 4.6).

4.3.9. Genotypic relatedness

In total, 93 ESBL-producing *E. coli* strains showing the most representative resistance profiles and originating from both pigs and humans of Cameroon and South Africa were subjected to genotyping by ERIC-PCR to determine their clonal relationships. ERIC-PCR allowed the differentiation of the 93 *E. coli* into 14 clusters named alphabetically from A-N (Table 4.7 and Fig 2). A batch of isolates in cluster M (PR210, PR212E*, PR209E2, PR246B1C and PN254E), collected from pigs of abattoir SH004 and SH005 in South Africa was considered to be closely related. Moreover, great interest was observed in cluster I, where one pair of animal strains, PR085E3 and PR209E1 isolated in abattoirs SH002 and SH004 in Cameroon and South Africa, respectively, showed 100% similarity, and were closely related with a human strain (HN503E2II) detected in abattoir SH001 in Cameroon (Fig 2).

Fig 2. Genotypic relationship of ESBL-*E. coli* strains (n=93) isolated from pigs and humans in Cameroon and South Africa. Dendrogram established by the biostatistical analysis software Bionumerics using the Dice similarity coefficient and UPGMA method on the basis of the ERIC-PCR profiles obtained with primers ERIC1 and ERIC2.

Table 4.7. Prevalence and distribution of extended-spectrum beta-lactamase producing-*E. coli* clusters per abattoir

Clusters	Resistance profiles	No. isolates	No. isolates (%)	Abattoir				
				SH001	SH002	SH003	SH004	SH005
A	AMP.TMP/SXT.CXM.CXM-A.CTX	5	5 (5)	0	0	0	0	5
B	AMP.TMP/SXT.CXM.CXM-A.CTX	1	3 (3)	2	0	0	0	1
	AMP.TMP/SXT.CXM.CXM-A.CTX.CAZ	1						
	AMP.TMP/SXT.CXM.CXM-A.CTX.CAZ.AMC.GEN.CIP	1						
C	AMP.CXM.CXM-A.CTX.TMP/SXT.GEN.AMC	2	3 (3)	0	1	0	0	2
	AMP.CXM.CXM-A.CTX.CIP.TMP/SXT	1						
D	AMP.TMP/SXT.CXM.CXM-A.CTX	10	12 (13)	0	0	3	0	7
	AMP.TMP/SXT.CXM.CXM-A.CTX.CAZ	1						
	AMP.CXM.CXM-A.CTX.CIP.TMP/SXT.AMC	1						
E	AMP.TMP/SXT.CXM.CXM-A.CTX	2	4 (4)	1	1	0	0	2
	AMP.TMP/SXT.CXM.CXM-A.CTX.CAZ	1						
	AMP.TMP/SXT.CXM.CXM-A.CTX.CAZ.FEP	1						
F	AMP.TMP/SXT.CXM.CXM-A.CTX	4	4 (4)	0	0	0	0	4
G	AMP.TMP/SXT.CXM.CXM-A.CTX.CAZ	2	3 (3)	3	0	0	0	0
	AMP.CXM.CXM-A.CTX.CAZ.CIP.TMP/SXT.AMC.TZP	1						
H	AMP.CXM.CXM-A.CTX.TMP/SXT.CAZ.FEP.AN.GEN.CS	1	4 (4)	2	1	0	0	1
	AMP.TMP/SXT.CXM.CXM-A.CTX.AMC.TZP.CAZ	1						
	AMP.TMP/SXT.CXM.CXM-A.CTX.CAZ.AMC.FEP.GEN.CIP	1						
	AMP.AMC.CXM.CXM-A.CTX.CIP	1						
I	AMP.TMP/SXT.CXM.CXM-A.CTX	2	18 (19)	13	2	2	1	0
	AMP.TMP/SXT.CXM.CXM-A.CTX.CAZ	2						
	AMP.TMP/SXT.CXM.CXM-A.CTX.CAZ.FEP	1						
	AMP.TMP/SXT.CXM.CXM-A.CTX.CAZ.GEN.CIP	1						
	AMP.TMP/SXT.CXM.CXM-A.CTX.CAZ.AMC.GEN.CIP	1						
	AMP.TMP/SXT.CXM.CXM-A.CTX.AMC.TZP.CAZ	5						
	AMP.CXM.CXM-A.CTX.FEP.TMP/SXT	1						
	AMP.CXM.CXM-A.CTX.CAZ	2						
	AMP.CXM.CXM-A.CTX.CIP.TMP/SXT.AMC	2						

	AMP.CXM.CXM-A.CTX.GEN.TMP/SXT	1						
J	AMP.TMP/SXT.CXM.CXM-A.CTX	1	8 (9)	7	0	1	0	0
	AMP.TMP/SXT.CXM.CXM-A.CTX.CAZ	3						
	AMP.TMP/SXT.CXM.CXM-A.CTX.CAZ.FEP	1						
	AMP.TMP/SXT.CXM.CXM-A.CTX.CAZ.GEN.CIP	1						
	AMP.TMP/SXT.CXM.CXM-A.CTX.AMC.TZP.CAZ	1						
	AMP.CXM.CXM-A.CTX.CIP.TMP/SXT.AMC	1						
K	AMP.TMP/SXT.CXM.CXM-A.CTX	1	12 (13)	9	3	0	0	0
	AMP.TMP/SXT.CXM.CXM-A.CTX.CAZ	1						
	AMP.TMP/SXT.CXM.CXM-A.CTX.CAZ.AMC	2						
	AMP.TMP/SXT.CXM.CXM-A.CTX.CAZ.AMC.GEN.CIP	1						
	AMP.TMP/SXT.CXM.CXM-A.CTX.AMC.TZP.CAZ	3						
	AMP.CXM.CXM-A.CAZ.CS	1						
	AMP.TMP/SXT.CXM.CXM-A.CTX.CAZ.AMC.TZP.FOX.FEP.GEN.CIP	1						
	AMP.CXM.CXM-A.CTX.CAZ.CIP.GEN.TMP/SXT.TZP.FEP	1						
	AMP.CXM.CXM-A.CTX.CAZ.CIP.GEN.TMP/SXT.TZP	1						
L	AMP.TMP/SXT.CXM.CXM-A.CTX	4	8 (9)	8	0	0	0	0
	AMP.TMP/SXT.CXM.CXM-A.CTX.CAZ	2						
	AMP.CXM.CXM-A.CTX.CIP.TMP/SXT	1						
	AMP.CXM.CXM-A.CTX.GEN.TMP/SXT	1						
M	AMP.TMP/SXT.CXM.CXM-A.CTX	4	7 (9)	0	0	0	4	4
	AMP.CXM.CXM-A.CTX	1						
	AMP.CXM.CXM-A.CTX.TMP/SXT.GEN	1						
	AMP.CXM.CXM-A.CTX.TMP/SXT.GEN.AMC	1						
N	AMP.TMP/SXT.CXM.CXM-A.CTX.CAZ	1	3 (3)	3	0	0	0	0
	AMP.TMP/SXT.CXM.CXM-A.CTX.CAZ.AMC	1						
	AMP.CXM.CXM-A.CTX.CAZ.FEP.GEN.TMP/SXT	1						
Grand Total		**93**	**93 (100)**	48	8	6	5	26

AMP: Ampicillin; AMC: Amoxicillin-clavulanate; AK: Amikacin; CXM: Cefuroxime; CXM-A: Cefuroxime-acetyl; CTX: Cefotaxime; CAZ: Ceftazidime; CS: Colistin; CIP: Ciprofloxacin; FEP: Cefepime; FOX: Cefoxitin; GM: Gentamicin; TMP/SXT: Trimethoprim-sulfamethoxazole; TZP: Piperacillin-tazobactam

4.4. Discussion

Enterobacteriaceae and especially ESBL-PE, were recently recognized as critical priority ARB by the WHO (3) and their emergence at the animal-human-environment interface leads to serious and multifaceted public health concern globally. In this study, the carriage, risk factors, antimicrobial resistance patterns and genetic relatedness of ESBL-PE isolated from healthy pigs and exposed workers in Cameroon and South Africa were determined.

The overall prevalence of ESBL-PE carriage in humans was 50% in hand and 45.75% nasal samples. Similar findings were reported by Magoue et al. (2013) in Cameroon, where the prevalence of ESBL-PE faecal carriage was 45% in outpatients in the region of Adamaoua (9). They are however higher than that reported by Dohmen et al. (2015) where a 27% prevalence of ESBL-PE carriage in faecal samples of people with daily exposure to pigs in Netherlands was described (10).

Our results are in contrast to a study of Fisher et al. (2016), where none of the 66.7% *Enterobacteriaceae* detected in the nares of participants were ESBL producers and where the authors concluded that nares were a negligible reservoir for colonization of ESBL-PE in pig's exposed workers (11). Our finding shows that the prevalence of ESBL-PE carriage in nasal samples substantially increased (8.33 vs 91.67%; $p<0.001$) and was significantly associated with their carriage on hand (OR 39.11; 95% CI 2.02-755.72; $p=0.015$). In addition, nasal ESBL-PE carriage was associated with inappropriate handwashing with high statistical significance (OR 4.71; 95% CI 2.28-9.70; $p<0.001$). This suggests, that nares might likely become reservoir of ESBL-PE when limited hygienic conditions prevail and biosecurity measures are not adequately implemented. It further reveals that, as with the transmission of nosocomial infections in hospital settings, hands constitute important vectors of ABR transmission in the food production industry and may not only drive the transfer from person-to-person but also the contamination of food products intended for the end consumer. Nasal ESBL-PE carriage reported in our study could also be attributed to airborne contamination as recently reported by Dohmen et al. (2017) who revealed that human CTX-M-gr1 carriage was statistically associated with presence of CTX-M-gr1 in dust (OR=3.5, 95% CI=0.6–20.9) and that inhalation of air might constitute another transmission route of ESBL-PE in the food chain (12).

The difference in the prevalence of ESBL-PE carriage in humans in both countries could be explained by the fact that South Africa not only has existing abattoir regulations in place, but also that implementation of Hazard Analysis Critical Control Points (HACCP) plans and

compliance with international food safety standard ISO 22000 were stringent in both abattoirs. In Cameroon, slaughterhouse/markets were principally low-grade, lacking in basic amenities, with sub-optimal hygienic conditions and minimal or non-existent biosecurity measures. The Food and Agriculture Organization for the United Nations (FAO) report on abattoir facilities in Central African countries including Cameroon, already underlined the gaps in term of biosecurity measures in these settings (13). Our findings, therefore, reinforce the importance of and the need to implement strict biosecurity procedures as when effective prevention and containment measures are implemented, the risk of ABR dissemination is reduced.

The overall prevalence of ESBL-PE in pigs was 71% and 75% in rectal and nasal pooled samples, respectively. The results are consistent with that reported by Le et al. (2015) in food animals and products in Vietnam where a 68.4% prevalence of ESBL-producing *E. coli* was described (14). They are however lower than that reported in pig farms in Germany, where 88.2% of ESBL-producing *E. coli* was detected (15), and higher than that reported in two other studies with prevalence ranging from 8.6 to 63.4% in food animals and products in Netherlands (16), and 8.4% in cattle in Switzerland (2).

The high rate of ESBL-PE carriage detected in both type of pooled samples in Cameroon may indicate that ESBL-PE are consistently widespread in food animals in Cameroon, disseminate in the farm-to-plate continuum and represent a serious food safety threat in the country. Similarly, the ESBL-PE prevalence detected in pigs in South Africa is not surprising, knowing that the use of antibiotics as growth promoters is legally approved in the country (17). These findings reveal gaps in the current state of knowledge about antibiotic use and ABR in food animals, and suggests that the debate about ABR-related consequences in the farm-to-plate continuum is neglected in Cameroon and South Africa and should be more seriously considered in these countries. Additionally, our study revealed a high frequency (95%) of ESBL-producing *E. coli*, emphasizing the relevance of this indicator bacteria as a serious public health issue.

ERIC analysis, demonstrated relative association between animal and human strains within and across countries. Some strains detected in humans were highly related with those isolated from pigs at the same or different slaughterhouse. These results suggest that occurrence of ESBL-PE in humans may be of animal origin or vice-versa, and that these pathogens may be transferred to human via the food chain, allowing their dissemination to the global population. Although not providing evidence on the transmission dynamics of the spread of ESBL-PE, our results nonetheless show an epidemiological link between strains isolated from animals and humans.

Hospitalization, antibiotic use and contact with (food) animals are known risk factors for human ESBL-PE carriage (18). Twenty-one workers or their close relatives had been hospitalized within a year of the sample collection, leading to a 39.29% and 71.43% prevalence of nasal and hand ESBL-PE, respectively (Table 1). Likewise, 55.26% and 71.05% of workers who had used antibiotics the month preceding the sampling were colonized by ESBL-PE in nasal and hand samples, respectively (Table 1). In this cross-sectional study, duration of ESBL-PE carriage was not investigated, and no association between human ESBL-PE carriage and contact with ESBL-PE carrying pigs was observed for all type of samples, although not statistically significant (Table 6). In contrast, a clear association between human ESBL-PE carriage and contact with other animals, especially poultry, was observed and with high statistical significance (Table 6). This suggest that more research is required on ESBL-PE carriage in high risk population and other food animals such as poultry in order to improve our knowledge about on the public health significance associated with the likely transmission of ESBL-PE through the farm-to-plate continuum.

4.5. Conclusion

To the best of our knowledge, this is the first report of ESBL-PE in animals and humans in both Cameroon and South Africa taking food safety perspective. The high prevalence of ESBL-PE found in pigs in both countries as well as in humans in Cameroon highlights the food safety issue associated with their presence in the farm-to-plate continuum. It demonstrates the urgent need to implement multi-sectorial, multi-faceted and sustainable collaboration and activities among all stakeholders involved in this continuum in order to reduce the prevalence and contain the dissemination of ESBL-PE and ABR in these countries.

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Conflict of interest

Professor Essack is a member of the Global Respiratory Infection Partnership sponsored by Reckitt and Benckiser. All other authors declare that there is no competing financial interest.

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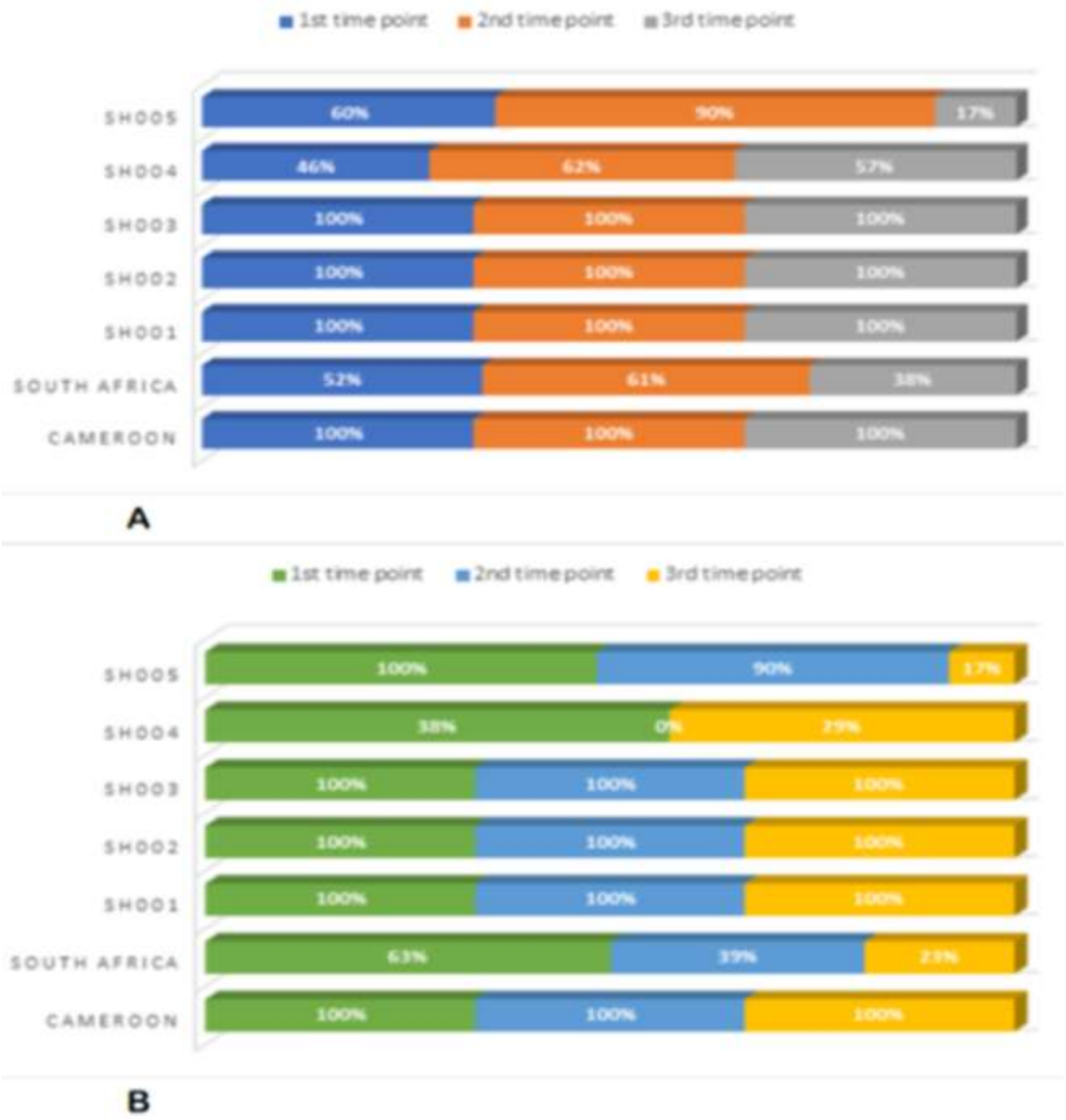

Figure 4.1. Overall prevalence of nasal (A) and rectal (B) ESBL-PE carriage in pigs per country, abattoir and time point.

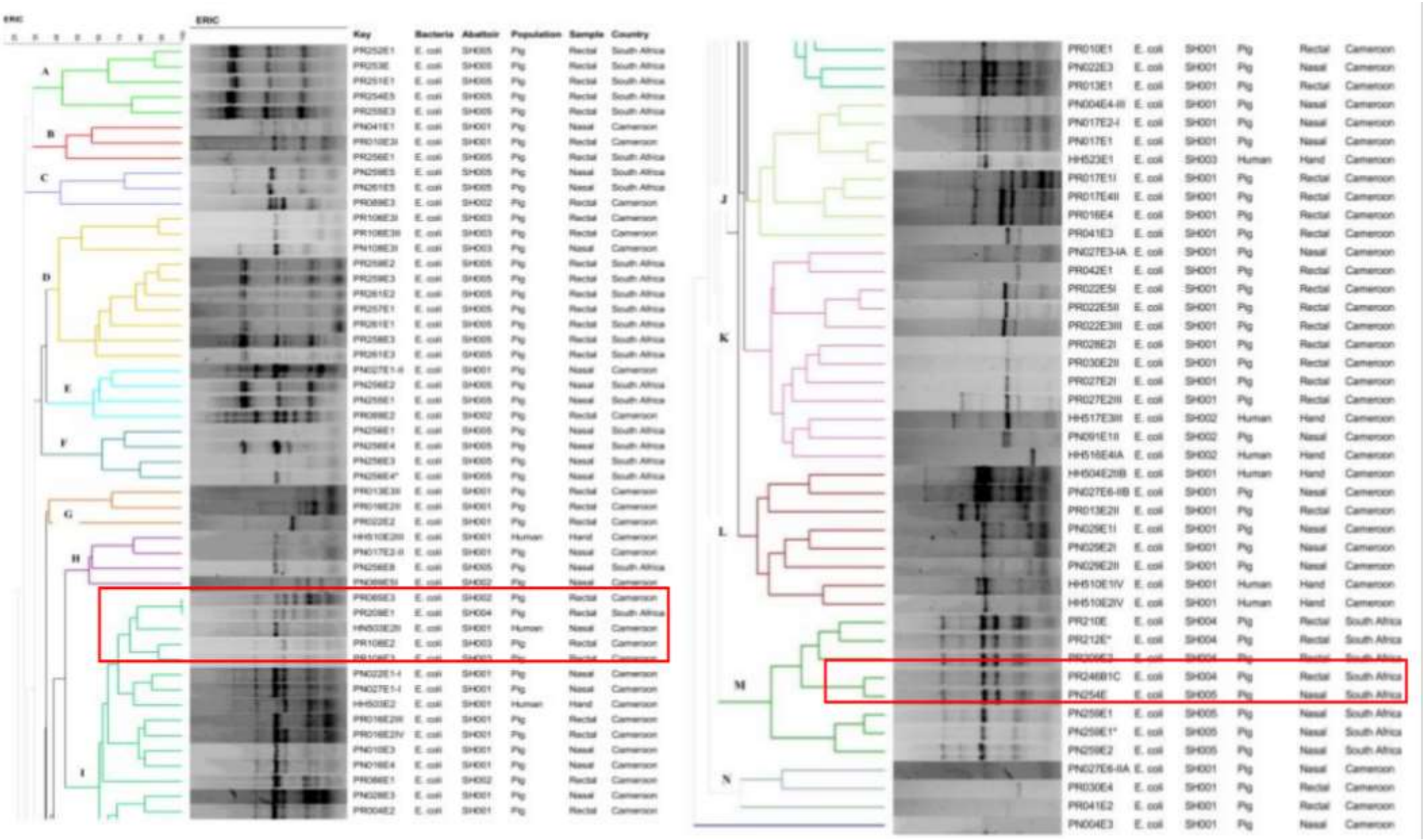

Figure 4.2. Genotypic relationship of ESBL-*E. coli* strains (n=93) isolated from pigs and humans in Cameroon and South Africa. Dendrogram established by* the biostatistical analysis software Bionumerics using the Dice similarity coefficient and UPGMA method on the basis of the ERIC-PCR profiles obtained with primers ERIC1 and ERIC2. Clusters were defined based on a similarity cut-off of 80%.

Supporting Information

S1 Table. Overall prevalence of extended spectrum beta-lactamase (ESBL) producing bacteria isolated from humans per country and specimen type

Bacteria	Cameroon		South Africa	
	Hand (%)	Nasal (%)	Hand (%)	Nasal (%)
E. coli	16 (41)	15 (47)	0	0
E. dissolvens	2 (5)	1 (3)	0	0
K. pneumoniae	2 (5)	6 (19)	0	0
Shigella sonnei	1 (3)	1 (3)	0	0
Others*	18 (46)	9 (28)	10 (50)	10 (50)
Total	39 (100)	32 (100)	10 (100)	10 (100)

**Acinetobacter baumannii, Pseudomonas aeruginosa, Sphingomonas spp., Pseudomonas fluorescens*

S2 Table. Overall prevalence of extended spectrum beta-lactamase (ESBL) producing bacteria isolated from animals per country and specimen type

Bacteria	Cameroon		South Africa	
	Nasal (%)	Rectal (%)	Nasal (%)	Rectal (%)
Bordetella bronchoseptica	0	0	33 (60)	2 (5)
C. freundii	3 (3)	0	0	0
E. coli	29 (32)	42 (63)	10 (18)	17 (42.5)
Enterobacter cloacae dissolvens	8 (9)	0	0	0
K. ozanae	1 (1)	0	0	0
K. pneumoniae	19 (21)	15 (22)	0	0
S. sonnei	1 (1)	4 (6)	0	0
Others*	29 (32)	6 (9)	12 (22)	21 (52.5)
Total	91 (100)	67 (100)	55 (100)	40 (100)

**Acinetobacter baumannii, Pseudomonas aeruginosa, Sphingomonas spp., Pseudomonas fluorescens*

CHAPTER 5

Article IV. Mannitol-Fermenting Methicillin-Resistant Staphylococci (MRS) in Pig Abattoirs in Cameroon and South Africa: A Serious Food Safety Threat

Mannitol-Fermenting Methicillin-Resistant Staphylococci (MRS) in Pigs and Abattoir Workers in Cameroon and South Africa: A Serious Food Safety Threat[5]

Author contributions

- Luria Leslie Founou, as the principal investigator, co-conceptualized the study, undertook sample collection, laboratory and statistical analyses, prepared tables and figures, and drafted the manuscript.
- Raspail Carrel Founou undertook sample collection, laboratory analyses, contributed to data analysis and vetting of the results, and reviewed the manuscript.
- Cyrille Finyom Djoko as co-supervisor, contributed materials, equipment and reagents, took part in the design of the study, coordinated the field implementation in Cameroon and reviewed the manuscript.
- Sabiha Yusuf Essack, as principal supervisor, co-conceptualized the study, contributed to vetting of the results and undertook critical revision of the manuscript.

Objectives met: This original research article reports on and compares the prevalence, risk factors as well as clonal relatedness of MRS circulating in Cameroonian and South African slaughterhouses and meets objectives Three, Four, Five, Six, Seven, Nine and Ten.

[5] This paper has been submitted to International Journal of Food Microbiology and is currently under review.

Mannitol-Fermenting Methicillin-Resistant Staphylococci (MRS) in Pig Abattoirs in Cameroon and South Africa: A Serious Food Safety Threat

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Abstract

Food animals can be reservoirs of methicillin-resistant staphylococci (MRS) and are involved in their zoonotic transmission through the food chain. In Africa, there is a dearth of information about the food safety issues associated with their dissemination in the farm-to-plate continuum. This study sought to determine and compare the carriage, antimicrobial resistance profiles and clonal relatedness of circulating MRS strains among pigs and exposed workers in Cameroon and South Africa.

A total of 288 nasal and rectal pooled samples collected from 432 pigs as well as nasal and hand swabs from 82 humans were cultured on mannitol salt agar supplemented with 6 mg/L cefoxitin. Presumptive MRS were screened for methicillin resistance using the cefoxitin disc test and confirmed with the VITEK 2 system. Selected isolates underwent genomic fingerprinting via REP-PCR. Univariate and multivariate logistic regression analyses were performed to identify risk factors for MRS carriage in humans from a questionnaire survey amongst slaughterhouse workers.

Overall, 75% and 70% of nasal and rectal pooled samples were respectively positive for MRS. The MRS prevalence in all pooled pig samples from Cameroon was higher than that of South Africa. MRS prevalence of carriage (nasal and hand) was higher in Cameroonian exposed workers compared to those from South Africa, with high statistical significance. Nasal MRS colonization was highly statistically associated with hand MRS (31.58% vs 86.21%; p=0.000; OR=13.54; 95% CI 3.99-45.95; p=0.015). Recent antibiotic use, previous hospitalization, occupation of relatives, years in the employment and contact with poultry were the main risk factors identified in the emergence and spread of MRS.

MRS are emerging as serious foodborne pathogens and present a food safety threat. There is an urgent need to implement stringent and effective prevention and containment measures to curb antibiotic resistance in the farm-to-plate continuum in Cameroon and South Africa.

Keywords: Antibiotic resistance, staphylococci, methicillin resistance, food safety, food chain, One Health approach

5.1.Introduction

The family of *Staphylococcaceae* encompasses 51 species and 27 sub-species of ubiquitous bacteria occurring in grapelike clusters of Gram-positive cocci. They are grouped into coagulase-positive (CoPS) and negative (CoNS) staphylococci due to their ability to produce the coagulase enzyme (Kluytmans, 2010; Osman et al., 2016). Staphylococcal species appear worldwide as commensal colonizers of skin of animals and humans. Additionally, they are found on mucous membrane of the upper respiratory and lower urogenital tracts, and transiently in the digestive tract (Kluytmans, 2010).

S. aureus is the most important human pathogenic specie of this bacterial family and constitutes together with *S. (pseud)intermedius* to be the main pathogenic CoPS in animals. Globally, *S. aureus* asymptomatically colonizes mucous membranes of the respiratory and intestinal tracts as well as other body surfaces, but it is also frequently involved in a broad range of diseases ranging from mild skin infections to life-threatening invasive infections in humans and animals. *S. aureus* became a worldwide health problem due to the emergence of methicillin resistant *S. aureus* (MRSA) (Njoungang et al., 2015). MRSA was first reported from animals in 1972 following its isolation in milk from mastitic cows (Kluytmans, 2010). Subsequently, it was isolated from various animal species including pets, horses, pigs, poultry, sheep, veal calves and dairy cows.

Although *S. aureus* is the most important pathogen among all staphylococcal species, CoNS have recently gained importance with increasing concerns in human and animal health due to the emergence and implications of resistant strains in several human and animal infections (Bhargava and Zhang, 2012; Njoungang et al., 2015). More significantly, methicillin-resistant CoNS (MRCoNS) have been detected worldwide from food animals including pigs, poultry, calves and cows as well as food and food products (Huber et al., 2011). There is a dearth of information about MRS in food animals and food products in Africa. This study therefore sought to determine and compare the carriage, antimicrobial resistance profiles and clonal relatedness of circulating MRS strains among pigs and abattoir workers in Cameroon and South Africa, in order to ascertain the food safety threat associated with these bacteria in the farm-to-plate continuum.

5.2.Materials and methods

5.2.1. Study design and site selection

A cross-sectional, multicentre study was conducted between March and October, 2016 in three slaughterhouses/markets in Cameroon and two abattoirs in South Africa, that were encoded for

ethical reasons as SH001, SH002, SH003 and SH004 and SH005, respectively. All slaughterhouses were visited at different time points to allow better representativeness of the pig population.

In Cameroon, samples were randomly taken at the two biggest pig abattoirs (SH001 and SH002) of Yaoundé, where more than 80% of pigs are slaughtered and with annual productivity scale ranging from 25 000 to 45 000 pigs. Samples from an auxiliary abattoir (SH003) were additionally collected to ensure a geographically representative distribution since animals originating from surrounding farms of Yaoundé are typically underrepresented in these slaughterhouses. These slaughterhouses/markets were located in three geographically distinct districts of Yaoundé. In South Africa, samples were taken at two of the biggest and most productive abattoirs of the province of KwaZulu-Natal (SH004 and SH005), with annual productivity ranging from 120 000 to 150 000 pigs.

5.2.2. Ethical considerations

Ethical approvals were received from the Animal Research Ethics Committee (**Ref. AREC/091/015D**) and Biomedical Research Ethics Committee (**Ref. BE365/15**) of the University of KwaZulu-Natal as well as from the National Ethics Committee for Research in Human Health of Cameroon (**Ref. 2016/01/684/CE/CNERSH/SP**) prior the implementation of the study. Ministerial approvals from the Cameroonian Ministry of Scientific Research and Innovation (**Ref. 015/MINRESI/B00/C00/C10/C14**) and Ministry of Livestock, Fisheries and Animal Industries (**Ref. 061/L/MINEPIA/SG/DREPIA/CE**) were also obtained.

5.2.3. Sampling procedures and questionnaire

a. Procedure for animal data

A randomized sampling method of apparently healthy and freshly slaughtered/stunned pigs was undertaken in both countries. Nasal (inner cavity of both anterior nares) and rectal swabs of pigs were collected using Amies swabs without charcoal (Copan Italia Spa, Brescia, Italia). Overall, 864 swabs (432 nasal and 432 rectal) were collected from 432 pigs in Cameroon (n=216) and South Africa (n=216), with the number of specimens from each slaughterhouse (SH001, n=129; SH002, n=57; SH003, n=30; SH004, n=120; SH005, n=96) being proportionally calculated to be representative of the number of pigs slaughtered annually per site.

b. Procedure for human subjects

Saturation of sampling was applied for human participants, i.e., all abattoir workers including slaughterers, transporters, wholesalers, farmers, butchers, veterinarians, food inspectors, older than 21 years old and willing to participate were included in the study after oral and written

informed consent. Upon this step, participants were asked to complete a questionnaire addressing socio-demographic (age, gender, educational level, average monthly income) and clinical (recent hospitalization, antibiotic use, medical history related to skin or nasal infection) information, as well as other potential risk factors associated with MRS colonization and dissemination. Data on abattoir-related factors including years in the employment, intensity of contact with pigs or other animals, measures of hygiene implemented, and training were also recorded. Information regarding areas of pig breeding were also collected from workers, from either abattoir' leader, supervisor, food safety inspector or veterinarian. Both anterior nares and hands (between fingers for each right and left hand) were swabbed with Amies media and all samples were processed within 4 h after collection.

5.2.4. Laboratory analysis

For microbiological analysis, three individual pig samples were pooled per slaughterhouse and according to the gender, sample type and area of breeding. This approach resulted in 144 pooled samples representing 432 original nasal and rectal samples respectively collected from 432 pigs.

Pooled samples from pigs and human swabs were streaked onto an in-house screening mannitol salt agar supplemented with 6 mg/L cefoxitin (MSA+FOX) and incubated for 18-24 h at 37°C for MRS screening. When no growth was observed, plates were incubated for another 18-24 h. Mannitol fermenting yellow colonies, presumptive of MRS were subsequently subjected to Gram staining, catalase and oxidase for phenotypic characterization of the isolates to the genus level.

a. Species identification and antimicrobial susceptibility testing

Each colony growing on MSA+FOX and with a unique morphotype was screened for methicillin resistance using the cefoxitin disk test as recommended by the Clinical Laboratory and Standards Institute (CLSI). An inhibition zone below 21 mm was regarded as positive for methicillin resistance (CLSI, 2014). Upon this two-step screening, a representative subset of isolates, underwent phenotypic identification via Vitek® 2 System (Biomérieux, Marcy l'Etoile, France). Using an 18 to 24 h fresh culture, 1 to 2 colonies were mixed with 3.70 ml of sterile saline solution, resulting into a 0.5 turbidity on the McFarland scale. Afterward, the minimum inhibitory concentrations (MIC) of benzylpenicillin, cefoxitin, oxacillin, clindamycin, erythromycin, gentamicin, ciprofloxacin, moxifloxacin, mupirocin, rifampicin, fusidic acid, teicoplanin, vancomycin, linezolid, tigecycline, trimethoprim-sulfamethoxazole, were determined by the broth micro-dilution method using Vitek® 2 System (BioMérieuX,

Marcy l'Etoile, France) and VITEK® 2 Gram Positive Susceptibility card (AST-P603) (bioMérieux, Marcy l'Etoile, France). The results were interpreted according to the CLSI guidelines (CLSI, 2014) and *S. aureus* ATCC 29213 was used as the control.

b. Genomic extraction

Genomic DNA was extracted from a subset of MRS strains selected on the basis of their antimicrobial resistance profiles. Samples were cultured in 3 ml of Tryptone soya broth with moderate shaking for 18 h at 37ºC in normal atmosphere. After incubation 1.5 ml of broth was centrifuged at 12,000 g for 10 min and the pellet was prepared for genetic analysis using the Thermo Scientific® GeneJet Genomic DNA purification kit (Thermo Fisher Scientific, South Africa) according to the manufacturer's instructions. DNA templates were stored at -20°C until used.

c. Genotypic relatedness determination of methicillin resistant staphylococci

i. Repetitive element palindromic-polymerase chain reaction (REP-PCR)

To establish the link of selected MRS strains from animals and humans within and between abattoirs and countries, the REP-PCR was performed with (GTG)5 primer 5'-GTGGTGGTGGTGGTG-3' (Švec et al., 2010). REP-PCR reactions were carried out in a 10 µl final solution containing 0.1 µl of (GTG)5 primer (100 µM), 5 µl Dream*Taq* Green Polymerase Master Mix 2× (Thermo Fisher Scientific, South Africa), 2.9 µl nuclease free water and 2 µl DNA template. Reactions were run in a 0.2 ml micro-centrifuge tube using a programmable thermal cycler (Bio-Rad, Johannesburg, South Africa) according to the following protocol: initial denaturation at 94°C for 7 min, 30 cycles consisting of a denaturation step at 94°C for 1 min, primer annealing at 40°C for 1 min, extension at 65°C for 8 min, a final extension step at 65°C for 16 min and final storage at 4°C. The amplicons were run on a 1.5% (wt/vol) agarose (Merck Diagnostic, Johannesburg, South Africa) gels along with the Quick Load® 1-kb DNA ladder (New England Biolabs, USA) in an electric field of 100 V for 1 h. Gels were subsequently stained in a solution containing 0.5 mg/ml ethidium bromide (10 mg/ml) for 15 min. Amplification products were visualized by UV trans-illuminator with images captured using a gel documentation system (Syngene, Johannesburg) and recorded for further analyses.

ii. Computer-Assisted DNA Fingerprint Analysis

DNA profiles were digitized for analysis using Bionumerics software (version 7.6, Applied Maths, TX, USA). All DNA fragment sizes within each gel were normalized using the Quick Load® 1-kb DNA molecular weight marker as external reference standard. The similarity

between each strain was determined from the homology matrix using Dice coefficient. Dendrograms were constructed based on the averaged similarity of the matrix using the algorithm Unweighted Pair-Group Method (UPGMA) with optimization and band tolerance set a 1% (version 7.6, Applied Maths, TX, USA). The cophenetic correlation value was calculated for the dendrogram, in order to measure the reliability of generated clusters. Clusters were defined based on a similarity cut-off of 80%.

5.2.5. Data analysis

Data was coded and entered into Excel spreadsheet (Microsoft Office 2016) and Epi Info (version 7.2, CDC, Atlanta, GA, USA), double-checked and verified with the questionnaire and analysed using Excel (Microsoft Office 2016) and STATA (version 14.0, STATA Corporation, TX, USA). A data set was created for individual human results and, aggregated animal and abattoir data. Abattoirs were classified as MRS-positive if an MRS strain was detected from at least one pooled sample (nasal or rectal). Prevalence of MRS was compared between categories (viz. type of sample, country and abattoir) using the chi square test. A p-value < 0.05 was considered as statistically significant. The relationship between MRS carriage in humans and pigs was calculated with the logistic regression analysis adjusted for clustering at abattoir level. Similarly, risk factors for MRS colonization including pig contamination, training, principal abattoir activities, previous hospitalization, antibiotic use, occupation of relatives and potential confounders such as age, gender, monthly income, educational level, and contact with other animals, were ascertained univariately and selected for multivariate analysis when the p-value was <0.2. Model fit was checked with the McFadden's pseudo R^2 statistic (maximum likelihood method) and the final model built included all determinants for which the pseudo R^2 was the most elevated with $p<0.05$ for each dependent variable.

5.3.Results

5.3.1. Participant characteristics

Out of 114 people contacted in the five abattoirs, 84 were enrolled for an overall participation rate of 73%. The response rate was greater in Cameroon (71%) than in South Africa (59%), with the most common reason for non-participation being lack of time or interest and fear of the sampling procedure and of the results. Seventy-seven filled out the questionnaire and provided both nasal and hand swabs, six provided questionnaire and hand swabs only, while one withdrew before the sampling step.

Table 1 shows nasal and hand MRS carriage of exposed workers in relation to individual, clinical and abattoir-related characteristics. The prevalence of nasal MRS carriage was significantly higher in Cameroonian workers than South African ones (92.45% vs 29.17; p=0.000) as was hand MRS carriage (100% vs 23.3%). Workers with a lower educational level, lower monthly income and without training for their profession were more likely to be colonized by MRS for both types of samples.

5.3.2. Risk factors of MRS carriage in humans

Table 5.2 (univariate and multivariate logistic regression) shows the association between MRS carriage in humans and the main potential risk factors. Recent antibiotic use, recent hospitalization, occupation of relatives, years in the employment, intensity of contact with other animals and contact with poultry, were identified via univariate logistic regression as the main risk factors for nasal and hand MRS carriage. In contrast, training, protective working clothes and convenient handwashing were found to significantly reduce the odds of being colonized by MRS for both nares and hands (Table 2).

Nasal MRS carriage was highly statistically associated with hand colonization of MRS (31.58% vs 86.21%; p=0.000) with an odds ratio (OR) of 13.54 (95% CI 3.99-45.95; p=0.015). The univariate analysis further reveals that nasal and hand MRS carriage were however not associated with rectal (OR=1.01, 95% CI 0.97-1.05; p=0.544; vs. OR=0.98, 95% CI 0.94-1.03; p=0.494) and nasal MRS contamination (OR=1.04, 95% CI 0.99-1.09; p=0.087 vs. OR=1.03, 95% CI 0.99-1.08; p=0.146) in pigs, although without statistical significance. In contrast, nasal and hand MRS carriage in humans were associated with contact with other animals, especially poultry for both samples (OR=3.6, 95% CI 0.94-19.70, p=0.060; vs OR=13.68, 95% CI 1.72-108.43, p=0.013; Table 5.2).

5.3.3. MRS status in humans

In total, out of 53 workers sampled in Cameroon, 49 (92%) and 53 (100%) were nasal and hand carriers of MRS, respectively. Due to financial constraint, further selection based on statistical analysis was carried out to select a subset of 21 non-duplicate (hand=11, nasal=10) human MRS isolates representative of the main population for phenotypic analysis. Out of these presumptive MRS isolated in Cameroon, 21 (100%) all were confirmed via VITEK 2 system as *Staphylococcus* spp. and antimicrobial susceptibility testing (with cefoxitin disc synergy and VITEK 2 system) revealed that 20/21 (95%) were MRS. The main species identified were *S. haemolyticus* (6), *S. epidermidis* (5), and *S. lentus* (4) (Table 5.3). In contrast, in South Africa,

MRS carriage was observed in 29% (7/24) and 23% (7/30) of nasal and hand swabs, respectively. All 14 (100%) isolates were phenotypically confirmed as *Staphylococcus spp.* with 100% being MRS and *S. haemolyticus* (n=9) being the main specie.

5.3.4. Epidemiological background of MRS in pigs

From the five abattoirs, 144 pooled nasal samples (three nasal swabs each, proportional to abattoir productivity, 72 per country) and 144 pooled rectal samples (three rectal swabs each, proportional to abattoir productivity, 72 per country) taken from 432 pigs were analysed. Altogether, MRS were isolated from 108/144 (75%) and 130/144 (90%) of the pooled nasal and rectal samples, respectively (Table 5.4). When comparing the results at country-level, a maximum level of MRS (100%) was detected in both type of pooled samples in Cameroon, whereas 50% (36/72) and 81% (58/72) MRS were isolated in nasal and rectal pooled samples, respectively, in South Africa with high statistical significance (p=0.000) (Table 5.4).

Upon further selection, 13 pig isolates (nasal=7 and rectal=6) representative of the main population and detected from 15 pooled samples in Cameroon were phenotypically confirmed as staphylococci. *S. lentus* (10) was the main species identified in both type of samples (Table 5.5). In South Africa, 30 presumptive MRS (9 nasal and 21 rectal) strains underwent phenotypic identification, with 24 (80%) being confirmed as MRS. *S. lentus* (14) and *S. aureus* (6) were the foremost species isolated in both type of pooled samples (Table 5.5).

5.3.5. Prevalence of MRS based on time points

Figures 5.1A and 5.1B illustrate the rectal and nasal MRS colonization in pigs per abattoir and at each time point, respectively. Overall, in South Africa, the prevalence of MRS was unsteady across time for both type of samples. At abattoir level, maximum prevalence of 91 and 90% MRS were recorded in SH005 during the first and second time point (p=0.000), respectively and for both sample type; whereas less than 40% prevalence was detected in SH004 at all time points for nasal sample (Figure 5.1). MRS positivity associated with rectal colonization was higher in the country at the second and third point of collection with 90 and 80% prevalence, respectively. In contrast, 100% MRS carriage was detected at all time-points and for both type of pooled samples in Cameroon.

5.3.6. Antimicrobial resistance patterns in pig MRS isolates

High resistance was observed for oxacillin (100%), cefoxitin (94%), penicillin (90%), tetracycline (90%), clindamycin (89%) and erythromycin (76%) with no resistance to ciprofloxacin, moxifloxacin, tigecycline, vancomycin, linezolid, trimethoprim/sulfamethoxazole and mupirocin in MRS isolated from pigs in South Africa.

Similarly, high resistance to oxacillin (100%), tetracycline (93%), penicillin (77%), cefoxitin (70%), fusidic acid (47%), erythromycin (30%) and clindamycin (30%) were detected in pig isolates originating from Cameroon, with one strain expressing resistance to teicoplanin, linezolid, and vancomycin (Table 5.5).

All *S. aureus* isolated from pigs in South Africa showed the resistance profile Penicillin.Cefoxitin.Oxacillin.Erythromycin.Clindamycin.Tetracycline whereas the unique strain detected in Cameroon expressed the pattern Penicillin.Cefoxitin.Oxacillin.Tetracycline (Table 5.6). The resistance profiles Penicillin.Cefoxitin.Oxacillin.Gentamicin.Erythromycin.Clindamycin.Tetracycline and Oxacillin.Erythromycin.Clindamycin.Tetracycline were prevalent among *S. lentus* in South Africa while Penicillin.Cefoxitin.Oxacillin.Tetracycline. Fusidic acid and Penicillin.Oxacillin.Erythromycin.Tetracycline.Fusidic acid were the predominant patterns detected in the same species in Cameroon. Moreover, two *S. lentus* strains isolated from rectal pooled samples in Cameroon showed the profiles Penicillin.Cefoxitin.Oxacillin. Erythromycin.Clindamycin.Linezolid.Teicoplanin.Vancomycin.Rifampicin.Fusidic acid and Cefoxitin.Oxacillin.Linezolid.Clindamycin.Rifampicin.Fusidic acid with resistance to seven and five classes of antibiotics, respectively, including the last resort linezolid, vancomycin and teicoplanin antibiotics (Table 5.6).

5.3.7. Antimicrobial resistance patterns in human MRS isolates

Human MRS isolated from South African abattoirs expressed high resistance to penicillin (100%), cefoxitin (100%), oxacillin (96%), trimethoprim-sulfamethoxazole (80%), gentamicin (49%) and tetracycline (33%) (Table 3). In addition, two strains isolated from hand swabs were resistant to teicoplanin (MIC= 8, 16 µg/ml). Likewise, high resistance to oxacillin (100%), penicillin (96%), cefoxitin (92%), tetracycline (84%), erythromycin (51%) and fusidic acid (52%) was detected in human MRS strains from Cameroonian workers. However, no resistance to vancomycin, mupirocin, tigecyclin, linezolid and rifampicin was observed in human strains from both countries (Table 5.3).

Table 5.7 presents the resistance patterns detected in MRS in humans in both countries. Penicillin.Cefoxitin.Oxacillin.Ciprofloxacin.Moxifloxacin.Erythromycin.Tetracycline.Trimethoprim-Sulfamethoxazole, Penicillin.Cefoxitin.Oxacillin.Gentamicin.Ciprofloxacin.Tetracycline.Trimethoprim-Sulfamethoxazole and Penicillin.Cefoxitin.Oxacillin.Gentamicin.Ciprofloxacin. Erythromycin.Tetracycline.Trimethoprim-Sulfamethoxazole.Fusidic acid,

Penicillin.Cefoxitin.Oxacillin.Ciprofloxacin.Gentamicin.Tetracycline.Trimethoprim-Sulfamethoxazole were the most relevant profiles observed in *S. haemolyticus* in hand and nasal samples, respectively, in Cameroon. Similarly, the most relevant resistance patterns detected in *S. haemolyticus* in South Africa were Penicillin.Cefoxitin.Ciprofloxacin. Erythromycin.Tetracycline.Trimethoprim-Sulfamethoxazole.Teicoplanin.Fusidic acid, Penicillin.Cefoxitin.Erythromycin.Tetracycline.Trimethoprim-Sulfamethoxazole and Penicillin.Cefoxitin.Oxacillin.Gentamicin.Tetracycline.Trimethoprim-Sulfamethoxazole.Fusidic acid, Penicillin.Cefoxitin.Oxacillin.Gentamicin.Ciprofloxacin.Erythromycin.Trimethoprim-Sulfamethoxazole in hand and nasal samples, respectively (Table 5.7).

5.3.8. Genotypic relatedness

All *S. aureus* and the most resistant *S. lentus* strains as the most prevalent CoPS and CoNS respectively, were genotyped by REP-PCR to determine their clonal relationships. REP-PCR allowed the differentiation of *S. aureus* strains into one major cluster and *S. lentus* into four clusters (Figure 5.2). One batch of *S. aureus* isolates (PN235B0, PN246B0, PN243B0 and PR243B0) detected in one South African abattoir (SH004) were closely related and share common ancestors as did a batch of *S. lentus* strains (PN085, PR226B0, and PR108) detected in pigs in two Cameroonian (SH002 and SH003) and one South African (SH004) abattoirs (Figure 5.2).

5.4. Discussion

S. aureus and especially MRSA, were recently recognized as high priority antibiotic resistant bacteria by the World Health Organization (WHO, 2017) as their existence at the animal-human-environment interface leads to grave socio-economic repercussions globally. In this study, the carriage, antimicrobial resistance patterns and clonal relatedness of MRS isolated from apparently healthy pigs and exposed workers in Cameroon and South Africa were compared. All MRS strains formed yellow colonies on MSA+FOX, characteristic of mannitol-fermenting isolates. Although the latter screening medium is intended for the detection of MRSA, our findings concur with a Nigerian study which reported that MRCoNS colonies were similar to MRSA ones (Ugwu et al. 2015). These results reinforce the need for appropriate diagnostic of mannitol-fermenting MRS.

5.4.1. MRS in humans

Among people working on pig abattoirs, absence of training, frequent contact with pigs and other animals were associated with MRS carriage. The overall prevalence of MRS carriage in

humans was highly statistically significant in hand [72% (60/84), p=0.000] and nasal samples [73% (56/77), p=0.000]. The prevalence reported in our study is consistent with a study from Switzerland where 49.3% of MRCoNS were detected among exposed workers (Huber et al., 2011). Our results are however, higher than that reported by Gulani et al. (2016) where a 13.5% prevalence of MRSA was detected in animal handlers in Nigeria. The prevalence of MRS carriage was significantly higher in Cameroonian workers than South African ones for both nasal (92.45% vs 29.17; p=0.000) and hand (100% vs 23.3%; p=0.000) samples. These discrepancies between both countries could be because that abattoir regulations, implementation of Hazard Analysis Critical Control Points (HACCP) plans and compliance with international food safety standard ISO 22000 are stringent in South Africa whereas Cameroonian slaughterhouses/markets were characterized by precarious hygienic conditions with minimal or non-existent biosecurity measures and absence of regulations. This has been confirmed by Ndebi et al. (2009) who already reported that the pig production industry in Cameroon is undermined by a mosaic of problems such as insufficient funds, poor sanitary and feeding requirements, sub-optimal transport conditions, lack of veterinarian control that leads to the emergence and spread of ABR in the farm-to-plate continuum. Our results therefore suggest that the implementation of effective food safety measures contributes to the containment of ABR dissemination.

The presence of MRS in nasal samples was highly statistically associated with hand colonization (31.58% vs 86.21%, p=0.000, OR=13.54; 95% CI 3.99-45.95; p=0.015). These findings reveal poor implementation of hygienic and sanitary conditions and suggest that these limited measures along with high prevalence of colonized food handlers represent an important food safety threat in both countries. It further suggests that food handlers represent a significant source of ABR dissemination for their relatives through person-to-person contact and for the general population through the contamination of food products occurring during food processing.

5.4.2. MRS in animals

The 75% and 70% positivity of all pooled nasal and rectal samples for MRS respectively, are higher than that reported from Nigeria where 31% and 52% of MRS were detected in pigs and pork, respectively (Ugwu et al., 2015; Igbinosa et al., 2016). They are however lower than that reported by Bhargava and Zhang (2012) where 100% of MRS were observed in nasal and rectal swabs of various food animals including pigs, sold during a livestock auction in Ohio. The high

prevalence reported in our study could be associated with extensive use of antimicrobials in the food production industry.

When comparing the results at country-level, animals sampled in Cameroon were statistically significantly more colonized than those collected in South Africa with a maximum prevalence of MRS (100%) for both type of samples versus 50% and 81% MRS in nasal and rectal pooled samples, respectively, in South Africa (p=0.000). The elevated prevalence of MRS carriage detected in both type of pooled samples in South Africa and Cameroon is not surprising, as the use of antibiotics as growth promoters is legally approved in the former (Department of Agriculture, Forestry and Fisheries, 1996) and, policies promoting rational drug use are non-existent in both animals and humans in the latter. Ndebi et al. (2009) already revealed that antibiotics of human medicine are frequently use for the prevention and treatment of infectious diseases in pig husbandry in Cameroon, that the current state of knowledge of antibiotic use and antibiotic resistance (ABR) in humans, (food) animals, and environment is minimal or non-existent and that the debate about ABR-related consequences is neglected in the country.

Our results further indicate that MRS are widespread in food animals and abattoirs in both countries, can spread actively in the farm-to-plate continuum and pose a serious food safety threat for these nations. The rectal colonization reported in our study could be attributed to widespread antibiotic use on farms either for therapeutic, prophylactic or growth promotion purposes whereas the nasal MRS carriage is likely associated with environmental contamination.

5.4.3. MRS based on time points

The overall high MRS prevalence observed across time points in both countries suggests that measures implemented in both countries are suub-optimal for the effective eradication and containment of MRS. This has been confirmed by the maximum MRS prevalence recorded during the first (91%) and second (90%) time point for rectal sample in SH005 whereas 92% and 86% of MRS was detected in SH004 at second and thrid time points for the same sample type in South Africa (Figure 1). Similarly, the high nasal MRS prevalence observed in both countries evidences the persistance of MRS not only in living food animals but also on environmental surfaces that leads to their dissemination across the animal-human-environmental interface. It further reveals that decontamination measures implemented in abattoirs should be updated and consider novel concepts that address environmental persistence of MRS.

5.4.4. Antimicrobial resistance patterns

The analyses of resistance profiles revealed more than 80% resistance to β-lactams including penicillin, cefoxitin and oxacillin in both countries and for both populations. The phenotype P.FOX.OX.E.CLI.TET showing resistance to six antibiotics was the most common in MRSA strains isolated from rectal and nasal pooled samples in South Africa. One *S. lentus* strain isolated from rectal pooled sample in Cameroon displayed the profile P.FOX.OX.E.CLI.LIN.TEI.VAN.FA.RIF with resistance to ten antibiotics including the following last resort antibiotics; teicoplanin, vancomycin and linezolid. This confirms MRS as significant food contaminants, reservoir of and potential vectors for dissemination of ABR in the food chain.

Co-resistance to non-beta-lactam antibiotics, including trimethoprim-sulfamethoxazole, erythromycin, clindamycin, fusidic acid, gentamicin and ciprofloxacin, was also observed. Such co-resistance to other antibiotic classes along with resistance to beta-lactams is frequent in MRS and could likely result from the indiscriminate and/or extensive antibiotic use in food animals in both countries (Asongalem et al., 2015). The presence of mobile genetic elements such as the *Staphylococcal Cassette Chromosome* (*Scc*) harbouring the *mec*A gene responsible for the methicillin resistance could also explained our results and be responsible of further resistance (Bhargava and Zhang, 2012). These results further reveal that MRS strains may transfer their resistance genes across susceptible species or genus through horizontal gene transfer in the farm-to-plate continuum.

5.4.5. Genotypic relatedness

REP-PCR analysis, demonstrated that isolates from pig origin exhibited varying levels of genetic variability and presented both related and unrelated patterns. The antibiotic resistance and REP patterns revealed relative association between animal and human strains within and across countries. Although no MRSA strains were detected in humans, some MRCoNS detected from humans share common ancestors with those isolated from pigs at the same or different abattoir. These results suggest that emergence of MRCoNS in humans may be of animal origin or vice-versa, and that these pathogens may be transferred to humans via the food chain, enabling them to subsequently enter community or healthcare settings. Similarly, MRSA strains detected in pigs revealed association within the same abattoir. Although not being an indication of MRS transmission dynamics, our results nonetheless show that some genetic relatedness exists between animal and human isolates due to similar clustering. This finding is of great concern as MRS originating from animals have been reported to be amongst the causative agents of clinical infections globally (Kluytmans, 2010).

5.4.6. Association of MRS carriage in humans and risk factors

Twenty-one workers or their close relatives had been hospitalized within a year of the sample collection, leading to an overall 95.24% (p=0.007) and 85.71% (p=0.112) prevalence of nasal and hand MRS, respectively (Table 5.1). Likewise, 92.11% (p=0.000) and 92.11% (p=0.000) of workers who had consumed antibiotics the month preceding the sampling were colonized by MRS in nasal and hand samples, respectively (Table 5.1). Although the duration of MRS carriage and resistance genes involved were not investigated, the study reveals that no association between human MRS carriage and contact with MRS carrying pigs was observed for all type of samples, although not statistically significant (Table 5.2). In contrast, a clear association between human MRS carriage and contact with other animals, especially poultry, was observed and with high statistical significance (Table 5.2). We showed that when workers have been trained to practice their profession, convenient handwashing facilities were available and wearing of protective clothes was implemented, the odds of being colonized by MRS were significantly reduced. Our findings also reveal that the implementation of food safety measures is imperative to contain the dissemination of ABR in general and MRS in particular.

The scarcity of similar studies precluded robust comparison of results. We therefore discussed our findings with data considered most appropriate and as close as possible to our own results. More research is required on MRS carriage in high risk human populations and other food animals such as poultry in order to improve our knowledge on the public health significance associated with the likely transmission of MRS through the farm-to-plate continuum.

5.5. Conclusion

Our findings indicate that MRS are emerging and serious foodborne pathogens of grave concern. The high prevalence of MRS reported in humans and animals in both Cameroon and South Africa highlights their active dissemination in the food chain, their contamination of food products and the subsequent food safety threat associated with these bacteria in these countries. It underscores the need for the implementation of appropriate and suitable food safety measures to contain their spread.

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Conflict of interest

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Table 5.1. Nasal and hand MRS carriage of exposed workers in relation to individual, clinical and abattoir-related characteristics[6].

Variables	Nasal sample				Hand sample			
	Frequency		Prevalence MRS (%)	Overall p-value	Frequency		Prevalence MRS (%)	Overall p-value
	n	%			n	%		
Individual characteristics								
Country								
Cameroon	53	69	92.45	0.000	53	64	100	0.000
South Africa	24	31	29.17		30	36	23.3	
Gender								
Female	9	12	88.89	0.247	12	12	75	0.821
Male	68	88	70.59		71	88	71.83	
Age								
[21-30]	31	40	61.29	0.231	32	39	59.38	0.229
[31-40]	26	34	76.92		28	34	78.57	
[41-50]	13	7	84.62		14	7	85.71	
[51-60]	5	6	100		6	7	83.33	
Above 60	2	3	50		3	3	66.67	
Educational level								
Never been to school	4	5	50	0.003	5	6	60	0.032
Primary school not completed	6	8	33.33		7	8	57.14	
Primary school	34	44	94.12		35	42	94.29	
Secondary school	27	35	59.26		27	33	55.56	
High school/university	6	8	66.67		8	10	62.50	
Average monthly income (US $)								
Below 55	8	11	100	0.025	8	14	100	0.019
55-110	14	19	85.75		14	29	85.71	
110-165	12	16	75		12	17	83.33	
165-220	10	13	80		10	17	80	
220-275	20	27	45		24	10	45.83	
Above 275	11	15	81.82		13	12	69.23	
Clinical factors								
Previous hospitalization (within the year of sampling)								
Yes	21	27	95.24	0.007	21	25	85.71	0.112

[6] Six out of the 84 enrolled workers refused the nasal sampling, one withdrew prior to the sample collection, leading to a total of 77 nasal and 83 hand samples collected. Few questions were not answered by workers while other could not recall the exact information leading to missing information that were not considered in the analysis.

No	56	73	64.29		62	75	67.74	
Nasal problem								
Yes	11	14	63.64	0.465	11	13	63.64	0.491
No	66	86	74.24		72	87	73.61	
Skin problem								
Yes	14	18	71.43	0.904	14	17	64.29	0.463
No	63	82	73.02		69	83	73.91	
Antibiotic use (within the month of sampling)								
Yes	38	49	92.11	0.000	38	64	92.11	0.000
No	39	51	53.85		45	36	55.56	
Relative working at hospital or with animal								
Yes	42	55	90.48	0.000	44	53	88.64	0.000
No	35	45	51.43		39	47	53.85	
Abattoir-related factors								
Proximity of abattoir with house								
Yes	32	42	59.38	0.027	14	33	64.71	0.199
No	45	58	82.22		28	67	75.55	
Abattoir								
SH001	21	27	100	0.000	21	25	100	0.000
SH002	19	25	89.47		19	23	100	
SH003	13	17	84.62		13	16	100	
SH004	4	5	50		10	12	40	
SH005	20	26	25		20	24	15	
Principal activity or working area								
Slaughterer	34	44	76.47	0.002	34	41	76.47	0.001
Transport of pig/pork	5	7	80		5	6	80	
Wholesaler	7	9	100		7	8	100	
Butcher	5	7	100		5	6	80	
Retailer of viscera*	7	9	85.71		7	8	100	
Retailer of grilled pork#	1	1	0		1	1	100	
Scalding of pigs	3	4	0		3	4	33.33	
Evisceration	8	10	37.50		14	17	28.57	
Transport of viscera/blood	1	1	0		1	1	0	
Veterinarian	5	7	100		5	6	100	
Meat inspector	1	1	0		1	1	0	
Training to practice profession								
Yes	28	36	39.29	0.000	34	41	32.35	0.000
No	49	64	91.84		49	59	100	
Year in profession								
[0-4]	31	43	51.61	0.007	31	39	51.61	0.011
[5-9]	6	8	83.33		8	10	100	
[10-14]	22	30	86.36		24	30	75	
Above 15	14	19	92.86		16	20	87.50	
Intensity of pig's contact								
Always	35	45	68.57	0.721	35	42	77.14	0.032
Almost always	32	42	75		38	46	60.53	
Sometimes	10	13	80		10	12	100	
Contact with other animals								
Yes	38	50	86.84	0.009	39	48	92.31	0.001
No	38	50	60.53		42	52	54.76	
Intensity animal contact								

Always	8	21	87.50	0.773	8	20	100	0.024
Almost always	9	24	77.78		10	26	70	
Sometimes	17	45	88.24		17	44	100	
Rarely	4	10	100		4	10	100	

retailer of viscera*: street-vendor purchasing pig's viscera from the abattoir, undertaking manual cleaning and sell ready-to-eat meal; #*retailer of grilled pork***: street-vendor purchasing pork at the abattoir and sell ready-to-eat grilled pork.

Table 5.2. Univariate and multivariate logistic regression for methicillin resistant staphylococci (MRS) carriage among exposed workers

Variables	Univariate Analysis				Multivariate Analysis			
	Nasal carriage of MRS		Hand carriage of MRS		Nasal carriage of MRS		Hand carriage of MRS	
	OR (95% CI)	*P*	OR (95% CI)	*P*	OR (95% CI)	*P*	OR (95% CI)	*P*
Abattoir	0.28 (0.16-0.48)	**0.000**	0.08 (0.03-0.26)	**0.000**	….		…..	
Hand MRS	13.54 (3.99-45.95)	**0.000**	…	…	…		…	
Nasal MRS	…	…	13.54 (3.99-45.95)	**0.000**	…		…	
Gender	0.3 (0.04-2.55)	0.271	0.85 (0.21-3.46)	0.682	….		….	
Educational level	0.98 (0.57-1.70)	0.960	0.76 (0.45-1.28)	0.306	….		….	
Monthly Income	0.67 (0.47-0.96)	0.031	0.60 (0.42-0.87)	**0.006**	…		…	
Training	0.06 (0.02-0.21)	**0.000**	1		…		…	
Principal Activities	0.71 (0.58-0.88)	0.001	0.7 (0.57-0.85)	**0.000**	…		…	
Occupation of relative [a]	8.97 (2.63-30.54)	**0.000**	6.68 (2.17-20.57)	**0.001**	7.09 (1.60-31.46)	**0.010**	3.51 (0.88-14.00)	0.076
Year in Profession	2.66 (1.30-5.43)	**0.007**	2.28 (1.27-4.11)	**0.006**	2.18 (1.01-4.70)	**0.046**	4.11 (1.53-11.00)	**0.005**
Age	1.45 (0.84-2.49)	0.177	1.39 (0.86-2.25)	0.178	…		…	
Recent hospitalization [b]	11.11 (1.38-89.06)	**0.023**	2.86 (0.75-10.84)	0.123	11.93 (1.23-115.76)	0.069	1.51 (0.28-7.98)	0.626
Recent antibiotic use [c]	9.99 (2.63-38.06)	**0.001**	9.33 (2.50-34.85)	**0.001**	…		…	
Skin problem	0.92 (0.25-3.34)	0.904	0.63 (0.19-2.15)	0.465	…		…	
Nasal problem	0.61 (0.16-2.33)	0.468	0.63 (0.16-2.38)	0.494	…		…	
Proximity of house with abattoir	0.31 (0.11-0.89)	0.030	0.53 (0.20-1.40)	0.201	…		…	
Protective working clothes	0.05 (0.01-0.22)	**0.000**	1		…		…	
Inadequate Handwashing	1		3.38 (0.40-28.70)	0.264	…		…	
Convenient handwashing	0.07 (0.02-0.25)	**0.000**	0.07 (0.02-0.21)	**0.000**	…		…	
Intensity of contact with pigs	1.36 (0.64-2.88)	0.422	1.15 (0.56-2.38)	0.696	…		…	
Contact with other animals	1.37 (0.50-3.75)	0.530	1.73 (0.47-6.34)	0.407	…		…	
Intensity of contact with other animals	4.30 (1.37-13.51)	**0.012**	9.91 (2.63-37.31)	**0.001**	…		…	
Contact with poultry	3.6 (0.94-13.70)	**0.060**	13.68 (1.72-108.43)	**0.013**	…		…	
Pig contamination Nasal MRS (yes or no)	1.04 (0.99-1.09)	0.087	1.03 (0.99-1.08)	0.146	1.12 (0.93-1.35)	0.219	1.28 (1.09-1.50)	**0.003**
Pig contamination Rectal MRS (yes or no)	1.01 (0.97-1.05)	0.544	0.98 (0.94-1.03)	0.494	0.91 (0.76-1.10)	0.352	0.77 (0.65-0.92)	**0.004**
Sow	1.09 (1.01-1.18)	**0.031**	1.05 (0.99-1.13)	0.112	…		…	
Boar	0.84 (0.76-0.93)	**0.001**	0.68 (0.57-0.83)	**0.000**	…		…	

a: Family members working at hospital, with food animals or crop production, b: Exposure within 12 months prior the date of sampling; c: Within one month prior the date of sampling

Table 5.3. Distribution of *Staphylococcus spp.* isolated from humans in Cameroon and South Africa according to their species diversity and multidrug resistance

***Staphylococcus spp.* (n=isolates)**	**Resistance to antibiotics (%)**															
	Penicillin	**Cefoxitin**	**Oxacillin**	**Gentamicin**	**Ciprofloxacin**	**Moxifloxacin**	**Erythromycin**	**Clindamycin**	**Linezolid**	**Teicoplanin**	**Vancomycin**	**Tetracycline**	**Tigecycline**	**Fusidic acid**	**Rifampicin**	**Trimethoprim-Sulfamethozaxole**
Cameroon (n=20)																
S. epidermidis (5)	100	100	100	60	0	0	80	0	0	0	0	80	0	20	0	40
S. haemolyticus (6)	100	100	100	83	100	17	50	0	0	0	0	50	0	17	0	83
S. hominis (2)	100	100	100	0	0	0	100	100	0	0	0	100	0	50	0	0
S. lentus (4)	75	50	100	25	0	0	25	0	0	0	0	75	0	75	0	25
S. sciuri (1)	100	100	100	0	0	0	0	0	0	0	0	100	0	100	0	0
S. warneri (2)	100	100	100	0	0	0	50	0	0	0	0	100	0	50	0	0
South Africa (n=14)																
S. epidermidis (1)	100	100	100	0	0	0	0	0	0	0	0	0	0	0	0	100
S. haemolyticus (9)	100	100	78	44	33	0	44	0	0	11	0	67	0	22	0	100
S. hominis (1)	100	100	100	0	0	0	0	0	0	0	0	0	0	0	0	0
S. lentus (2)	100	100	100	100	100	0	100	0	0	0	0	100	0	0	0	100
S. lugdunensis (1)	100	100	100	100	0	0	0	0	0	0	0	0	0	0	0	100

0; >0-10; >10-20; >20-30; >30-40; >40-50; >50-60; >60-70; >70-80; > 80-90; >90-100

Table 5.4. Methicillin resistant staphylococci in nasal and rectal pooled samples

Characteristics	Frequency Pooled samples, n (%)	Nasal MRS, n (%)	Overall p-value	Frequency Pooled samples, n (%)	Rectal MRS, n (%)	Overall p-value
Country						
Cameroon	72 (50)	72 (100)	0.000	72 (50)	72 (100)	0.000
South Africa	72 (50)	36 (50)		72 (50)	58 (81)	
Gender						
Sow	79 (55)	67 (85)	0.003	79 (55)	76 (96)	0.008
Boar	65 (45)	41 (63)		65 (45)	54 (83)	
Abattoir						
SH001	43 (30)	43 (100)	0.000	43 (30)	43 (100)	0.001
SH002	19 (13)	19 (100)		19 (13)	19 (100)	
SH003	10 (7)	10 (100)		10 (7)	10 (100)	
SH004	40 (28)	12 (30)		40 (28)	30 (75)	
SH005	32 (22)	24 (75)		32 (22)	28 (88)	
Time point						
First	42 (29)	32 (76)	0.221	42 (29)	35 (83)	0.102
Second	54 (38)	44 (82)		54 (38)	52 (96)	
Third	48 (33)	32 (67)		48 (33)	43 (90)	

Table 5.5. Distribution of *Staphylococcus spp.* isolated from pigs in Cameroon and South Africa according to their species diversity and multidrug resistance

***Staphylococcus spp.* (n=isolates)**	**Resistance to antibiotics (%)**																
	Penicillin	**Cefoxitin**	**Oxacillin**	**Gentamicin**	**Ciprofloxacin**	**Moxifloxacin**	**Erythromycin**	**Clindamycin**	**Linezolid**	**Teicoplanin**	**Vancomycin**	**Tetracycline**	**Tigecycline**	**Mupirocin**	**Fusidic acid**	**Rifampicin**	**Trimethroprim-Sulfamethoxazole**
Cameroon (n=13)																	
S. aureus (1)	100	100	100	0	0	0	0	0	0	0	0	100	0	0	0	0	0
S. lentus (10)	80	60	100	0	0	0	40	40	20	10	10	80	0	0	90	20	10
S. sciuri (2)	50	50	100	0	0	0	50	50	0	0	0	100	0	0	50	50	50
South Africa (n=24)																	
S. aureus (6)	100	100	100	0	0	0	100	100	0	0	0	100	0	0	0	0	0
S. epidermidis (1)	100	100	100	100	0	0	100	100	0	100	0	100	0	0	0	0	0
S. hominis (2)	100	100	100	50	0	0	100	100	0	0	0	50	0	0	0	0	0
S. lentus (14)	50	70	100	14	0	0	79	43	0	0	0	100	0	0	57	14	0
S. warneri (1)	100	100	100	0	0	0	0	100	0	0	0	100	0	0	0	0	0

0; >0-10; >10-20; >20-30; >30-40; >40-50; >50-60; >60-70; >70-80; > 80-90; >90-100

Table 5.6. Antimicrobial resistance profiles of methicillin resistant staphylococci (MRS) isolated from pigs

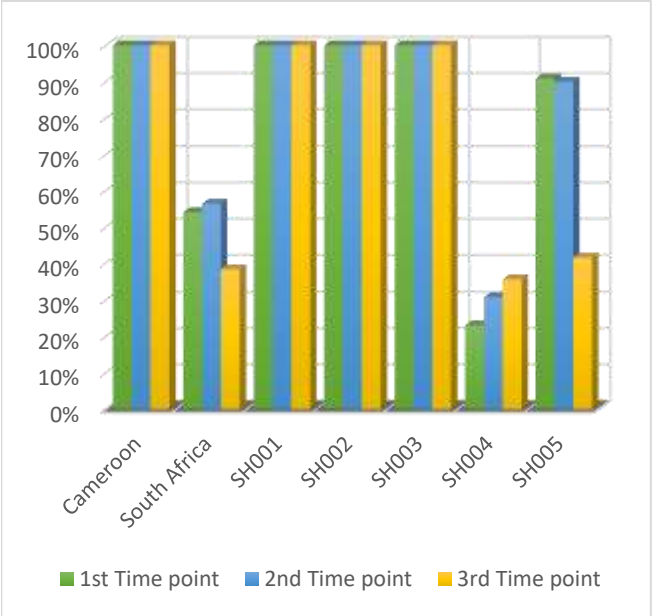
Staphylococcus spp. **(n=isolates)**	**Phenotypic resistance patterns**	**N° Antibiotics**	**No. Classes**	**Cameroon**		**South Africa**	
				Nasal (%)	**Rectal (%)**	**Nasal (%)**	**Rectal (%)**
S. aureus (7)	P.FOX.OX.TET	4	2	1 (14)	0	0	0
	P.FOX.OX.E.CLI.TET	6	4	0	0	3 (33)	3 (20)
S. lentus (24)	P.FOX.OX.TET	4	2	0	0	1 (11)	2 (13)
	OX.E.CLI.TET	4	4	0	0	0	1 (7)
	P.FOX.OX.GEN.E.TET	6	4	0	0	0	1 (7)
	FOX.OX.E.TET.FA	5	4	0	0	0	1 (7)
	FOX.OX.E.TET.FA.CLI	6	5	0	0	1 (11)	1 (7)
	OX.CLI.TET.FA	4	4	0	0	0	1 (7)
	OX.CLI.TET.FA.RIF	5	5	0	0	0	1 (7)
	P.FOX.OX.GEN.E.CLI.TET	7	5	0	0	1 (11)	0
	OX.E.TET.FA	4	4	0	0	1 (11)	0
	P.FOX.OX.TET.FA	5	3	1 (14)	2 (43)	0	0
	P.OX.E.TET.FA	5	4	2 (28)	0	0	0
	FOX.OX.TET.FA.RIF	5	4	1 (14)	0	0	0
	FOX.OX.LIN.CLI.FA.RIF	6	5	1 (14)	0	0	0
	P.FOX.OX.E.CLI.LIN.TEI.VAN.FA.RIF	10	7	0	1 (14)	0	0
	P.FOX.OX.E.CLI.TET.FA.TMP/SXT	8	6	0	1 (14)	0	0
	P.FOX.OX.CLI.TET	5	3	0	1 (14)	0	0
	P.FOX.OX.E.CLI.TET.FA.RIF	8	6	0	0	1 (11)	0
	FOX.OX.E.CLI.TET.FA	6	5	0	0	1 (11)	0
S. hominis (2)	P.FOX.OX.E.CLI	5	3	0	0	0	1 (7)
	P.FOX.OX.GEN.E.CLI.TET	7	5	0	0	0	1 (7)
S. epidermidis (1)	P.FOX.OX.GEN.E.CLI.TET	7	5	0	0	0	1 (7)
S. warneri (1)	P.FOX.OX.CLI.TET	5	3	0	0	0	1 (7)
S. sciuri (2)	P.FOX.OX.E.CLI.TET.FA.RIF.TMP/SXT	9	7	0	1 (14)	0	0
	OX.TET.FA	3	3	1 (14)	0	0	0
Grand Total				7 (100)	6 (100)	9 (100)	15 (100)

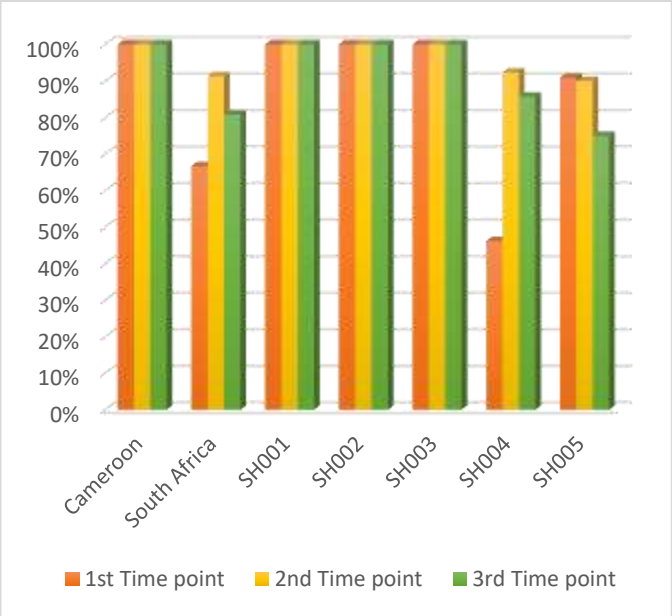
PEN: benzylpenicillin, OX: oxacillin, GEN: gentamicin, CIP: ciprofloxacin, MOX: moxifloxacin, E: erythromycin, CLI: clindamycin, LIN: linezolid, TEI: teicoplanin, VAN: vancomycin, TIG: tigecycline, FA: fusidic acid, RIF: rifampicin, SXT: trimethoprim/sulfamethoxazole

Table 5.7. Antimicrobial resistance profiles of methicillin resistant staphylococci (MRS) isolated from humans

Staphylococcus spp. (n=isolates)	Phenotypic resistance patterns	N° Antibiotics	N° Classes	Cameroon		South Africa	
				Nasal (%)	Hand (%)	Nasal (%)	Hand (%)
S. haemolyticus (15)	P.FOX.OX.TMP/SXT.E.TET	6	4	0	0	1 (13)	0
	P.FOX.OX.GEN.CIP.E.TMP/SXT	7	5	0	0	1 (13)	0
	P.FOX.OX.GEN.TMP/SXT.FA.TET	7	5	0	0	1 (13)	0
	P.FOX.OX.GEN.TMP/SXT.TET	6	4	0	0	1 (13)	0
	P.FOX.OX.TET.TMP/SXT	5	3	0	0	1 (13)	0
	P.FOX.OX.GEN.CIP.TMP/SXT	6	4	0	0	1 (13)	0
	P.FOX.OX.TMP/SXT	4	2	0	0	0	1 (17)
	P.FOX.CIP.E.TEI.TET.FA.TMP/SXT	8	7	0	0	0	1 (17)
	P.FOX.E.TMP/SXT.TET	5	4	0	0	0	1 (17)
	P.FOX.OX.CIP.MOX.E.TET.TMP/SXT	8	5	0	1 (9)	0	0
	P.FOX.OX.GEN.CIP.TET.TMP/SXT	7	5	0	1 (9)	0	0
	P.FOX.OX.TMP/SXT.GEN.CIP	6	4	1 (11)	0	0	0
	P.FOX.OX.GEN.CIP.E	6	4	1 (11)	0	0	0
	P.FOX.OX.TMP/SXT.GEN.CIP.TET	7	5	1 (11)	0	0	0
	P.FOX.OX.TMP/SXT.GEN.CIP.E.TET.FA	9	7	1 (11)	0	0	0
S. epidermidis (6)	P.FOX.OX.TMP/SXT	4	2	0	0	0	1 (17)
	P.FOX.OX.E.TET.TMP/SXT	6	4	0	1 (9)	0	0
	P.FOX.OX.GEN.E.TET.FA.TMP/SXT	8	6	0	1 (9)	0	0
	P.FOX.OX.GEN	4	2	0	1 (9)	0	0
	P.FOX.OX.GEN.E.TET	6	4	0	1 (9)	0	0
	P.FOX.OX.E.TET	5	3	0	1 (9)	0	0
S. hominis (3)	P.FOX.OX	3	1	0	0	0	1 (17)
	P.FOX.OX.TET	4	2	1 (11)	0	0	0
	P.FOX.OX.TET.FA	5	3	1 (11)	0	0	0
S. lentus (6)	P.FOX.OX.GEN.CIP.E.TET.TMP/SXT	8	6	0	0	1 (13)	1 (17)
	P.FOX.OX.GEN.TET	5	3	1 (11)	0	0	0
	P.OX.TET.FA.TMP/SXT	5	4	1 (11)	0	0	0
	P.FOX.OX.TET.FA	5	3	0	1 (9)	0	0
	OX.E.FA	3	3	0	1 (9)	0	0
S. lugdunensis (1)	P.FOX.OX.GEN.TMP/SXT	5	3	0	0	1 (13)	0
S. sciuri (1)	P.OX.TET.FA	4	3	0	1 (9)	0	0
S. warneri (2)	P.FOX.OX.TET.FA	5	3	0	1 (9)	0	0
	P.FOX.OX.E.TET	5	3	1 (11)	0	0	0
Grand Total				9 (100)	11 (100)	8 (100)	6 (100)

PEN: benzylpenicillin, OX: oxacillin, GEN: gentamicin, CIP: ciprofloxacin, MOX: moxifloxacin, E: erythromycin, CLI: clindamycin, LIN: linezolid, TEI: teicoplanin, VAN: vancomycin, TIG: tigecycline, FA: fusidic acid, RIF: rifampicin, SXT: trimethoprim/sulfamethoxazole





1A. **1B.**

Figure 5.1. Overall prevalence of MRS carriage (A: nasal; B: rectal) in pigs per country, abattoir and time point.

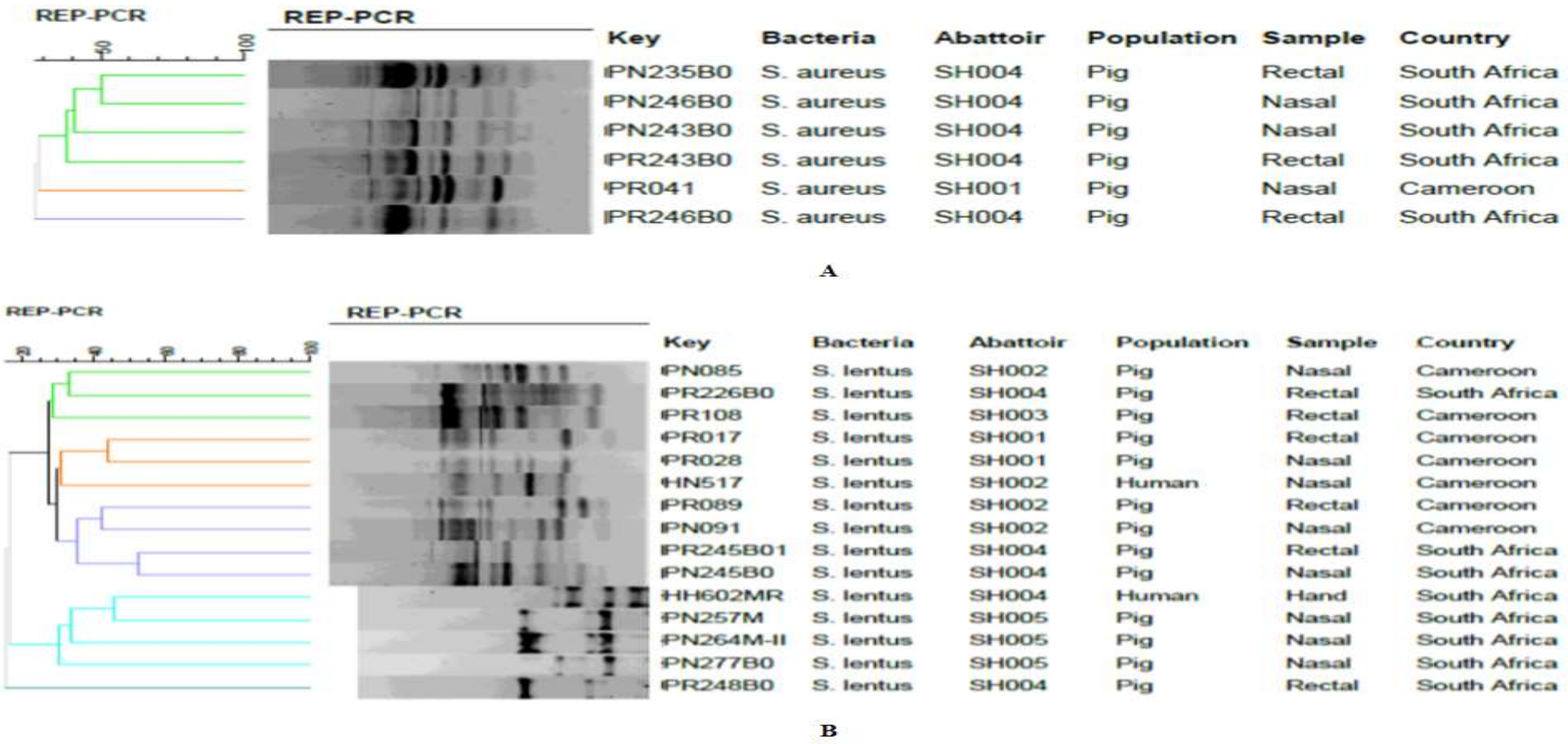


Figure 5.2. Genotypic relationship of methicillin resistant *S. aureus* (A) and *S. lentus* (B) isolated from pigs and humans in Cameroon and South Africa. Dendrogram established by the biostatistical analysis software Bionumerics using the Dice similarity coefficient and UPGMA method on the basis of the REP-PCR profiles obtained with (GTG)5 primer. Clusters were defined based on a similarity cut-off of 80%.

CHAPTER 6

Article V. Genome Analysis of Methicillin-Resistant *Staphylococcus aureus* Isolated from Pigs: Emergence of the Clonal Lineage ST398 in Cameroon and South Africa

Genome Analysis of Methicillin-Resistant *Staphylococcus aureus* Isolated from Pigs: Emergence of the Clonal Lineage ST398 in Pigs in Cameroon and South Africa[7]

Author contributions

- Luria Leslie Founou, as the principal investigator, co-conceptualized the study, undertook sample collection, microbiological laboratory analyses, prepared tables and figures, contributed to bioinformatics analysis, interpreted results and drafted the manuscript.
- Raspail Carrel Founou undertook sample collection, microbiological laboratory analyses, contributed to bioinformatics analysis and vetting of the results.
- Mushal Allam, undertook bioinformatics analyses.
- Arshad Ismail, undertook whole genome sequencing analysis.
- Cyrille Finyom Djoko as co-supervisor, took part in the design of the study, provided materials, equipment and reagents, coordinated the field implementation in Cameroon and reviewed the manuscript.
- Sabiha Yusuf Essack, as principal supervisor, co-conceptualized the study, contributed to vetting of the results and undertook critical revision of the manuscript.

Objectives met: This original research article investigates the antibiotic resistance genes, virulence factors, mobile genetic elements and genetic lineages of circulating MRSA strains isolated from pigs in Cameroonian and South African abattoirs using whole genome sequencing (WGS) and addresses objectives Eight and Nine.

[7] This paper has been submitted to Frontiers in Microbiology and is currently under review. It will reference the "Mannitol-Fermenting Methicillin-Resistant Staphylococci (MRS) in Pig Abattoirs in Cameroon and South Africa: A Serious Food Safety Threat" paper, which has been submitted and is expected to be published first.

Genome Analysis of Methicillin-Resistant *Staphylococcus aureus* Isolated from Pigs: Emergence of the Clonal Lineage ST398 in Cameroon and South Africa

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Keywords: *Staphylococcus aureus*; Methicillin resistance; Antibiotic resistance; LA-MRSA; Whole genome sequencing; Food chain

Number of Figures: 3

Number of Tables: 4

Word counts: 2852

Abstract

Background and objectives: Food animals are considered reservoirs of methicillin resistant *Staphylococcus aureus* (MRSA) and are implicated in their zoonotic transmission in the farm-to-plate continuum. MRSA sequence type (ST) 398, the prototype of livestock-associated MRSA (LA-MRSA), that first emerged in pigs, is an important public health threat worldwide due to its global zoonotic dissemination and implications in human infections and outbreaks. In this study, whole genome sequencing was used to describe the genetic environment (resistance mechanisms, virulence factors and mobile genetic elements), investigate the genetic lineages and determine the putative public health threat of circulating and closely related MRSA strains isolated from pigs in Cameroonian and South African abattoirs.

Methods: During March–October 2016, 288 nasal and rectal pooled samples from 432 pigs as well as nasal and hand swabs from 82 humans were collected. Genomic DNA of MRSA strains was sequenced using an Illumina MiSeq platform. Generated reads were *de novo* assembled using the Qiagen CLC Genomics Workbench and SPAdes. The assembled contigs were annotated and antibiotic resistance genes, virulence factors, plasmids and phage elements identified with ResFinder, Virulence Finder, PlasmidFinder and PHAST, respectively.

Results: A lower MRSA prevalence was observed in pigs in Cameroon (0.07%) compared with South Africa (16.66%) and none of the workers were colonized by MRSA. Genome analysis identified various antibiotic resistance genes associated with resistance to β–lactams (*mec*A, *bla*Z), fluoroquinolones (*nor*A), macrolide, lincosamide and streptogramins [*erm*(B), *erm*(C)] and tetracycline [*tet*(M), *tet*(K), *tet*(L)]. All circulating MRSA isolates belong to the clonal lineage ST398 and showed chromosomal integration of several plasmid replicon types.

Conclusion: Our study shows that the livestock-associated MRSA clonal lineage ST398 is already present in both Cameroon and South Africa and is probably underestimated in the absence of molecular epidemiological studies. It reveals the serious food safety and public health threat associated with this animal strain and underscores the urgent need for interventions to contain its zoonotic transmission.

6.1. Introduction

Livestock-associated methicillin-resistant *Staphylococcus aureus* (LA-MRSA) is a bacterium that first emerged in pigs in the Netherlands in the early 2000s (Voss et al., 2005; van den Broek et al., 2008; Price et al., 2012; Chen, 2013). Some reports suggested that LA-MRSA originated from human methicillin-susceptible *S. aureus* (MSSA) sequence type (ST) 398, underwent a jump from humans to food animals where it has subsequently acquired tetracycline and methicillin resistance (Weese, 2010; Price et al., 2012; Mehndiratta and Bhalla, 2014). In fact, all LA-MRSA strains exhibit resistance to tetracycline and are normally susceptible to trimethoprim/sulfamethoxazole. They rarely harbor the Panton-Valentine Leukocidin (*PVL*) genes that characterize human community-acquired-MRSA (CA-MRSA) (Weese, 2010; Mehndiratta and Bhalla, 2014). Although (food) animals are often colonized by LA-MRSA ST 398, clinical diseases associated with this resistant pathogen have rarely been reported (van Den Broek et al., 2008; Chen, 2013). Nasal and rectal asymptomatic carriage of the MRSA ST 398, the prototype of LA-MRSA, has been frequently detected in pigs (Wulf and Voss, 2008; Wagenaar et al., 2009; Van Cleef et al., 2010; Graveland et al., 2011) which are considered the most important reservoir although other clonal lineages such as ST9 (Fang et al., 2014), ST5 (Hau et al., 2015) and ST97 (Spoor et al., 2013) have also been identified. LA-MRSA ST398 is largely a pig- or veal-calf-associated lineage characterized by its high capacity to colonize multiple hosts such as poultry (Murray et al., 2017), sheep (Weese et al., 2010), horses (Abdelbary et al., 2014) and pets (Weese et al., 2010). It is highly prevalent in Europe (van Den Broek et al., 2008; Harrison et al., 2017) and Northern America (Tegegne et al., 2017). LA-MRSA ST9 is a pandemic livestock-associated clone detected in pigs and exposed workers in Asian countries, including Thailand (Sinlapasorn et al., 2015), China (Ye et al., 2016) and Taiwan (Fang et al., 2014) and LA-MRSA ST5 is mostly associated with poultry globally (Murray et al., 2017).

The asymptomatic carriage of LA-MRSA increases the risks of its zoonotic transmission among exposed workers, other animals and the environment, thus contributing to the complex variations, exchanges, and hosts adaptabilities (Wulf et al., 2008a; Van Cleef et al., 2010, 2011; Price et al., 2012; Köck et al., 2013; Smith, 2015). Graveland et al. (2011) revealed that four hours of narrow contact with colonized or sick animals were sufficient to infect or permanently colonize humans. Several studies thus concluded that pig farmers, abattoir workers, food handlers and veterinarians, have high occupation/profession-associated risks for LA-MRSA

colonization and infection (Van Cleef et al., 2010; Graveland et al., 2011; Price et al., 2012). These humans may in turn likely become reservoirs of LA-MRSA and drive its spread into the community and healthcare settings leading to the emergence of genetically diverse CA-MRSA and hospital-acquired-MRSA both representing serious global public health threat (Wulf and Voss, 2008; Price et al., 2012; Mehndiratta and Bhalla, 2014).

Several reports have described the origin and evolution of MRSA of animal origin in the human population, highlighting its adaptation to various hosts, and subsequent spread of new clones widely into the general population (van Loo et al., 2007; van Den Broek et al., 2008; Graveland et al., 2011; Köck et al., 2013). In an international study of nine countries, Wulf et al., (2008a) reported a 12.5% (34/272) prevalence of MRSA in professionals (other than farmers and veterinarians) in contact with pigs. The study showed that 91% (31/34) of these strains were ST398, and were previously isolated from Dutch pigs, pig farmers and veterinarians (Wulf et al., 2008a). It further concluded that these strains could represent an important source of community-acquired MRSA (CA-MRSA) not only in Europe, but also worldwide, if their spread is not contained (Wulf et al., 2008a).

Even though LA-MRSA virulence is almost null or likely lesser than in other human MRSA clones, numerous lineages of LA-MRSA including ST398, ST5, ST97, ST30, ST9 have been involved in outbreaks and human infections in hospitals particularly in regions with a high density of pig production activities (Wulf et al., 2008a, Wagenaar et al., 2009, Köck et al., 2013). For instance, two hospital-acquired outbreaks of MRSA of livestock origin have been reported in Dutch health care settings and in nursing homes demonstrating the putative spread of LA-MRSA in the general population (Wulf et al., 2008a). Invasive LA-MRSA ST398 infections have been associated with negative clinical outcomes ranging from mild to life-threatening. Resistant strains originating from animals, are serious threats to the world as they could lead to the emergence of new strains with greater resistance, virulence and mobility (Wulf and Voss, 2008; Weese, 2010; Price et al., 2012).

Understanding the epidemiology, molecular characteristics and clonal lineages of *S. aureus* at the animal, human and environmental interface is thus imperative to correlate the genetic diversity associated with the genetic exchange and spread of resistant bacteria through the farm-to-plate continuum. This is especially important in sub-Saharan African countries, such as Cameroon and South Africa where policies on antimicrobial use and antimicrobial stewardship programs in the food production industry are limited. The purpose of this study

was to investigate the antibiotic resistance genes, virulence factors, mobile genetic elements and genetic lineages of circulating MRSA isolated from pigs in Cameroonian and South African abattoirs using whole genome sequencing (WGS).

6.2. Materials and methods

6.2.1. Ethical considerations

Ethical approvals from the Biomedical Research Ethics Committee (**Ref. BE365/15**) and Animal Research Ethics Committee (**Ref. AREC/091/015D**) of the University of KwaZulu-Natal as well as from the National Ethics Committee for Research in Human Health of Cameroon (**Ref. 2016/01/684/CE/CNERSH/SP**) were obtained prior the implementation of the study. Ministerial approvals from the Cameroonian Ministry of Livestock, Fisheries and Animal Industries (**Ref. 061/L/MINEPIA/SG/DREPIA/CE**) and Ministry of Scientific Research and Innovation (**Ref. 015/MINRESI/B00/C00/C10/C14**) were also granted.

6.2.2. Study design and bacterial isolates

From March to October 2016, a multi-center study was carried out in three slaughterhouses/markets in Cameroon and two abattoirs in South Africa, encoded for ethical reasons as SH001, SH002, SH003 and SH004 and SH005, respectively. Three individual samples were pooled to yield 144 nasal and 144 rectal pools representing 432 individual nasal and rectal samples respectively collected from 432 pigs. A total of 288 swabs from the 144 nasal and 144 rectal pools constituted the final sample. Nasal and hand swabs were also collected from 82 humans in both Cameroon and South Africa. All samples were cultured on Mannitol Salt agar supplemented with 6 mg/L cefoxitin and incubated for 18-24 h at 37^0C. All putative methicillin resistant staphylococci (MRS), were subjected to Gram staining, and the catalase and oxidase tests for phenotypic characterization of the isolates to the genus level. The isolates were thereafter phenotypically confirmed using the Vitek® 2 System (BioMérieuX, Marcy l'Etoile, France). The strains sequenced in this study were isolated from five pooled samples, one in Cameroon (PR041) and four in South Africa (PR243B0, PN243B0, PN246B0, PN235B0). The isolates, PN243B0, PN246B0 and PN235B0, were collected from the nares of pigs processed in abattoir SH004 in South Africa. Likewise, the strains, PR041 and PR243B0, originated from two different abattoirs, SH001 and SH004, respectively and were both collected from the rectum. These strains were the unique MRSA strains isolated in a previous study and were observed to be closely related via repetitive-palindromic-polymerase chain

reaction (REP-PCR) analysis. All MRSA isolates were subjected to WGS analysis in order to describe the genetic environment (resistance mechanisms, virulence factors and mobile genetic elements), investigate whether they were of livestock origin, and determine their putative threat for human health.

6.2.3. Screening for methicillin resistance

The cefoxitin disk test was used for the screening of the methicillin resistance as recommended by the Clinical Laboratory and Standards Institute (CLSI) (CLSI, 2016). An inhibition zone below 21 mm was regarded as putative proof for methicillin resistance. The Vitek® 2 System (BioMérieux, Marcy l'Etoile, France) was further used for phenotypic confirmation of methicillin resistance. *S. aureus* ATCC 29213 and ATCC 43300 were used as controls.

6.2.4. Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed to determine the resistance patterns of the selected strains. The minimum inhibitory concentrations (MIC) of benzylpenicillin, cefoxitin, oxacillin, clindamycin, erythromycin, gentamicin, ciprofloxacin, moxifloxacin, mupirocin, rifampicin, fusidic acid, teicoplanin, vancomycin, linezolid, tigecycline, trimethoprim-sulfamethoxazole, were determined by broth micro-dilution method using Vitek® 2 System (BioMérieuX, Marcy l'Etoile, France) and Vitek® 2 Gram Positive Susceptibility card (AST-P603) (BioMérieux, Marcy l'Etoile, France). The results were interpreted according to the CLSI guidelines (CLSI, 2016).

6.2.5. DNA isolation

Genomic DNA (gDNA) was extracted using GenElute® bacterial genomic DNA kit (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions. The quantification of extracted gDNA was determined on a NanoDrop spectrophotometer with verification by agarose gel electrophoresis and fluorimetric analysis (Qubit®).

6.2.6. Genome sequencing

Multiplexed paired-end libraries (2×300 bp) were prepared using the Nextera XT DNA sample preparation kit (Illumina, San Diego, CA, USA) and followed by sequencing on an Illumina MiSeq platform with a depth of 100× per sample at the National Institute of Communicable Diseases Sequencing Core Facility, South Africa.

6.2.7. Genome assembly

The resulting raw reads were checked for quality, trimmed and mapped to the reference genome of *S. aureus* RIVM3897 (CP013621) using the CLC Genomics Workbench version 10 (CLC, Bio-QIAGEN, Aarhus, Denmark). *De novo* assembling was further performed for all genomes with CLC Genomics and SPAdes version 3.5. (Bankevich et al., 2012) to overrule any inherent shortfall from both assembler.

6.2.8. Genome analysis

The *de novo* assembled contigs were uploaded to GenBank and annotated using NCBI prokaryotic genome annotation pipeline and RAST 2.0 server (http://rast.nmpdr.org; Aziz et al., 2008) which identified encoding proteins, rRNA and tRNA, assigned functions to the genes and predicted subsystems represented in the genome. The bacterial analysis pipeline of GoSeqIt tools was also used to annotate and identify known acquired antibiotic resistant genes via ResFinder (Zankari et al., 2012), virulence factors using VirulenceFinder (Joensen KG et al., 2014) and mobile genetic elements through PlasmidFinder (Carattoli et al., 2014). The RAST SEED viewer was used to identify the presence of transposases, integrons and mobilization proteins flanking the resistance genes (Overbeek et al., 2014). PHAge Search Tool (PHAST) server was used for the identification, annotation and visualization of prophage sequences (Zhou et al., 2011). The multilocus sequence type (MLST) of the isolates was determined from the WGS data based on seven housekeeping genes (arc, aroe, glpf, gmk, pta, tpi, yqil).

6.2.9. Whole genome phylogenetic analyses

The assembled contigs were aligned against a reference genome of LA-MRSA ST398 (CP013621) using the progressive Mauve genome alignment package version 2.3.1. Study genomes were contextualized against a collection of four LA-MRSA ST398 and one LA-MRSA ST5, accession numbers AM990992, CP003808, CP020019, CP013621 and CP017090, respectively. Phylogenetic analysis was based on the core genomes and performed using the Rapid large-scale prokaryote pan genome analysis (Roary) (Page et al., 2015). The maximum likelihood phylogenetic tree encompassing, country, population, sample type metadata and MLST type was generated, edited and visualized using FastTree version 2.1.7. In addition, the contigs were mapped against the complete genome of *S. aureus* RIVM3897 (CP013621) for visualization of the genomic organization using CGView server (Grant et al., 2012).

6.2.10. Nucleotide sequence accession number

This whole-genome shotgun project PRJNA412434 of LA-MRSA strains PR041, PR243B0, PN243B0, PN246B0 and PN235B0 has been deposited at DDBJ/EMBL/GenBank under accession numbers PDVL00000000, PDVA00000000, PDUZ00000000, PDVB00000000 and PDUY00000000, respectively. The versions described in this paper are the versions PDVL00000000.1, PDVA00000000.1, PDUZ00000000.1, PDVB00000000.1 and PDUY00000000.1, respectively.

6.3. Results

6.3.1. Phenotypic analyses

Out of the 288 pooled samples (144 nasal and 144 rectal pools) taken from 432 pigs in the five abattoirs, methicillin resistant staphylococci (MRS) were isolated from 108/144 (75%) and 102/144 (70%) of the pooled nasal and rectal samples, respectively. In South Africa, 50% (36/72) and 81% (58/72) MRS were isolated in nasal and rectal pooled samples, whereas a maximum level of MRS (100%) was detected in both type of pooled samples in Cameroon. Despite all being mannitol-fermenting colonies, only one (0.07%) and four (16.66%) MRSA were detected in Cameroon and South Africa, respectively, whereas none of the workers were colonized by MRSA (unpublished data). Table 6.1 summarizes relevant population data, specimen source, and, phenotypic and genotypic characteristics. All South African strains (PN235B0, PN243B0, PR243B0 and PN246B0) exhibited the same phenotypic resistance profile P.FOX.OX.E.CLI.TET whereas the Cameroonian strain displayed the profile P.FOX.OX.TET with susceptibility to all other antibiotics tested (unpublished data). The MICs results of the MRSA isolates are summarized in Table 6.2, with the resistance observed being corroborated with WGS analyses.

6.3.2. Genotypic analyses

The WGS analyses revealed the presence of resistance genes and virulence factors in all isolates. Beta-lactamase encoding genes, especially *blaZ* and *mec*A were identified in all except one strain PN246B0 that did not harbor *blaZ* gene. Likewise, the co-presence of *erm*(B) and *erm*(C) encoding for resistance to macrolide, lincosamide, streptogramin B resistance (MLS_B) was observed in all strains as were tetracycline resistance genes *tet*(M) and *tet*(K), with one strain PR041, additionally carrying the *tet*(L) gene (Tables 6.1 and 6.2). Although not

phenotypically resistant to fluoroquinolone, all MRSA strains harbored genes encoding related resistance products including the fluoroquinolone efflux transporter protein *nor*A as well as two sub-unit topoisomerase IV (*par*C, *par*E) and DNA gyrase (*gyr*A, *gyr*B). In all strains, the *tet*(M) gene was flanked downstream by a plasmid recombination, MobE mobilization protein suggesting that this gene could likely be horizontally transferred to other species.

Six virulence genes including aureolysin (*aur*), beta-hemolysin (*hlb*), gamma-hemolysin chain II precursor (*hlg*A), gamma-hemolysin component B precursor (*hlg*B), gamma-hemolysin component B precursor (*hlg*C) and enterotoxin B (*seb*) were identified in all isolates with the *lukS*-PV being detected in only one strain, PR243B0 (Table 6.1).

6.3.3. *In silico* detection of plasmids and prophage sequences

PlasmidFinder revealed that the plasmid replicon types repL(pDLK1), rep(SAP101A) and repC(Cassette) were detected in all strains. Two isolates PN235B0 [repL(pDLK1); rep(pKH13); rep(SAP101A); rep(SAP071A); repC(Cassette)] and PR041 [repL(pDLK1); rep(pKH13); rep(SAP101A); rep(pWBG754); repC(Cassette); repB(pUB110)] concomitantly harbored five plasmid replicon types. The three remaining isolates each harbored four plasmid replicon types (Table 6.3). A plasmid rolling-circle replication (RCR) was further hosted on all strains.

Similarly, PHAST server revealed that all strains harbored prophage regions that were either intact or incomplete (Table 6.4). The *Staphylococcus* phage phiJB (phage accession NC_028669) and bacteriophage 47 (phage accession NC_007054), were hosted intact on the genome of three isolates, namely PN235B0, PR243B0 (Figure 1) and PN243B0. The phage Staphy_StauST398-2 was also detected but incomplete in two strains (PN235B0 and PN246B0).

6.3.4. Multi-locus sequence typing

MLST-analyses assigned all MRSA strains to ST398 with 100% identity among all seven housekeeping genes. The South African strains were isolated from three nasal and one rectal pooled samples from one of the biggest abattoir located in the KwaZulu-Natal province, while the Cameroonian strain originated from one rectal pooled sample from the principal abattoir of Yaoundé (Table 6.1).

6.3.5. Phylogenetic analysis

The phylogenetic analysis revealed that all our strains fall within a clade of the known LA-MRSA ST398. Figure 6.2 demonstrates that considerable similarity exists between our collection of LA-MRSA and two strains AM990992 and CP0220019 isolated from a human with endocarditis in Netherlands and fattened pig in Germany, respectively. On the other hand, a certain phylogenetic difference was observed when compared with LA-MRSA CP013621 and CP003808 detected in human samples in Netherlands and Canada, respectively. Figure 6.3 depicts the genomic organization of the MRSA PR243B0 mapped against the complete genome of *S. aureus* RIVM3897 (CP013621).

6.4. Discussion

In this study, whole genome sequencing was used to investigate antibiotic resistance genes, virulence factors, mobile genetic elements and genetic lineages of five circulating and closely related MRSA strains isolated from pigs in Cameroonian and South African abattoirs. Our findings reveal that the MRSA clonal lineage ST398 is present in pigs with a relatively low prevalence in both Cameroon (0.07%) and South Africa (16.66%) although none of the exposed workers were colonized. This concurs with a 2017 study from England, where a 0.82% LA-MRSA prevalence was detected in humans during a one year-period (Harrison et al., 2017). To the best of our knowledge, this is the first report of a porcine LA-MRSA ST398 reservoir in these two nations. Moreover, the first report of MRSA ST398 harboring *luk*S-PV, one of the pro-toxin subunit encoding for PVL gene is also reported herein.

This lineage was primarily detected in Europe where it colonized pigs and occupationally exposed workers (Armand-Lefevre et al., 2005; Voss et al., 2005; van Loo et al., 2007; Van Cleef et al., 2011) before being subsequently identified with various prevalence and clonal lineage in the USA (Smith, 2015), Canada (Khanna et al., 2008; Golding et al., 2010), Australia (Groves et al., 2013), South America (Arriola et al., 2011) and Asia (Wagenaar et al., 2009; Lim et al., 2012). In the African continent, the prevalence of MRSA colonization in animals is very low, ranging from 0% to 3% (Lozano et al., 2016) with only two studies emanating from Tunisia reporting the clonal lineage ST398 on the continent (Chairat et al., 2015; Elhani et al., 2015). Chairat et al. (2015) reported a 1.2% (2/169) prevalence of MRSA in raw meat with only one being ST398 and harboring in addition to beta-lactam encoding genes, *tet(M)*, *erm(C)*, and four virulence genes (including *sen*, *hla*, *hlg* and *hlgv*), while Elhani et al. (2015) revealed

that one strain out of 24 tetracycline-resistant MRSA belonged to the ST398 and was isolated from nasal sample of a hospitalized farmer patient in Tunisia.

The arsenal of *S. aureus* virulence factors is extensive, with both secreted and structural products involved in its pathogenicity and its ability to circumvent the host immune system (Otto, 2014). An interesting finding of this study, is the detection of various virulence determinants in all our isolates. In fact, the staphylococcal enterotoxin B (*seb*), considered a biological warfare weapon, was identified along with β-hemolysins (*hlb*) and γ-hemolysins (*hlg*A, *hlg*B and *hlg*C). Staphylococcal enterotoxins induce the release of serotonin into the intestine and thereby play a causative role in staphylococcal food poisoning while hemolysins are pore-forming exotoxins causing lysis and subsequently, death of blood cells including erythrocytes, endothelial cells, platelets and leukocytes (Fluit, 2012; Otto, 2014). The success of several community-acquired (CA)-MRSA clones such as the USA300 (ST8), the Taiwan clone USA1000 (ST59), the European clone (ST80) has been attributed to the presence of staphylococcal enterotoxin and hemolysins (David and Daum, 2010; Sowash and Uhleman, 2014; Li et al., 2016). Although we did not quantify the production levels and mobilization of the virulence determinants, we postulated that, as with the global success of CA-MRSA, the repertoire of virulence factors identified in the LA-MRSA isolates in this study could signal their potential high pathogenicity if they become human pathogens. Moreno et al. (2016) already described a vancomycin intermediate LA-MRSA ST398 strain SA7112 responsible of exudative epidermitis in swine in Brazil (Moreno et al., 2016). Similarly, Lima et al. (2017) reported the genome of a multidrug-resistant LA-MRSA ST398 from a patient with cystic fibrosis in the same country. Interestingly, these Brazilian vancomycin intermediate and multi-drug resistant LA-MRSA isolates displayed similar virulence characteristics to that of our isolates with the presence of aureolysin (*aur*), staphylococcal β-hemolysins (*hlb*) and γ-hemolysins (*hlg*A, *hlg*B and *hlg*C). The above-mentioned studies give credence to our hypothesis that the detection of LA-MRSA in livestock is a public health threat with the potential for dissemination to humans via the food chain in Cameroon and South Africa. They further attest to the broad host range and rapid genetic evolution of LA-MRSA clones.

The acquisition of the human virulence mechanism *lukS*-PV, subunit of the synergistic PVL toxin, by the LA-MRSA strain PR243B0 is another great concern. In a multicenter study involving five African towns, including Yaoundé, Breurec et al. (2010) showed that the three predominant clones detected in Yaoundé, were the major PVL-positive lineages ST5, ST8 and ST88, closely related to the hypervirulent USA300 (ST8). They harbored several virulence genes, including *luk*-PV, *hlb* and *seb* (Breurec et al., 2010). As with the LA-MRSA ST5, that

showed human-to-poultry host transition events, accompanied by host-adaptive evolution, genetic changes, including acquisition of novel mobile genetic elements (MGEs) and spread to poultry populations (Murray et al., 2017), it is highly plausible that the selective pressure and intimate contact between humans and animals will be driving forces behind the genetic exchanges and emerging fluidity of virulence genes that will enable LA-MRSA ST398 to undergo similar host-adaptive evolution and become a well-established human pathogen. It is therefore of paramount importance to closely monitor food animals and food products to alleviate the risk of development and contain the spread of emerging virulent and resistant strains.

The typical presence of the *mec*A gene encoding methicillin resistance and complete resistance to the β-lactam family was evident in this clone along with several *S. aureus* genes supporting resistance against various antibiotic families that enhanced antibiotic resistance of these animal strains. The genomic analysis of our isolates attributed tetracycline resistance to the concomitant presence of *tet*(K) and *tet*(M) while the presence of *erm*(B) and *erm*(C) resulted in macrolide and lincosamide resistance in five out of six strains. This finding concurs with the study by Harrison et al. (2017) where *erm*(C), *tet*(K) and *tet*(M) were all present in the single ST398 detected in clinical isolates in England. All *tet*(K) gene detected in our study, were flanked by a plasmid rolling-circle replication (RCR) and a mobilization MobE protein, a conjugative mobilization protein that generally enables plasmid recombination in site specific manner. The presence of these genetic elements may contribute to *tet*(K) mobilization, especially when considering the extensive use of tetracycline in livestock. Wassenaar et al. (2016) showed that the *qac*C gene encoding resistance to quaternary ammonium compounds in staphylococci, was mobilized and transferred to rolling-circle plasmids in the absence of other genetic elements such as insertion sequences, plasmids or transposons and postulated rolling-circle plasmids as a novel gene transfer mechanism in staphylococci (Wassenaar et al., 2016).

Of further interest, is the presence of *tet*(L) along with *tet*(M) and *tet*(K) within the genome of the Cameroonian isolate PR041. While *tet*(M) and *tet*(K) are widespread and quite constant among LA-MRSA isolate, *tet*(L) is an uncommon resistance gene in staphylococci. In fact, it is a plasmid-mediated tetracycline resistance gene that first emerged in *Bacillus spp.* and subsequently spread to staphylococci via horizontal gene transfer (HGT). In this isolate (PR041), the *tet*(L) gene was hosted on a plasmid that had 100% homology with an, as yet, unnamed plasmid encoding tetracycline determinants *tet*(M) and *tet*(L), detected in a clinical isolate of *Enterococcus faecium* strain UW 8175 (accession number CP011830.1). This

suggests that HGT occurs not only within LA-MRSA and staphylococcal species but also between different bacterial species.

Phenotypic characterization showed that all isolates were susceptible to ciprofloxacin (MIC ≤0.5 µg/ml) and moxifloxacin (MIC ≤0.25 µg/ml). This initially led us to not suspect that they might contain gene encoding for resistance to fluoroquinolones. However, *nor*(A), *par*C, *par*E, *gyr*A and *gyr*B were detected in all isolates. Similarly, although not expressing phenotypic resistance to erythromycin (MIC ≤0.25 µg/ml) and clindamycin (MIC ≤0.25 µg/ml), the Cameroonian isolate PR041, harbored *erm*(B) and *erm*(C) genes, encoding for resistance to macrolides, lincosamides and streptogramin B. This finding thus indicate that these genes might be "silent" in circulating LA-MRSA in pigs in Cameroon and South Africa, and could likely be activated in response to the selective pressure of extensive antibiotic use. Although, novel or uncommon resistance genes such as the multi-resistant gene *cfr*, the phenicol exporter gene *fex*A, the lincosamide–streptogramin A–pleuromutilin resistance genes *vga*(C) and *vga*(E), the macrolide–lincosamide–streptogramin B resistance gene *erm*(T), the apramycin resistance gene *apm*A and the trimethoprim resistance gene *dfr*K, were not detected, the presence of several other resistance genes along with various virulence factors could contribute to the pathogenicity of the clonal lineage ST398. Even though none of the exposed workers were colonized by MRSA strains in this study, this porcine LA-MRSA ST398 reservoir highlights the high risk of foodborne infectious diseases and pandemic potential of this clone. Ateba et al. (2010) and Yannick et al. (2013) reported the presence of MRSA in raw milk (100%) and cooked pork (81.8%) in South Africa and Cameroon, respectively, suggesting dissemination of this pathogen throughout the food chain. The greatest threat in this respect, will therefore be the emergence and human adaptation of LA-MRSA ST398 with major virulence factors along with various resistance genes.

The constant evolving, versatility and virulence mechanisms of *S. aureus* as well as the potential for genetic exchanges between LA-MRSA and other bacteria among several hosts are of significant issues. In fact, its successful pathogenicity and versatile host adaptation is facilitated by the acquisition of virulence factors encoded in MGEs such as prophages, plasmids, integrons, and genomic islands (Moon et al., 2014). Prophages play a role in rapid evolution, trigger adaptation to new environments and host species, and increase the pathogenicity of LA-MRSA ST398 (Diene et al, 2017). The φJB prophage of the family of Siphoviridae was detected in three out of five isolates. Varga et al. (2016) showed that φJB has efficient plasmid transduction abilities and can easily transfer antibiotic resistance genes with high frequency (Varga et al., 2016). The detected φJB showed a putative phage attachment site

which is involved in the transduction process. Although we did not specifically address the dissemination of resistance genes and virulence factors through the prophages detected in our LA-MRSA isolates, we hypothesized that with the various evolutionary changes and host adaptations inherent to LA-MRSA, mobilization of these genetic elements within and between bacterial species with ongoing acquisition of virulence and multiple antibiotic resistant genes will likely happen in a near future and cause more severe infections in animals and humans.

In addition, three plasmids, repL(pDLK1), rep(SAP101A) and repC (Cassette), were conserved within our isolates. *In silico* analysis of these plasmids revealed that all rep(SAP101A) had 99% homology with the *S. aureus* plasmid pRIVM4296 (accession number CP013626.1) detected in LA-MRSA ST398 in human clinical samples in Netherlands. Likewise, the repL(pDLK1), shared 100% homology with the *Staphylococcus chromogenes* 3688 plasmid pPV141 (accession number U82607.1), which is a plasmid associated with erythromycin resistance while the repC(Cassette) detected here, hosted all the *tet*(K) genes and was similar to a plasmid recombination protein (accession number WP 014532526.1). These findings reveal the ability of these strains to easily acquire either resistance and/or virulence genes, although further work is required to delineate whether these plasmids could be easily horizontally transferred to other epidemic MRSA strains. Accordingly, studies are needed around the world to correlate the genetic diversity associated with the epidemiological shifts and dissemination of LA-MRSA strain. Such studies are especially needed in low-and-middle-income countries where policies about antimicrobial use and antimicrobial stewardship programs in food animal production are particularly limited.

6.5. Conclusion

Our study shows that the livestock-associated MRSA clonal lineage ST398 is already present in both Cameroon and South Africa and is probably underestimated considering the absence of molecular epidemiological studies in the farm-to-plate continuum. The emergence of LA-MRSA ST398 is of great concern as it could disseminate throughout the food chain and contaminate other animals and humans, and potentially spread to healthcare settings where human infections would be inevitable. In keeping with this, routine screening for MRSA colonization in food animals, exposed workers, farms, abattoirs and food products is recommended for its effective containment. Continuous efforts and further well-designed epidemiological and molecular works not only for the conservation of antibiotics for future generations but also for understanding of the MRSA epidemiology, transmission dynamics and pathways, risk factors, extent of and related public health implications associated with the food

animal reservoir have still to be implemented to identify and design effective interventions measures for its containment.

Author contributions

LF co-conceptualized the study, undertook sample collection, microbiological laboratory analyses, prepared tables and figures, contributed to bioinformatics analysis, interpreted results and drafted the manuscript. **RF** undertook sample collection, microbiological laboratory analyses, contributed bioinformatics analysis, vetting of the results and writing of the manuscript. **MA** undertook bioinformatics analyses. **AI** performed whole genome sequencing analysis. **CD** took part in the design of the study, provided material, equipment and reagents, undertook critical revision of the manuscript and coordinated the field implementation in Cameroon. **SE** co-conceptualized the study and undertook critical revision of the manuscript. All authors read and approve the manuscript.

Conflict of interest

Professor Essack is a member of the Global Respiratory Infection Partnership sponsored by an unrestricted educational grant from Reckitt and Benckiser. All other authors declare that there is no competing financial interest.

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Table 6.1. Summary of sample type, phenotypic and genotypic characteristics of MRSA isolates from pigs

Isolate name	Country	Sample type	Abattoir	Antibiotic resistance genes	Virulence factors	MLST
PN235B0	South Africa	Nasal pooled sample	SH004	*mec*A, *bla*Z, *erm*(B), *erm*(C), *tet*(M), *tet*(K), *nor*(A), *par*C, *par*E, *gyr*A, *gyr*B	*aur*, *hlb*, *hlg*A, *hlg*B, *hlg*C, *seb*	ST398
PN243B0	South Africa	Nasal pooled sample	SH004	*mec*A, *bla*Z, *erm*(B), *erm*(C), *tet*(M), *tet*(K), *nor*(A), *par*C, *par*E, *gyr*A, *gyr*B	*aur*, *hlb*, *hlg*A, *hlg*B, *hlg*C, *seb*	ST398
PR243B0	South Africa	Rectal pooled sample	SH004	*mec*A, *bla*Z, *erm*(B), *erm*(C), *tet*(M), *tet*(K), *nor(*A), *par*C, *par*E, *gyr*A, *gyr*B	*aur*, *hlb*, *hlg*A, *hlg*B, *hlg*C, *seb*, *luk*S-PV	ST398
PN246B0	South Africa	Nasal pooled sample	SH004	*mec*A, *erm*(B), *erm*(C), *tet*(M), *tet*(K), *nor*(A), *par*C, *par*E, *gyr*A, *gyr*B	*aur*, *hlb*, *hlg*A, *hlg*B, *hlg*C, *seb*	ST398
PR041	Cameroon	Rectal pooled sample	SH001	*mec*A, *bla*Z, *erm*(B), *erm*(C), *tet*(M), *tet*(K), *tet*(L), *nor*(A), *par*C, *par*E, *gyr*A, *gyr*B	*aur*, *hlb*, *hlg*A, *hlg*B, *hlg*C, *seb*	ST398

Table 6.2. Minimum inhibitory concentration (MIC) of β-lactam and non-β-lactam antibiotics tested against individual MRSA isolates

Isolate name	β-lactams			non-β-lactam antibiotics													Antibiotic resistance genes
	PEN	FOX	OX	GEN	CIP	MOX	E	CLI	LIN	TEI	VAN	TET	TIG	FA	MUP	RIF	TMP/SXT	
PN235B0	≥0.5	≥4	≥4	≤0.5	≤0.5	≤0.25	≥8	≥8	1	≤0.5	≤0.5	≥16	≤0.12	≤0.5	≤2	≤0.5	≤10	*mec*A, *bla*Z, *erm*(B), *erm*(C), *tet*(M), *tet*(K), *nor*(A), *par*C, *par*E, *gyr*A, *gyr*B
PN243B0	≥0.5	≥4	≥4	≤0.5	≤0.5	≤0.25	≥8	≥8	1	≤0.5	1	≥16	≤0.12	≤0.5	≤2	≤0.5	≤10	*mec*A, *bla*Z, *erm*(B), *erm*(C), *tet*(M), *tet*(K), *nor*(A), *par*C, *par*E, *gyr*A, *gyr*B
PR243B0	≥0.5	≥4	≥4	≤0.5	≤0.5	≤0.25	≥8	≥8	1	≤0.5	1	≥16	≤0.12	≤0.5	≤2	≤0.5	≤10	*mec*A, *bla*Z, *erm*(B), *erm*(C), *tet*(M), *tet*(K), *nor*(A), *par*C, *par*E, *gyr*A, *gyr*B
PN246B0	≥0.5	≥4	≥4	≤0.5	≤0.5	≤0.25	≥8	≥8	1	≤0.5	1	≥16	≤0.12	≤0.5	≤2	≤0.5	≤10	*mec*A, *erm*(B), *erm*(C), *tet*(M), *tet*(K), *nor*(A), *par*C, *par*E, *gyr*A, *gyr*B
PR041	≥0.5	≥4	≥4	≤0.5	≤0.5	≤0.25	≤0.25	≤0.25	2	≤0.5	≤0.5	≥16	≤0.12	≤0.5	≤2	≤0.5	≤10	*mec*A, *bla*Z, *erm*(B), *erm*(C), *tet*(M), *tet*(K), *tet*(L), *nor*(A), *par*C, *par*E, *gyr*A, *gyr*B

PEN: benzylpenicillin, OX: oxacillin, GEN: gentamicin, CIP: ciprofloxacin, MOX: moxifloxacin, E: erythromycin, CLI: clindamycin, LIN: linezolid, TEI: teicoplanin, VAN: vancomycin, TIG: tigecycline, FA: fusidic acid, RIF: rifampicin, SXT: trimethoprim/sulfamethoxazole

Table 6.3. Distribution of plasmid replicon types identified among the five MRSA isolates

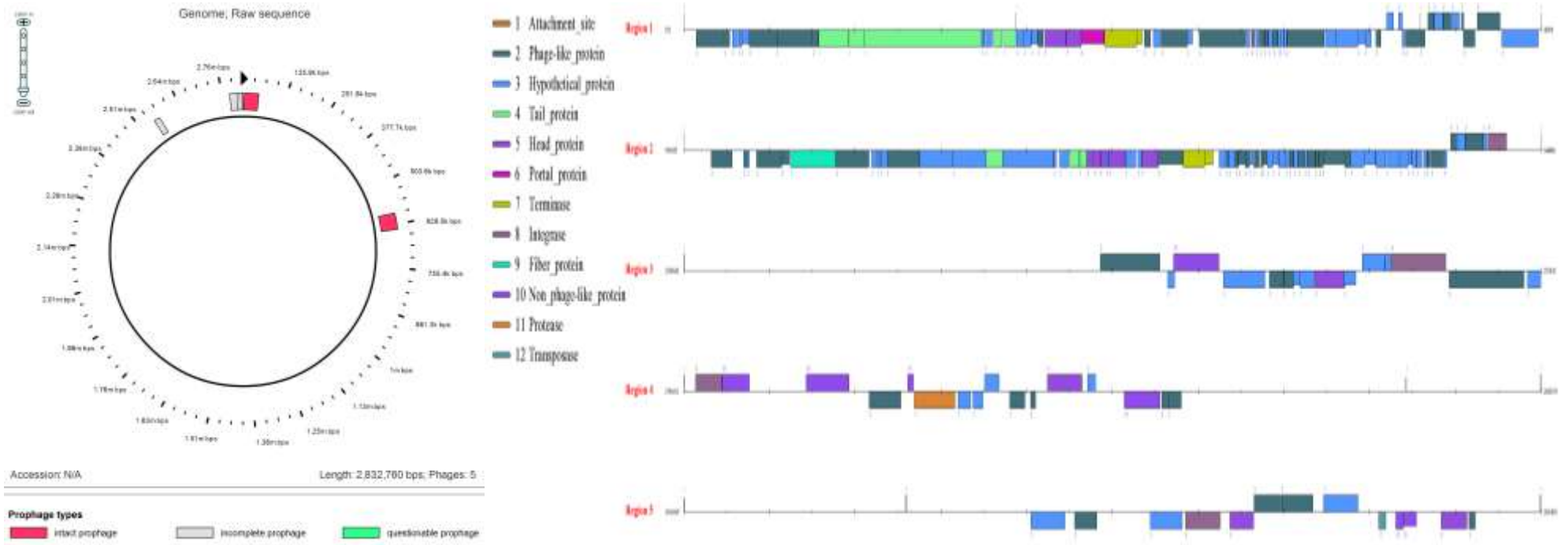
Replicons	PN235B0		PN243B0		PR243B0		PN246B0		PR041	
	Location nt*	% identity	Location nt	% identity	Location nt	% identity	Location nt	% identity	Location nt	% identity
repL(pDLK1)	2173-2649	100	1261-1737	100	2380-2856	100	3676-4152	100	946-1422	100
rep(pKH13)	3788-4627	91.91	3766-4605	91.91	3775-4614	91.91				
rep(SAP101A)	1346-2054	87.47	1324-2032	87.45	1333-2041	87.45	1449-2157	87.45	1449-2157	87.45
rep(SAP071A)	2-470	83.80								
repC (Cassette)	3674-4628	98.85	3836-4790	98.85	3650-4604	98.85	3650-4604	98.85	3650-4604	100
rep(pWBG754)							3825-4823	89.69	3825-4823	89.69
repB(pUB110)									273-980	100

*****nt: nucleotide

Table 6.4. Distribution of the prophage regions among the MRSA strains

Isolate name	Region*	Length# (kb)	Completeness	No. CDS	GC%	Phage (hit genes count)[&]
PN235B0	1	58.1	Intact	60	33.89	Staphy 47 (28)
	2	59.1	Intact	70	34.56	Staphy phiJB (26)
	3	26.2	Incomplete	15	31.88	Acineto phiAbaA1 (3)
	4	10.3	Incomplete	10	30.13	Staphy Stau 398-2 (2)
PN243B0	1	23.8	Incomplete	17	31.52	Lactoc PLgT-1 (4)
	2	58	Intact	60	33.89	Staphy 47 (28)
	3	57.2	Intact	60	34.53	Staphy phiJB (27)
	4	17.1	Incomplete	17	32.32	Mycoba Phrann (4)
	5	6.6	Incomplete	10	29.76	Entero lato (2)
PR243B0	1	45.3	Intact	58	31.52	Staphy 47 (27)
	2	48.7	Intact	70	33.89	Staphy phiJB (26)
	3	18.4	Incomplete	14	34.53	Acineto phiAbaA1 (3)
	4	25.9	Incomplete	16	32.32	Entero lato (2)
	5	15.7	Incomplete	13	29.76	Entero lato (5)
PN246B0	1	22.6	Incomplete	16	30.14	Staphy Stau 398-2 (2)
	2	27.7	Incomplete	16	31.30	Gordon GAL1 (3)
	3	30.1	Incomplete	19	36.07	Strept phiARI0460 (3)
PR041	1	20.2	Incomplete	9	32.89	Bacill SP 15 (1)
	2	32	Incomplete	14	33.93	Bacill AR9 (4)

*Intact and incomplete prophage region; #Region length of prophage regions; &Phage with the highest number of CDS in the region and the number of gene counts in brackets.



A **B**

Figure 6.1. Graphical representation (A: circular view and B: linear view) of the prophage regions hosted in the MRSA strain PR243B0. Putative genes are coloured according to the predicted functions of their products.

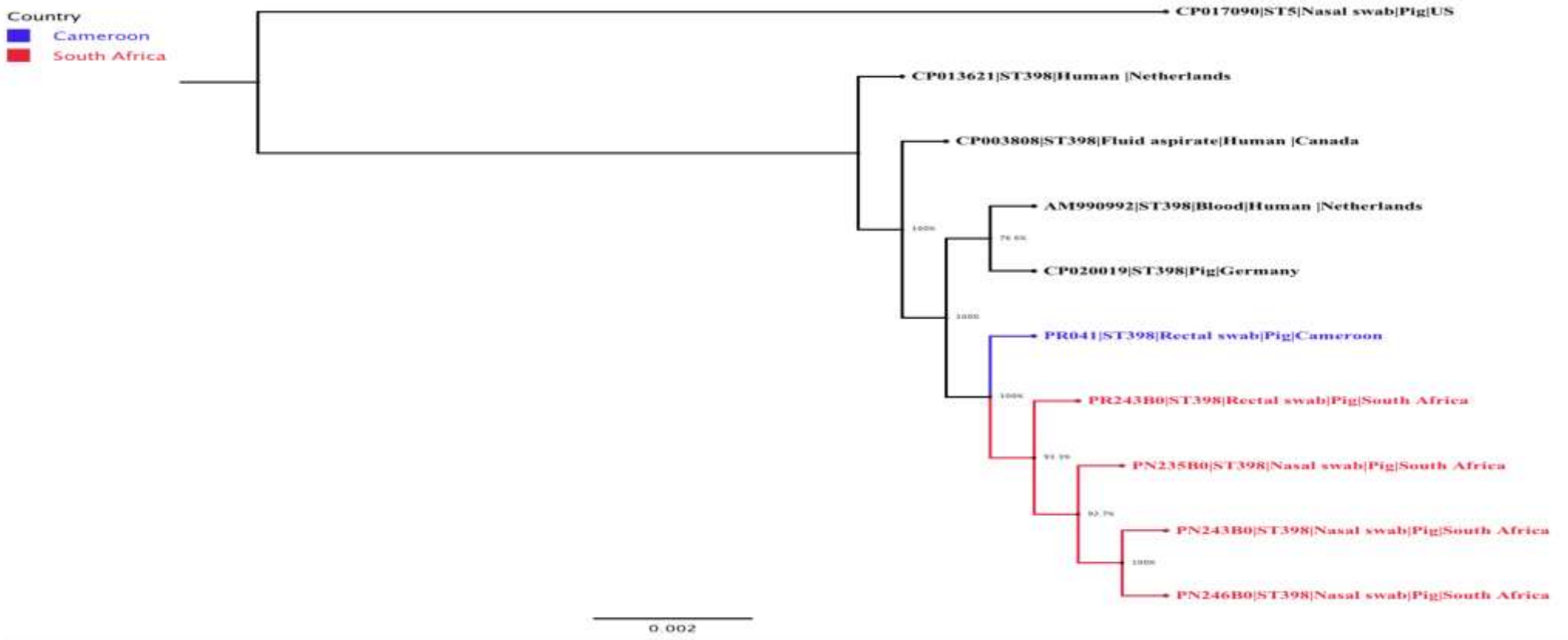


Figure 6.2. A maximum-likelihood phylogenetic tree of LA-MRSA ST398 isolates generated using FastTree version 2.1.7. The South African and Cameroon isolates are coloured in red and blue, respectively. The following information is also provided for each isolated: name/reference, sequence type, type of sample, populati and country. Clusters were defined based on a similarity cut-off of 80%.

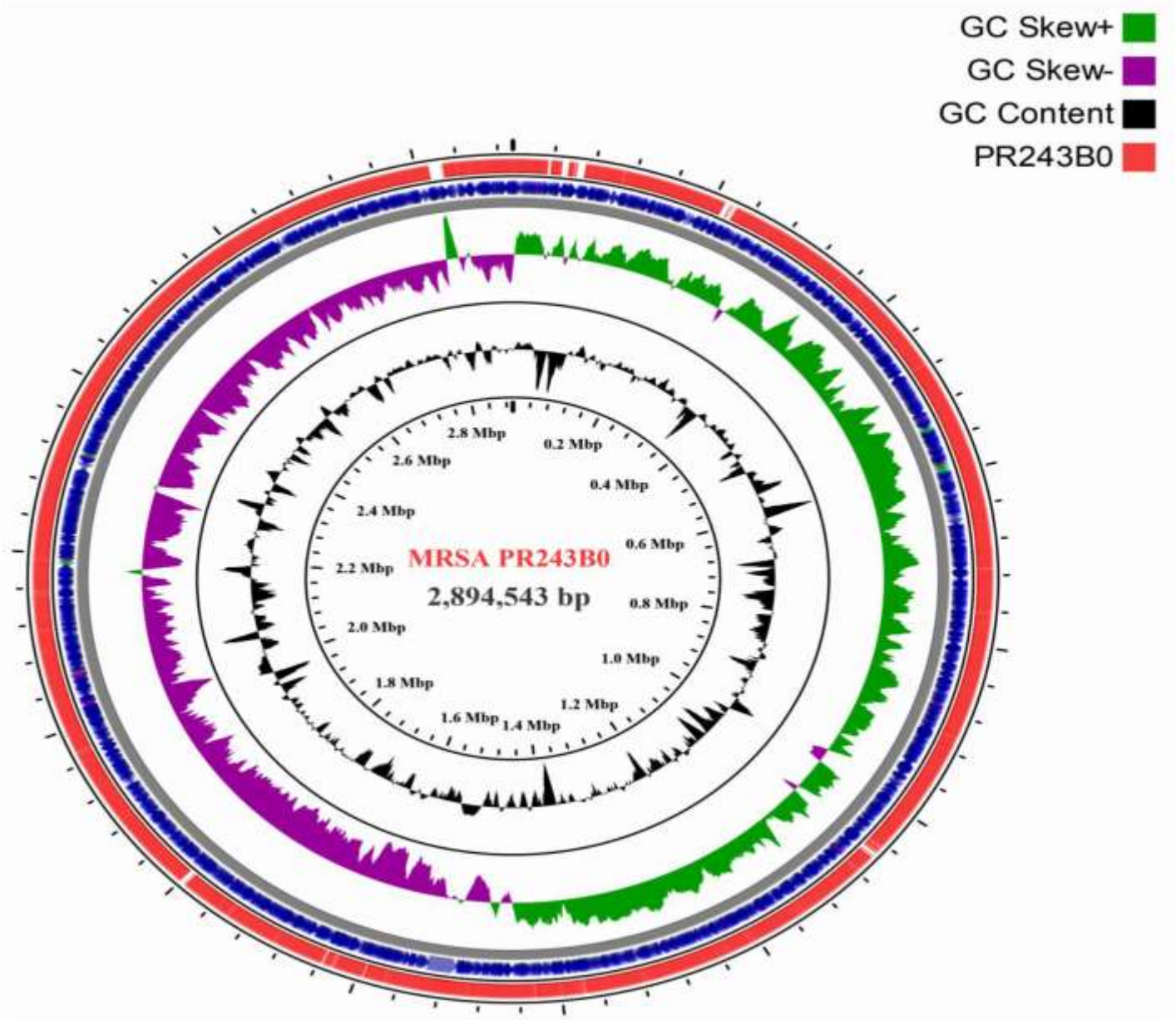


Figure 6.3. MRSA PR243B0 ring representation using CGView Server V 1.0 (Grant et al. 2012). The inner ring displays the percent of identity comparing MRSA PR243B0 and the finished genome of *S. aureus* RIVM3897 (CP013621). The next two (inner) rings show the GC content and GC skew. The next ring alternating blue and green regions shows the contig delimitations of PR243B0. The last outer ring indicates the genome PR243B0.

CHAPTER 7

Article VI. Whole Genome Sequencing of Extended-Spectrum Beta-Lactamase (ESBL)-Producing *Klebsiella pneumoniae* Isolated from Pigs and Abattoir Workers in Cameroon

Whole Genome Sequencing of Extended-Spectrum Beta-Lactamase (ESBL)-Producing *Klebsiella pneumoniae* Isolated from Pigs and Abattoir Workers in Cameroon[8]

Author contributions

- Luria Leslie Founou, as the principal investigator, co-conceptualized the study, undertook sample collection, microbiological laboratory and data analyses, prepared tables and figures, interpreted results, contributed to bioinformatics analysis, and drafted the manuscript.
- Raspail Carrel Founou undertook sample collection, microbiological laboratory analyses, contributed bioinformatics analysis, vetting of the results and writing of the manuscript.
- Mushal Allam, undertook bioinformatics analyses.
- Arshad Ismail, undertook whole genome sequencing analysis.
- Cyrille Finyom Djoko as co-supervisor, took part in the design of the study, provided materials, equipment and reagents, coordinated the field implementation in Cameroon and reviewed the manuscript.
- Sabiha Yusuf Essack, as principal supervisor, co-conceptualized the study, contributed to vetting of the results and undertook critical revision of the manuscript.

Objectives met: This original research article investigates the antibiotic resistance genes, virulence factors, mobile genetic elements and genetic lineages of circulating ESBL-producing *K. pneumoniae* strains isolated from pigs and exposed workers in Cameroonian abattoirs using whole genome sequencing (WGS) and addresses objectives Eight and Nine.

[8] This paper has been submitted to Frontiers in Microbiology and is currently under review. It will reference the "Emergence and Spread of Extended Spectrum Beta-Lactamase-Producing *Enterobacteriaceae* (ESBL-PE) in Pigs and Exposed Workers: A Multicentre Comparative Study Between Cameroon and South Africa" paper, which has been submitted and is expected to be published first.

Whole Genome Sequencing of Extended-Spectrum Beta-Lactamase (ESBL)-Producing *Klebsiella pneumoniae* Isolated from Pigs and Abattoir Workers in Cameroon

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Abstract

Background and objectives: Extended-spectrum β-lactamase (ESBL)-producing *Klebsiella pneumoniae* is a serious public health issue globally. In this study, the antibiotic resistance genes, virulence factors, mobile genetic elements and genetic lineages of circulating ESBL-producing *K. pneumoniae* strains isolated from pigs and humans in Cameroonian abattoirs were investigated using whole genome sequencing (WGS), in order to ascertain zoonotic transmission (viz. from animals to humans and/or vice-versa) in the food chain.

Methods: During March–October 2016, 288 nasal and rectal pooled samples from 432 pigs as well as nasal and hand swabs from 82 humans were collected. Seven circulating ESBL-producing *K. pneumoniae* were selected and their genomic DNA sequenced using an Illumina MiSeq platform. Generated reads were *de novo* assembled using the Qiagen CLC Genomics Workbench and SPAdes. The assembled contigs were annotated using RAST and antibiotic resistance genes, virulence factors, plasmids and bacteriophages were identified with ResFinder, Virulence Finder, PlasmidFinder and PHAST, respectively.

Results: ESBL-producing *K. pneumoniae* were detected in pigs (28%) and exposed workers (18%). The circulating *K. pneumoniae* strains were dominated principally by the sequence type (ST) 14 and 39. In addition, the "high-risk" ST307 clone and two novel STs assigned ST2958 and ST2959 were detected. Genomic analysis identified various antibiotic resistance genes associated with resistance to β–lactams, aminoglycosides fluoroquinolones, macrolide, lincosamide and streptogramins, rifampicin, sulphonamide, trimethoprim, phenicol and tetracycline. Intermingled *K. pneumoniae* populations were observed between pig- and human-source within and across abattoirs in the country.

Conclusion: Our study shows that ESBL-producing *K. pneumoniae* is actively disseminating in the food chain in Cameroon and is probably underestimated in the absence of molecular epidemiological studies. It suggests pigs, abattoir workers and food products as potential reservoirs and sources of foodborne ESBL-producing *K. pneumoniae* infections in Cameroon. It underlines the existence of an unheeded food safety and public health threat associated with these resistant strains and reinforces the crucial importance of implementing appropriate food safety measures and promoting rational antibiotic use.

7.1. Introduction

Klebsiella pneumoniae is an important Gram-negative bacillus associated with several clinical infections in humans (Perovic et al., 2014). Of particular concern is the emergence of extended-spectrum β-lactamase (ESBL) producing *K. pneumoniae* in hospital settings (Perovic et al., 2014) which has considerably increased during the last decade in response to the selection pressure of extensive antibiotic use. This resistant strain is considered a significant public health issue due to the limited therapeutic options and increased morbidity and mortality associated with it (WHO, 2017). However, the concern of ESBL-producing *K. pneumoniae* goes beyond healthcare settings to affect various ecological niches including (food) animals, food products, soil and wastewater. In fact, humans may become colonized or infected by ESBL-producing *K. pneumoniae* upon contact with blood, saliva, feces and urine of ESBL carrier animals or consumption of contaminated water or food products (Founou et al., 2016).

The most frequent and clinically relevant ESBL genes belong to Temoneira (TEM), Sulfhydryl-variable (SHV) and Cefotaximase-München (CTX-M) families, with CTX-M enzymes emerging as the predominant type. CTX-M is divided into five groups namely CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9 and CTX-M-25 according to their amino-acid identities (Perovic et al., 2014). *K. pneumoniae* commonly produces all three groups of enzymes but the latest public health concern has been the emergence of carbapenemase-producing *K. pneumoniae* and colistin-resistant *K. pneumoniae* (Hudson et al., 2014; Perovic et al., 2016). These resistance genes are generally carried on mobile genetic elements (MGEs) facilitating their dissemination within and between bacterial species (Founou et al., 2016). The presence of MGEs likely increases the proportion of serious difficult-to-treat *K. pneumoniae* infections.

The true prevalence of ESBL is not well-known in Africa and probably underestimated due to the gaps encountered in their detection on the continent. Nevertheless, some studies, have confirmed the global distribution and the high prevalence of ESBL-producing organisms in the region, albeit focusing on human health sector and ignoring the animal one (Gangoue-Pieboji et al., 2005; Breurec et al., 2013; Belbel et al., 2014; Lyonga et al., 2015; Jacobson et al., 2015; Nzalie et al., 2016). In Cameroon, antibiotics are used without restriction not only in the healthcare sector but also in the food production industry, where epidemiology of antibiotic resistant bacteria in food animals and associated public health implications is neglected. This study investigated the antibiotic resistance genes, virulence factors, mobile genetic elements and genetic lineages of circulating ESBL-producing *K. pneumoniae* strains isolated from pigs

and humans in Cameroonian abattoirs using whole genome sequencing (WGS), to ascertain zoonotic transmission (viz. from animals to humans and/or vice-versa) of ESBL-producing *K. pneumoniae* in the food chain.

7.2. Materials and methods

7.2.1. Ethical approvals

Ethical approvals were obtained from the Biomedical Research Ethics Committee (**Ref. BE365/15**) and Animal Research Ethics Committee (**Ref. AREC/091/015D**) of the University of KwaZulu-Natal as well as from the National Ethics Committee for Research in Human Health of Cameroon (**Ref. 2016/01/684/CE/CNERSH/SP**) prior to the implementation of the study. Ministerial approvals were also obtained from the Cameroonian Ministry of Livestock, Fisheries and Animal Industries (**Ref. 061/L/MINEPIA/SG/DREPIA/CE**) and Ministry of Scientific Research and Innovation (**Ref. 015/MINRESI/B00/C00/C10/C14**).

7.2.2. Study design and bacterial isolates

From March to October 2016, a multi-center study was carried out in three slaughterhouses/markets in Cameroon and two abattoirs in South Africa, encoded for ethical reasons as SH001, SH002, SH003 and SH004 and SH005, respectively (unpublished data). Three individual samples were pooled to yield 144 nasal and 144 rectal pools representing 432 original nasal and rectal samples respectively collected from 432 pigs. A total of 288 swabs from the 144 nasal and 144 rectal pools constituted the final sample. Nasal and hand swabs were also collected from 82 humans in both Cameroon and South Africa. All samples were cultured on MacConkey agar supplemented with 2 mg/L cefotaxime and incubated for 18-24 h at 37^0C in normal atmosphere (unpublished data). All putative ESBL-producers, were subjected to Gram staining, and the catalase and oxidase tests for phenotypic characterization of the isolates to the genus level. The isolates were thereafter phenotypically confirmed using the Vitek® 2 System (BioMérieuX, Marcy l'Etoile, France). The strains sequenced in this study were isolated from four pig pooled samples (PN030E4, PN089E1, PN085E1IA and PR042E3) and three exposed workers (HH510E2I, HH517E1II and HN523E1II) in Cameroon (unpublished data). The pig isolates, PN30E4 and PR042E3, originated from the same abattoir (SH001), although the former was collected from the nares and the latter from rectum. Likewise, the strains, PN089E1 and PN085E1IA, were both collected from the nares of pigs processed in abattoir SH002. The human strains, HH510E2I, HH517E1II and HN523E1II,

originated from three different abattoirs, SH001, SH002 and SH003, respectively, with HH510E2I and HH517E1II being collected from hands and HN523E1II from the nares. These strains were identified in a previous study, to be highly closely related via enterobacterial-repetitive-polymerase chain reaction (ERIC-PCR) analysis where they were grouped into five clusters (unpublished data). Given that we aimed to highlight and provide evidence of zoonotic transmission (i.e. from animals to humans and vice-versa) of ESBL-*K. pneumoniae* in the food chain, within each generated cluster, only representative isolates of intermingled strains (i.e. strains isolated from animal and/or human of the same abattoir, having high genetic relationship with those from another abattoir) were considered for WGS.

7.2.3. Screening for ESBL

The standard double disk synergy test (DDST), using cefotaxime and ceftazidime, alone and in combination with clavulanic acid as recommended by the Clinical Laboratory and Standards Institute (CLSI) was performed for ESBL screening. An increase in size of the inhibition zone of more than 5 mm in the presence of clavulanic acid was regarded as proof for ESBL production (CLSI, 2016).

7.2.4. Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed to determine the resistance patterns of the selected strains. The minimum inhibitory concentrations (MIC) of ampicillin, amoxicillin + clavulanic acid, cefuroxime, cefotaxime, ceftazidime, cefoxitin, cefepime, ertapenem, imipenem, meropenem, gentamicin, amikacin, ciprofloxacin, tigecycline, nitrofurantoin, piperacillin/tazobactam, colistin and trimethoprim-sulfamethoxazole, were determined by broth microdilution method using Vitek® 2 System (BioMérieuX, Marcy l'Etoile, France) and Vitek® 2 Gram Negative Susceptibility card (AST-N255) (BioMérieux, Marcy l'Etoile, France). The results were interpreted according to the CLSI guidelines (CLSI, 2016) with the exception of colistin, amoxicillin + clavulanic acid, piperacillin/tazobactam, amikacin that were based on EUCAST breakpoints (EUCAST, 2016) with *E. coli* ATCC 25922 and *K. pneumoniae* ATCC700603 being used as controls.

7.2.5. DNA isolation and sequencing

Genomic DNA (gDNA) was extracted using GenElute® bacterial genomic DNA kit (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions. The quantification

of extracted gDNA was determined on a NanoDrop spectrophotometer with verification by agarose gel electrophoresis and fluorimetric analysis (Qubit®). Multiplexed paired-end libraries (2×300 bp) were prepared using the Nextera XT DNA sample preparation kit (Illumina, San Diego, CA, USA) and sequences determined on an Illumina MiSeq platform with 100× coverage at the National Institute of Communicable Diseases Sequencing Core Facility, South Africa.

7.2.6. Genome assembly

The resulting raw reads were checked for quality, trimmed and *de novo* assembled into contigs using CLC Genomics Workbench version 10 (CLC, Bio-QIAGEN, Aarhus, Denmark) and SPAdes version 3.11 (Bankevich et al., 2012) to overrule any inherent shortfalls from both assemblers.

7.2.7. Genome analysis

The *de novo* assembled reads were uploaded in GenBank and annotated using NCBI prokaryotic genome annotation pipeline and RAST 2.0 server (http://rast.nmpdr.org; Aziz et al., 2008) which identified encoding proteins, rRNA and tRNA, assigned functions to the genes and predicted subsystems represented in the genome. The bacterial analysis pipeline of GoSeqIt tools was also used to annotate and identify known acquired antibiotic resistant genes via ResFinder (Zankari et al., 2012), virulence factors using VirulenceFinder (Joensen et al., 2014) and mobile genetic elements through PlasmidFinder (Carattoli et al., 2014). The RAST SEED viewer was used to identify the presence of transposases and integrons flanking the beta-lactamase genes (Overbeek et al., 2014). PHAge Search Tool (PHAST) server was used for the identification, annotation and visualization of prophage sequences (Zhou et al., 2011).

7.2.8. Multilocus sequence typing (MLST)

In silico MLST-analyses was performed using the scheme of Diancourt et al. (2005), which consider allelic variation amongst seven housekeeping genes (gapa, infb, mdh, pgi, phoe, rpob and tonb) to assign STs. WGS data were used to generate a *K. pneumoniae* MLST assignment for each isolate with new or unknown STs being sent for curation at the *Klebsiella pneumoniae* MLST database at the Pasteur Institute (http://bigsdb.pasteur.fr/klebsiella/klebsiella.html).

7.2.9. Whole genome phylogenetic analyses

The assembled contigs were aligned against the complete genome of *K. pneumoniae* KPN528 (CP020853) using the progressive Mauve genome alignment package version 2.3.1. Study genomes were contextualized against a collection of five relevant assembled *K. pneumoniae* genomes (accession numbers AYQE00000000, CP006918, CP020853, CP020847, CP020841). Phylogenetic analysis was based on the core genomes and performed using the Rapid large-scale prokaryote pan genome analysis (Roary) (Page et al., 2015). The maximum likelihood phylogenetic tree encompassing, country, population, sample type metadata and MLST type was generated, edited and visualized using FastTree version 2.1.7 (http://www.microbesonline.org/fasttree/). In addition, the contigs were mapped against the complete genome of *K. pneumoniae* KPN528 (CP020853) for visualization of the genomic organization.

7.2.10. Nucleotide sequence accession number

This whole-genome shotgun project PRJNA412434 of *K. pneumoniae* strains PN030E4, HH510E2I, HN523E1II, PN089E1, PR042E3, HH517E1II and PN085E1IA has been deposited at DDBJ/EMBL/GenBank under accession numbers PDVM00000000, PDVF00000000, PDVG00000000, PDVC00000000, PDVE00000000, PDVU00000000 and PDVD00000000, respectively. The versions described in this paper are the versions, PDVM00000000.1, PDVF00000000.1, PDVG00000000.1, PDVC00000000.1, PDVE00000000.1, PDVU00000000.1 and PDVD00000000.1, respectively.

7.3. Results

7.3.1. Phenotypic and analyses

Out of the 144 pooled nasal samples (three nasal swabs each) and 144 pooled rectal samples (three rectal swabs each) taken from 432 pigs in Cameroon and South Africa, ESBL-producing *Enterobacteriaceae* were detected in 108/144 (75%) and 102/144 (71%) nasal and rectal pools, respectively (unpublished data). Carriage of ESBL-producing *Enterobacteriaceae* was observed in Cameroonian workers only. Multiple colonies (up to ten) were isolated from both populations and countries, but due to financial constraints, post-stratification allowed the selection of 158 ESBL-PE in pigs and 71 in exposed workers, for phenotypic analysis (unpublished data). Out of these, ESBL-producing *K. pneumoniae* were only detected in Cameroon with 21.52% (34/158) and 11.26% (8/71) detected in pigs and exposed workers,

respectively (unpublished data). None of the South African pig or human samples were positive for ESBL-producing *K. pneumoniae*. Table 7.1 summarizes relevant population data, specimen source, phenotypic and genotypic characteristics for these isolates. All isolates displayed reduced susceptibility to the amino-penicillins, cephalosporins, trimethoprim-sulfamethoxazole, with various resistance to gentamicin (n=5; 71%), ciprofloxacin (n=1; 14%) and nitrofurantoin (n=1; 14%). The antimicrobial susceptibility results of the ESBL-producing *K. pneumoniae* isolates are summarized in Table 7.2, with the resistance observed being corroborated with WGS analyses.

7.3.2. Genotypic analyses

All isolates carried sulphonamide (*sul*1), fosfomycin (*fos*A) and quinolone (*oqx*A and *oqx*B) resistance genes. Various beta-lactamase encoding determinants were detected with $bla_{\text{CTX-M-15}}$ (86%), $bla_{\text{TEM-1B}}$ (57%) and $bla_{\text{SCO-1}}$ (43%) being the most prevalent. Four (PN089E1, PN085E1IA, HH517E1II and HH510E2II) and three (PR042E3, PN030E4 and HN523E1II) isolates concomitantly harbored four and three beta-lactamase encoding genes. Likewise, six (86%) strains harbour dfrA15 gene responsible for trimethoprim resistance, while co-presence of *str*A and *str*B encoding for aminoglycoside resistance was observed in five (71%) isolates as was the *tet*(A) gene responsible for tetracycline resistance. Interestingly, $bla_{\text{OXA-9}}$, bla_{LEN12}, $bla_{\text{SHV-134}}$, $bla_{\text{TEM-1A}}$ were observed in the unique *K. pneumoniae* ST307 strain as were *aac(6')Ib-cr*, *cat*A2, *sul*2, *tet*(D), *drf*A27 and ARR-3 encoding resistance to aminoglycosides, phenicols, sulphonamides, tetracycline, trimethoprim and rifampicin respectively. Similarly, the β-lactamase genes $bla_{\text{TEM-116}}$ and $bla_{\text{SHV-28}}$, and plasmid mediated quinolone resistance (PMQR) genes QnrB1 were only identified in the two *K. pneumoniae* ST14 strains.

7.3.3. Multilocus sequence typing

In silico MLST-analyses revealed that *K. pneumoniae* strains belonging to five different sequence types, namely ST14 (n=2), ST39 (n=2), a single-locus variant ST307 and two new sequences. The two *K. pneumoniae* ST14 strains were isolated from a pig pooled nasal sample (PN30E4) and a human nasal swab (HN523E1II) located in two different abattoirs, SH001 and SH003, respectively (Table 7.1). The *K. pneumoniae* ST39 strains, HH517E1II and PN085E1IA, were also detected from an exposed worker and pooled nasal samples but both from the same abattoir SH002. In our collection of seven ESBL-producing *K. pneumoniae* isolates, two strains, PR042E3 and PN089E1 isolated from pigs in two different abattoirs

(SH001 and SH002), had a novel combination of known *K. pneumoniae* MLST alleles, that were assigned as ST2958 (n=1) and ST2959 (n=1), respectively. The sole *K. pneumoniae* ST307 strain was isolated from hand of an exposed worker and harbored a total of 22 resistance determinants encoding resistance to nine antibiotic classes.

7.3.4. Phylogenetic analysis

The phylogenetic analysis confirmed the intermingled reservoir of ESBL-producing *K. pneumoniae* strains and revealed that our strains fall within two clades of international *K. pneumoniae* isolates. Figure 7.2 demonstrates considerable similarity between our collection of *K. pneumoniae* ST14 and three strains CP020841 (ST37), CP006918 (ST258) and CP020847 (ST906), all isolated from clinically ill humans in United States. Similarly, the *K. pneumoniae* ST39 strains isolated from nares of healthy pig (PN085E1IA) and the hand of healthy human (HH517E1II) in abattoir SH002, were closely related *K. pneumoniae* ST307 (HH510E2I) isolated from hand of a human in SH001 and a *K. pneumoniae* ST336 isolated in clinically ill patient in Lebanon (Figure 7.2). Figure 7.3 shows the genomic organization of the ESBL-producing *K. pneumoniae* HN523E1II mapped against the complete genome of *K. pneumoniae* KPN528 (CP020853).

7.3.5. Detection of plasmids and phage-associated regions

PlasmidFinder revealed that the colRNAI plasmid replicon type was identified in all strains whereas the IncFIB(K) plasmid incompatibility group was detected in six (86%) isolates. The two *K. pneumoniae* ST14 strains, PN030E4 and HN523E1II, concomitantly harbored two colRNAI plasmid replicon types as well as two IncFIA(HI1) and IncFIB(K) plasmid incompatibility groups, with the strain PN030E4 additionally harboring the IncY plasmid. Likewise, the *K. pneumoniae* ST307 harbor two colRNAI replicons as well as FIA (pBK30683) and IncR plasmid incompatibility groups while the *K. pneumoniae* ST 39 carries four colRNAI replicons along with IncHI1B and IncFIB(K) plasmid incompatibility groups. *In silico* plasmid MLST-analyses assigned the IncF plasmid incompatibility group as belonging to various sequence types including [K1:A13-like:B-], [K-:A10-like:B-], [K-:A13:B-] while the IncH plasmid belongs to a non-typeable ST.

With regard to the phage-associated regions, all strains hosted at least one intact bacteriophage. Shigel SfII, pseudo JBD44, Entero lato and Escher HK639 were the predominant intact bacteriophages. Six phage regions were identified in one *K. pneumoniae* ST14 (HN523E1II)

isolated from hand of a worker using PHAST algorithm, while the other ST14 (PN030E4) and one ST39 (PN085E1IA) observed in pigs hosted three phage regions each.

7.4. Discussion

In this study, antibiotic resistance genes, virulence factors, mobile genetic elements and genetic lineages of seven circulating and closely related ESBL-producing *K. pneumoniae* strains isolated from pigs and occupationally exposed workers in Cameroonian abattoirs were investigated using whole genome sequencing.

ESBL-producing *K. pneumoniae* have been observed at diverse rates in clinical samples in the Ivory Coast (16%), Morocco (13%), Cameroon (10%) and Madagascar (9%) (Breurec et al., 2013). They were also responsible of community-acquired urinary tract infections in a Cameroonian city with an incidence of 16.4% (Nzalie et al., 2016). Our study, similarly revealed ESBL-producing *K. pneumoniae* strains in pigs (27.64%) and exposed workers (16.66%) in Cameroon as the first report ESBL-producing *K. pneumoniae* isolates in food animals and occupationally exposed workers in the country. The ESBL-producing *K. pneumoniae* strains were mainly circulating in two clonal lineages since four out of seven isolated strains belong to the ST14 (n=2) and ST39 (n=2). To the best of our knowledge, this is the first evidence of a porcine ESBL-producing *K. pneumoniae* reservoir in this country. Moreover, the first reports of *bla*$_{\text{CTX-M-15}}$, *bla*$_{\text{TEM-1B}}$, *bla*$_{\text{SHV-11}}$, *bla*$_{\text{SCO-1}}$ producing *K. pneumoniae* ST39 and *bla*$_{\text{CTX-M-15}}$, *bla*$_{\text{TEM-116}}$, *bla*$_{\text{SHV-28}}$ producing *K. pneumoniae* ST14 in pigs and humans in Cameroon are presented here.

The *K. pneumoniae* ST14 isolates were found to be resistant to ampicillin, cefuroxime, cefuroxime-axetil, cefotaxime, ceftazidime and trimethoprim-sulfamethoxazole. This resistance phenotype was corroborated by the identification of the CTX-M-15, SHV-28 and TEM-116 genes by WGS which also elucidated with multiple resistant determinants to non-β-lactams antibiotic, notably the aminoglycoside resistant genes (*str*A, *str*B), plasmid-mediated quinolone resistance genes (*Qnr*B1, *oqx*A, *oqx*B), fosfomycin resistant gene (*fos*A), and sulphonamide resistant gene (sul1 and sul2) which were not phenotypically evident. Although the two *K. pneumoniae* ST39 isolates displayed similar phenotypic profiles these were attributed to different resistance gene permutations. For example, the pig strain PN085E1IA harbored genes encoding for aminoglycoside [*aad1*, *aac(3)-IIa*], fluoroquinolones (*oqx*A, *oqx*B), fosfomycin (*fos*A), tetracycline [*tet*(A)], trimethoprim (*dfr*A15) and sulphonamide (*sul*1, *sul*2) resistance alongside blaCTX-M-15, *bla*$_{\text{TEM-1B}}$ and *bla*$_{\text{SHV-1}}$ while *aad*1, *aac*(3)-IIa, *oqx*A, *oqx*B, fosA, *sul*1, *tet*(A), *dfr*A15, *bla*$_{\text{TEM-1B}}$, *bla*$_{\text{CTX-M-15}}$, *bla*$_{\text{SCO-1}}$, *bla*$_{\text{SHV-11}}$ were

observed in the human strain HH517E1II. CTX-M-15 detection is consistent with a multicenter study conducted in five African (including Yaoundé) and two Vietnamese towns where it was detected in 74% of isolates and was the predominant ESBL among the African isolates (Breurec et al., 2013). This study further reported the predominance of *Qnr*B determinant among the African strains (Breurec et al., 2013). In addition, CTX-M-15-producing *K. pneumoniae* hosted on a plasmid has already been reported in patients with clinical urinary tract infections in Cameroon (Gangoue-Pieboji et al., 2005), confirming the widespread dissemination of this ESBL type. The preponderance of CTX-M-15 as the main ESBL genes (85.71%) in our *K. pneumoniae* isolates confirms that CTX-M-15 is currently the most largely distributed CTX-M enzyme worldwide. The potential role of *K. pneumoniae* as a reservoir for β-lactam and non-β-lactam resistance determinants is a major concern in countries with inadequate antibiotic resistance (ABR) surveillance, prevention and containment measures such as Cameroon. Key factors favouring the emergence and spread of ABR in the food chain in the country include irrational antibiotic use on farms, poor sanitary and feeding practices, sub-optimal transport conditions, lack of veterinarian control, inadequate diagnostic facilities, substandard quality of antibiotics, lack of antimicrobial monitoring and poor biosecurity measures (Ndebi et al., 2009).

Our study showed that both ST14 and ST39 demonstrated overlap and intermingled populations between pig- and human-sources within and across abattoirs (Figure 1). Specifically, the *K. pneumoniae* ST14 strains colonized nares of both human and pigs located in two different abattoirs (SH001 and SH003) whereas *K. pneumoniae* ST39 was detected in nares of pigs and the hand of a worker present in the same abattoir, SH002 (Figure 1). This could be associated with neglected hygienic practices prevailing during production, transport, storage and/or retail stages. The virtual absence of physical barriers between community and healthcare settings in this country along with poverty and limited education may also be important contributory factors.

The ST14 and ST39 clonal lineages are a major cause of nosocomial infections and outbreak situations around the world, although their evolutionary emergence is somewhat poorly documented in developing countries. In fact, OXA-181-producing *K. pneumoniae* ST14 was detected in South Africa where it was responsible of an outbreak of among hospitalized patients in a tertiary hospital (Jacobson et al., 2015), whereas, a multidrug resistant and biofilm producing *K. pneumoniae* strain belonging to the ST14 was detected in India also at tertiary care (Rafiq et al., 2016). Similarly, the *K. pneumoniae* ST39 was responsible for an outbreak in a pediatric hospital in Algeria (Belbel et al., 2014). The isolation of these STs always urged

the implementation of stringent infection and control measures and ongoing surveillance of antibiotic resistance in hospital settings. Similar strict interventions should thus be undertaken in the food production industry if we are to successfully contain their clonal dissemination in the food chain.

The detection of the international *K. pneumoniae* ST307 in a human isolate is further evidence of the wide and increasing spread of ESBL-producing bacteria in the country. The *K. pneumoniae* ST307 has been recognized as candidate for becoming one of the prevalent high-risk and clinically relevant clones since its worldwide emergence during the last five years (Villa et al., 2017). The *K. pneumoniae* ST307 lineage is generally capsulated, displays higher resistance to complement-mediating killing, has novel virulence arrays and is associated with CTX-M-15 and KPC encoding plasmids (Villa et al., 2017). Accordingly, *Klebsiella pneumoniae* Carbapenemase (KPC)-producing *K. pneumoniae* ST307 carrying a self-transferable plasmid (IncX3-type) was detected among clinical specimens during a nosocomial outbreak in South Korea (Kim et al., 2017) as was KPC-producing *K. pneumoniae* ST307 harboring pKPN-307 plasmid (Villa et al., 2017). In contrast to these reports, the *K. pneumoniae* ST307 detected in our study did not harbor the CTX-M-15 and KPC enzymes nor the IncX plasmid but rather TEM-1A, LEN12, OXA-9 and SHV-134 as β-lactamases, and colRNAI, IncR and FIA (pBK30683) plasmid incompatibility groups, suggesting a different phylogenetic evolution. The ability of this clonal lineage to acquire novel genetic features may contribute to its increased persistence in the environment and highlights its potential public health threat.

Only *K. pneumoniae* ST11 and ST15 were detected from clinical samples in Yaoundé, Cameroon to date. The detection of two new genome sequences *K. pneumoniae* ST2958 and ST2959 in pigs with different antibiotic resistance profiles and genes, improve our understanding and scope of the molecular epidemiology and evolution of resistant bacteria in the country. The emergence of these ESBL-producing *K. pneumoniae* in pigs and exposed workers within and between abattoirs in Cameroon is of great significance as it confirms their active clonal dissemination via direct contact, and suggests their indirect spread throughout the food chain in the country. These findings further suggest that pigs, pork, and abattoir workers represent a potential reservoir and source of foodborne ESBL-producing *K. pneumoniae* infections in Cameroon and reinforce the crucial importance of implementing appropriate food safety measures and promoting rational antibiotic use.

All isolates except HH510E2I (MIC ≥4 µg/ml) were susceptible to ciprofloxacin (MIC ≤0.5 µg/ml) suggesting that they might not contain gene encoding for resistance to fluoroquinolones,

but *oqx*A and *oqx*B were detected in all isolates. Similarly, although not expressing phenotypic resistance to gentamicin (MIC ≤1 µg/ml), amikacin (MIC ≤ 2 µg/ml) and ciprofloxacin (MIC ≤0.25 µg/ml), the pig and human isolates, PN030E4 and HN523E1II, harbored *str*A and *str*B encoding for resistance to aminoglycoside together with the fluoroquinolone resistance genes *oqx*A, *oqx*B and *Qnr*B1. This finding thus indicate that these genes might be "silent" in ESBL-producing *K. pneumoniae* circulating in pigs and humans in Cameroon, and could likely be triggered in response to the selective pressure of widespread antibiotic use.

FosA gene a glutathione *S*-transferase that causes enzymatic inactivation of and resistance to fosfomycin, was detected in six out of seven isolates in our study with 100% homology. Fosfomycin is a broad-spectrum antibiotic used extensively in Europe and Africa for treatment of uncomplicated urinary tract infections (Xu et al., 2011). It is receiving renewed interest globally as a therapeutic option for the treatment of infections caused by carbapenem-resistant *Enterobacteriaceae*. Our finding of chromosomal fosA gene in 85.71% our isolates concurs with a report which revealed that several Gram-negative species including *K. pneumoniae, K. oxytoca, Enterobacter cloacae, Enterobacter aerogenes, Serratia marcescens,* carry *fos*A gene on their chromosome and are species with intrinsic reduced susceptibility or resistance to fosfomycin (Ito et al., 2017). The widely distribution of *fos*A gene in our study further suggests that *K. pneumoniae* could serve as a reservoir for this gene and facilitate its dissemination to species lacking *fos*A such as *E. coli* in the country. Although the contextualization of this finding is difficult due to the scarcity of molecular epidemiological studies in Africa, this finding concurs with several studies from Asian (Wachino et al., 2010; Hou et al., 2013; Chan et al., 2014) and European countries (Benzerara et al., 2017) which showed the presence of multiple lineages of fosA producing ESBL-*E. coli.* Any interest in repurposing use of old antibiotics should thus be considered with caution and in light of existing environmental reservoirs of resistance genes.

The CGview analyses showed that major parts of the *K. pneumoniae* ST14 (HN523E1II), are present within the reference genome KPN528 (CP020853) (Figure 2). However, some discrepancies in both identity and coverage were observed, as part of the rings are lighter colored or missing, implicating the presence of other genetic elements or independent evolution of this isolate. This suggests that closure of our genomes would be essential to decipher and understand the evolutionary biology of ESBL-producing *K. pneumoniae*. Besides, investigation of the resistance genes and comparison along with the phage-associated regions harboured by our isolates, did not reveal the presence of a prophage-encoding resistance genes.

This suggests that prophages are unlikely to act as vectors for the dissemination of resistance within our isolates. Nonetheless, the acquisition of mobile genetic elements including the plasmids and bacteriophages contribute to the phenotypic and genetic plasticity of their bacterial host and can act as vector for the transfer of resistance determinants and virulence factors leading to increased prevalence. Routine screening for ESBL-producing *K. pneumoniae* colonization in food animals, exposed workers, farms, abattoirs and food products is thus essential for its effective containment.

Even though our findings may not be extrapolated to the overall situation of the country, it is noteworthy that these highlight a serious food safety threat as the study took place in the main abattoirs of the country's capital. Further high-resolution genotyping studies of ESBL-producing *K. pneumoniae* collected over larger temporal and spatial scales are required to better understand the evolution, molecular epidemiology, and transmission dynamics of these resistant isolates.

7.5. Conclusion

Our study shows that ESBL-producing *K. pneumoniae* is actively disseminating in pigs and occupationally exposed workers in Cameroonian abattoirs and is probably under-estimated considering the absence of molecular epidemiological studies. It underlines the existence of a potential unheeded food safety and public health threat associated with these resistant strains in the country especially if they spread to susceptible people such as immunocompromised. Ongoing efforts and further well-designed epidemiological studies to understand the epidemiology, transmission dynamics and pathways, risk factors and public health implications associated with the food animal reservoir of ESBL-producing bacteria are essential to inform effective interventions for their containment.

Conflict of interest

Professor Essack is a member of the Global Respiratory Infection Partnership sponsored by an unrestricted educational grant from Reckitt and Benckiser. All other authors declare that there is no competing financial interest.

Author contributions

LF co-conceptualized the study, undertook sample collection, microbiological laboratory and data analyses, prepared tables and figures, interpreted results, contributed to bioinformatics analysis, and drafted the manuscript. **RF** undertook sample collection, microbiological

laboratory analyses, contributed bioinformatics analysis and vetting of the results. **MA** undertook bioinformatics analyses. **AI** performed whole genome sequencing analysis. **CD** took part in the design of the study, provided material, equipment and reagents, undertook critical revision of the manuscript and coordinated the field implementation. **SE** co-conceptualized the study and undertook critical revision of the manuscript. All authors read and approve the final manuscript.

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Table 7.1. Summary of population, sample type, phenotypic and genotypic characteristics of ESBL-producing *K. pneumoniae* isolates

Isolate name	Country	Sample type	Abattoir	Antibiotic resistance genes	Plasmids	MLST
PN030E4	Pig	Nasal pooled sample	SH001	*str*A, *str*B, *bla*$_{\text{TEM-116}}$, *bla*$_{\text{SHV-28}}$, *bla*$_{\text{CTX-M-15}}$, *oqx*A, *oqx*B, *Qnr*B1, *fos*A, *sul*1, *sul*2, *tet*(A), *dfr*A15	ColRNAI, IncFIB(K), IncFIA(HI1), IncY	ST14
HN523E1II	Human	Nasal swab	SH003	*str*A, *str*B, *bla*$_{\text{TEM-116}}$, *bla*$_{\text{SHV-28}}$, *bla*$_{\text{CTX-M-15}}$, *oqx*A, *oqx*B, *Qnr*B1, *fos*A, *sul*1, dfrA15	ColRNAI, IncFIB(K), IncFIA(HI1)	ST14
HH517E1II	Human	Hand swab	SH002	aac(3)-IIa , *aad*A1, *bla*$_{\text{TEM-1B}}$, *bla*$_{\text{SHV-11}}$, *bla*$_{\text{CTX-M-15}}$, *bla*$_{\text{SCO-1}}$, *oqx*A, *oqx*B, *fos*A, *sul*1, *tet*(A), *dfr*A15	ColRNAI, IncFIB(K), IncHI1B	ST39
PN085E1IA	Pig	Nasal pooled sample	SH002	aac(3)-IIa , *aad*A1, strA, strB, *bla*$_{\text{TEM-1B}}$, *bla*$_{\text{SHV-27}}$, *bla*$_{\text{SCO-1}}$, *bla*$_{\text{CTX-M-15}}$, *oqx*A, *oqx*B, *fos*A, *cat*A1, *sul*1, *sul*2, *tet*(A), *dfr*A15	ColRNAI, IncFIB(K), IncFII(K), FIA(pBK30683)	ST39
PR042E3	Pig	Rectal pooled sample	SH001	aac(3)-IIa , *aad*A1, *str*A, *str*B, *bla*$_{\text{TEM-1B}}$, *bla*$_{\text{SHV-1}}$, *bla*$_{\text{CTX-M-15}}$, *oqx*A, *oqx*B, *fos*A, *sul*1, *tet*(A), *dfr*A15	ColRNAI, IncFIB(K)	ST2958
PN089E1	Pig	Nasal pooled sample	SH002	aac(3)-IIa , *aad*A1, *str*A, *str*B, *bla*$_{\text{TEM-1B}}$, *bla*$_{\text{SHV-27}}$, *bla*$_{\text{SCO-1}}$, *bla*$_{\text{CTX-M-15}}$, *oqx*A, *oqx*B, *fos*A, *mph*(A), *cat*A1, *sul*1, *sul*2, *tet*(A), *dfr*A15	ColRNAI, IncFIB(K), IncR, ColE10	ST2959
HH510E2I	Human	Hand swab	SH001	aac(6')-Ib, *str*A, *str*B, aac(3)-IId, aac(6')Ib-cr*, *aad*A1, *aad*A16, *bla*$_{\text{OXA-9}}$, *bla*$_{\text{LEN-12}}$, *bla*$_{\text{SHV-134}}$, *bla*$_{\text{TEM-1A}}$, *oqx*A, *oqx*B, *fos*A, *cat*A2, *ARR*-3, *sul*1, *sul*2, *tet*(D), *dfr*A27	ColRNAI, IncR, FIA(pBK30683)	ST307

*aac(6')Ib-cr: confers concomitant resistance to aminoglycosides and fluoroquinolones

Table 7.2. Minimum inhibitory concentrations (MICs) of selected β-lactam and non-β-lactam antibiotics for individual ESBL-producing *K. pneumoniae* isolates

Isolate name	β-lactam antibiotics										non-β-lactam antibiotics							Antibiotic resistance genes
	AMP	AMC	TZP	CXM	CTX	CAZ	FEP	ETP	MEM	IMP	GEN	AN	CIP	TGC	FT	CS	TMP/SXT	
HH510E2I	≥32	16	≤4	16	4	≥64	≤1	≤0.5	≤0.25	≤0.25	≥16	≥16	≥4	≤0.5	≤16	≤0.5	≥320	*aac(6')-Ib, strA, strB, aac(3)-IId, aac(6')Ib-cr, aadA1, aadA16, bla$_{OXA-9}$, bla$_{LEN-12}$, bla$_{SHV-134}$, bla$_{TEM-1A}$, oqxA, oqxB, fosA, catA2, ARR-3, sul1, sul2, tet(D), dfrA27*
HH517E1II	≥32	16	8	≥64	≥64	4	≤1	≤0.5	≤0.25	≤0.25	≥16	≤2	≤0.25	1	128	≤0.5	≥320	*aac(3)-IIa , aadA1, bla$_{TEM-1B}$, bla$_{SHV-11}$, bla$_{CTX-M-15}$, bla$_{SCO-1}$, oqxA, oqxB, fosA, sul1, tet(A), dfrA15*
PN085E1IA	≥32	8	≤4	≥64	≥64	16	2	≤0.5	≤0.25	≤0.25	≥16	≤2	≤0.25	1	32	≤0.5	≥320	*aac(3)-IIa , aadA1, strA, strB, bla$_{TEM-1B}$, bla$_{SHV-27}$, bla$_{SCO-1}$, bla$_{CTX-M-15}$, oqxA, oqxB, fosA, catA1, sul1, sul2, tet(A), dfrA15*
PR042E3	≥32	4	≤4	≥64	≥64	2	2	≤0.5	≤0.25	≤0.25	≥16	≤2	≤0.25	1	32	≤0.5	≥320	*aac(3)-IIa , aadA1, strA, strB, bla$_{TEM-1B}$, bla$_{SHV-1}$, bla$_{CTX-M-15}$, oqxA, oqxB, fosA, sul1, tet(A), dfrA15*
PN089E1	≥32	4	≤4	≥64	≥64	8	2	≤0.5	≤0.25	≤0.25	≥16	≤2	≤0.25	1	32	≤0.5	≥320	*aac(3)-IIa , aadA1, strA, strB, bla$_{TEM-1B}$, bla$_{SHV-27}$, bla$_{SCO-1}$, bla$_{CTX-M-15}$, oqxA, oqxB, fosA, mph(A), catA1, sul1, sul2, tet(A), dfrA15*

PN030E4	≥32	4	≤4	≥64	8	8	2	≤0.5	≤0.25	≤0.25	≤1	≤2	≤0.25	≤0.5	≤16	≤0.5	≥320	*strA, strB, bla$_{TEM-116}$, bla$_{SHV-28}$, bla$_{CTX-M-15}$, oqxA, oqxB, QnrB1, fosA, sul1, sul2, tet(A), dfrA15*
HN523E1II	≥32	4	≤4	≥64	≥64	8	2	≤0.5	≤0.25	≤0.25	≤1	≤2	≤0.25	≤0.5	64	≤0.5	≥320	*strA, strB, bla$_{TEM-116}$, bla$_{SHV-28}$, bla$_{CTX-M-15}$, oqxA, oqxB, QnrB1, fosA, sul1, dfrA15*

AMP: Ampicillin, AMC: Amoxicillin-clavulanic acid; TZP: Piperacillin-tazobactam; CXM: Cefuroxime; CTX: Cefotaxime; CAZ: Ceftazidime; ETP: Ertapenem; MEM: Meropenem; IMP: Imipenem; GEN: Gentamicin; AN: Amikacin; CIP: Ciprofloxacin; TGC: Tigecycline; FT: Nitrofurantoin; CS: Colistin; TMP/SXT: Trimethoprim-Sulfamethoxazole

Table 7.3. Distribution of intact prophage regions among the ESBL-producing *K. pneumoniae* strains

Isolate name	Region[a]	Length[b] (kb)	No CDS	GC%	Phage (hit genes count)[c]
HH510E2I	1	37.6	47	51.42	Salmon 103203 sal5 (13)
HH517E1II	1	39.3	52	52.13	Salmon 64795 sal3 (15)
HN523E1II	1	30.3	37	51.02	Entero mEp390 (6)
	2	17.1	20	48.37	Entero lato (7)
	3	27.6	12	53.30	Shigel SfII (8)
	4	17.4	26	54.84	Shigel SfII (18)
	5	58.6	44	53.75	Escher HK639 (18)
	6	29.8	35	51.86	Entero c 1 (9)
PN030E4	1	29.6	35	51.54	Brucel BiPBO1 (6)
	2	23	28	53.03	Shigel SfII (20)
	3	48.6	49	53.27	Escher HK639 (19)
PN085E1IA	1	27.4	28	52.12	Pseudo JBD44 (9)
	2	31.4	25	52.93	Entero c 1 (12)
	3	33.1	12	49.24	Entero lato (7)
PR089E1	1	47.6	41	50.80	Salmon 118970 sal3 (11)
	2	27	34	55.25	Salmon Fels 2 (22)
PR042E3	1	53.1	57	52.17	Pseudo JBD44 (13)

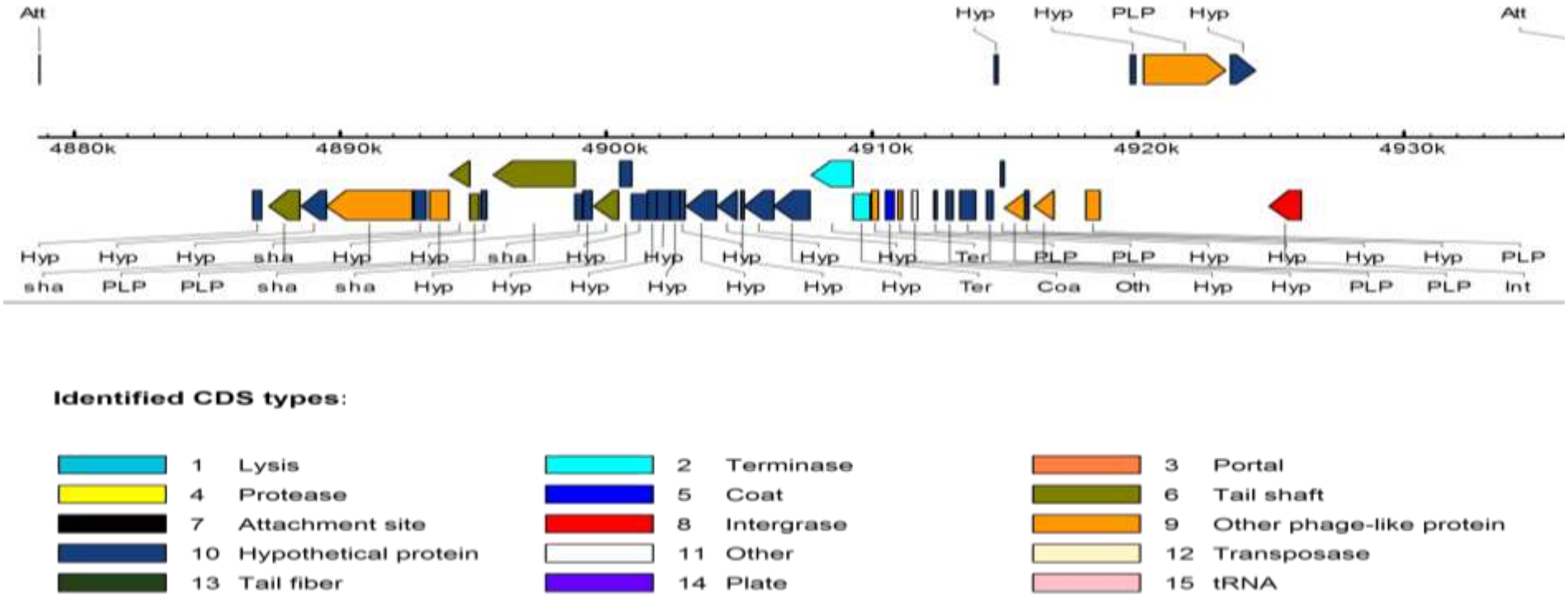

Figure 7.1. Linear view of the prophage Escher HK639 isolated from the *K. pneumoniae* ST14 (HN523E1II). Putative genes are coloured according to the predicted functions of their products.

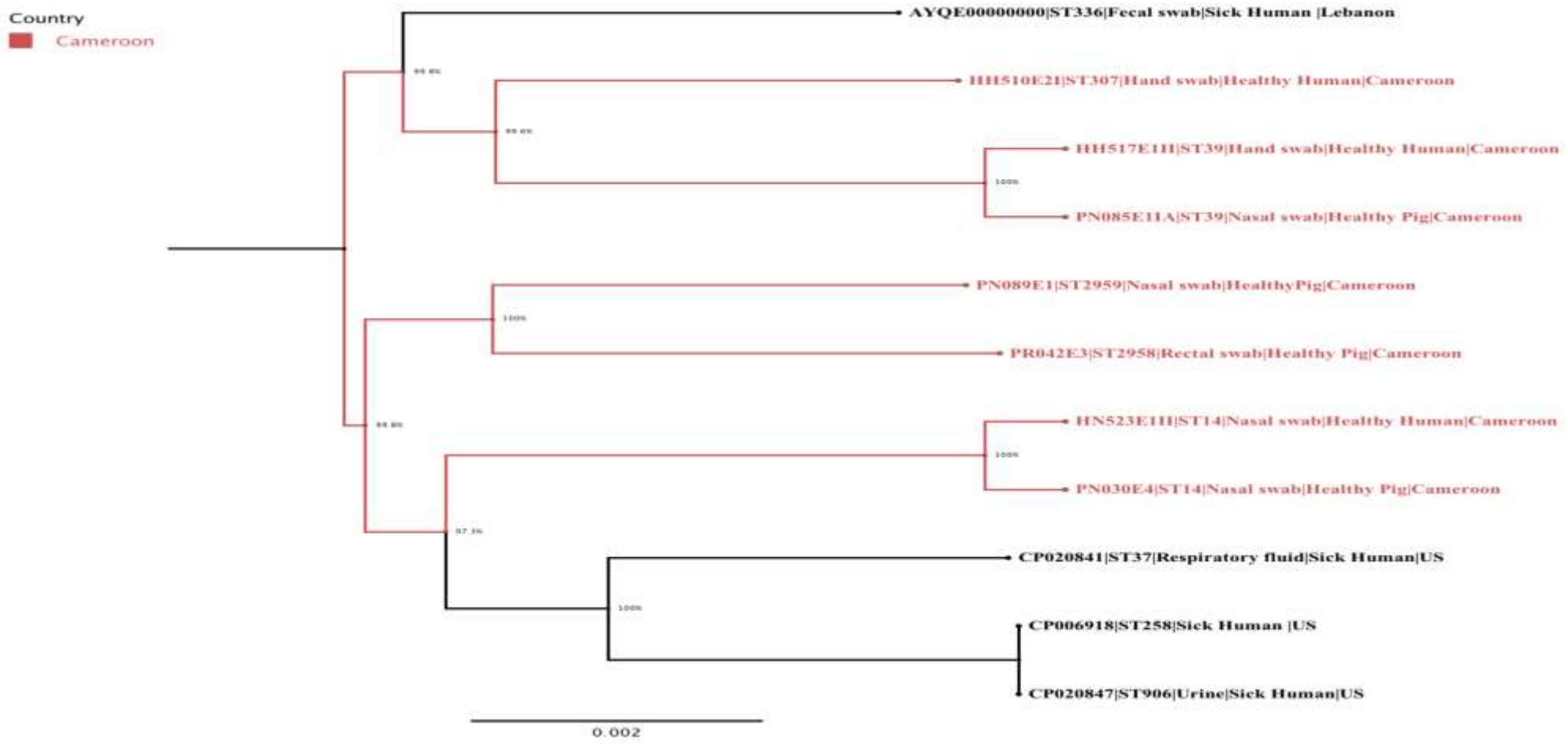

Figure 7.2. A maximum-likelihood phylogenetic tree of ESBL-producing *K. pneumoniae* isolates generated using FastTree version 2.1.7. The Cameroonian isolates are coloured in red. The following information is also provided for each isolate: name/reference, sequence type, type of sample, population, and country.

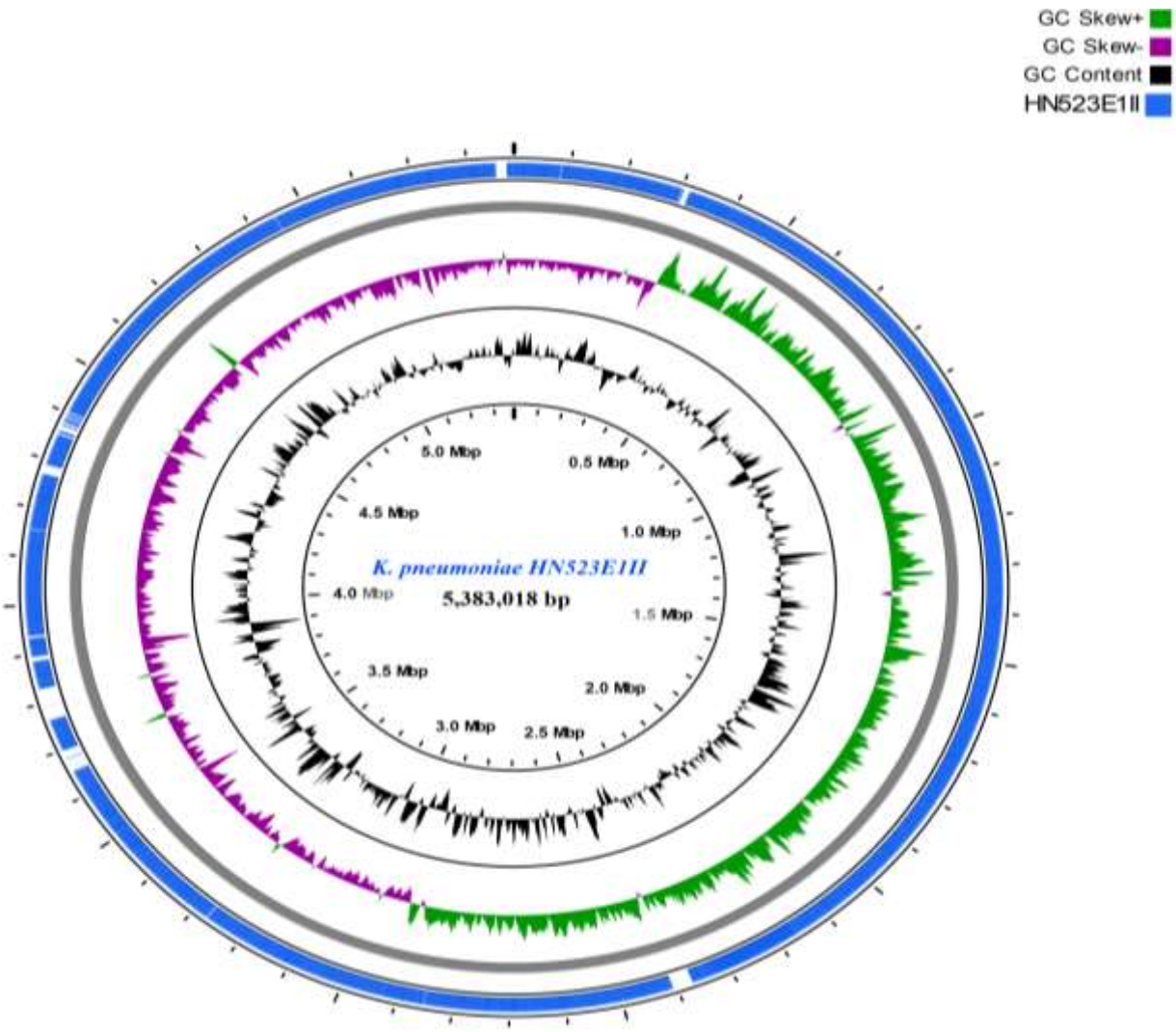

Figure 7.3. ESBL-producing *K. pneumoniae* HN523E1II ring representation using CGView Server V 1.0 (Grant et al., 2012). The inner ring displays the percent of identity comparing *K. pneumoniae* HN523E1II and the complete genome of *K. pneumoniae* KPN528 (CP020853). The two next (inner) rings display the GC content and GC skew, respectively. The last outer ring indicates the genome *K. pneumoniae* HN523E1II.

CHAPTER 8
CONCLUSIONS

I. Introduction and general findings

Antimicrobial resistance is a major public health concern for human and animal health. In the context of globalization, increasing trade and travel, this phenomenon can spread very quickly and pose a serious threat globally. In Africa, particularly in sub-Saharan African countries such as Cameroon and South Africa, antibiotic use in food animals is not necessarily well-regulated, and there is a dearth of studies relating to the zoonotic spread of ABR in both animal and human populations (Vila, 2010; Laxminarayan et al., 2013; WHO, 2014a,b).

Between March and October 2016, ESBL-PE and MRSA were screened from the 288 nasal and rectal pooled samples collected from 432 pigs as well as from nasal and hand swabs sampled from 82 humans in both Cameroon and South Africa. Carriage, risk factors, antimicrobial resistance profiles and clonal relatedness of circulating ESBL-PE and MRSA in pigs and humans were investigated using various microbiological methods and compared using statistical tools including, the chi-square test, and, univariate and multivariate logistic regression analyses. WGS was further used to delineate the current molecular epidemiology, resistance genes, virulence factors, plasmids, phage-related regions and circulating clonal lineages of these isolates.

The following main conclusions emanated from the study with respect to the study objectives:

- ABR in the food chain affects both developed and developing countries. As there are no geographic bounders to impede its global dissemination, multi-faceted, comprehensive and integrated preventive and containment measures complying with the One Health approach should be implemented locally, nationally and regionally in other to ensure food safety and security, effectively combat infectious diseases, curb the emergence and spread of ABR and preserve the efficacy of antibiotics for future generations.
- Studies investigating ABR in food animals in Africa are rare but overall pooled estimates showed high level of resistance (86%; p=0.000) and multi-drug resistance (73%; p=0.003) in food animals in Africa. The vast majority of studies were carried out in Nigeria, with a small number conducted in Eastern, Northern, Western and Southern Africa.
- ESBL-PE prevalence in animal samples from Cameroon was higher than for South Africa and ESBL-PE carriage was observed in Cameroonian workers only. Human nasal ESBL-PE colonization was statistically significantly associated with hand ESBL-PE (21.95% vs 91.67%; p=0.000; OR=39.11; 95% CI 2.02-755.72; p=0.015).

- MRS prevalence of carriage in all pooled pig samples and exposed workers (nasal and hand) from Cameroon was higher than that of South Africa. Human nasal MRS colonization was highly statistically associated with hand MRS (31.58% vs 86.21%; p=0.000; OR=13.54; 95% CI 3.99-45.95; p=0.015).
- Low level of education, previous hospitalization, recent antibiotic use, and contact with poultry were the common risk factors identified for ESBL-PE and MRS carriage in humans
- A low MRSA prevalence was observed in Cameroon (0.07%) and South Africa (16.66%) in pigs and none of the workers was colonized by MRSA.
- Genome analysis identified various antibiotic resistance genes and revealed that all circulating MRSA strains isolated in Cameroon and South Africa, belong to the clonal lineage ST398.
- This is the first report of LA-MRSA ST398 in pigs in Cameroon and South Africa.
- ESBL-producing *K. pneumoniae* were detected in pigs (28%) and exposed workers (18%) in Cameroon.
- The circulating *K. pneumoniae* strains were dominated by the sequence type (ST) 14 and 39. The "high-risk" clone ST307 and two novel STs assigned ST2958 and ST2959 were also detected.
- Intermingled *K. pneumoniae* populations were observed between pig- and human-source within and across abattoirs in Cameroon suggesting their zoonotic transmission.

The elevated ESBL-PE and MRSA prevalence among pigs in both Cameroon and South Africa demonstrates that food animals are potential reservoir and sources of foodborne ESBL-PE and MRSA infections and that occupationally exposed workers are at high risk of colonization in Cameroon and South Africa. The study shows that ESBL-PE and LA-MRSA are actively disseminating in the food chain in both Cameroon and South Africa and are probably underestimated considering the absence of molecular epidemiological studies in the food chain in these countries. The study further suggests that farmers, abattoir workers and food handlers, besides being at high risk of colonization, represent a significant source of ABR dissemination for their relatives through person-to-person contact and for the general population through the contamination of food products occurring during food processing. This finding is of great concern as ESBL-PE and MRSA originating from animals have been reported to be the main causative agents of foodborne infections globally (Huijdens et al., 2006; Cuny et al., 2013; de Balogh et al., 2013; Kock et al., 2013, Nordstrom et al., 2013; Cuny et al., 2015).

The study also highlights the food safety threat associated with the presence of ESBL-PE and LA-MRSA ST398 in the food chain and confirms that these bacteria are serious global public health threats requiring urgent, holistic, and stringent interventions in line with the One Health approach. It finally underlines the existence of an unheeded food safety and public health threat associated with these resistant strains and reinforces the imperative of implementing appropriate food safety measures and promoting rational antibiotic use in Cameroon and South Africa.

II. Significance of the study

Resistant foodborne infections are amongst the main public health issues associated with the threat of ABR in the food chain. This global concern equally affects developed and developing countries, and may cause outbreaks and pandemic situations (Padungtod et al., 2008). The problem is more serious in the developing world, where resistant infections significantly increase morbidity and mortality rates, whereas in developed countries, these infections will increase therapeutic costs (Harbarth et al., 2015).

This work sought to investigate and provide evidence for the containment of the zoonotic transmission of multi-drug resistant bacteria through the implementation of the One Health approach, compliant with the directives of the WHO Global Action Plan on Antimicrobial Resistance (WHO, 2015), The FAO Action Plan on Antimicrobial Resistance 2016-2020 (FAO, 2016) and the OIE Strategy on Antimicrobial Resistance and the Prudent Use of Antimicrobials (OIE, 2016). This was undertaken in two distinct countries where farming and intensive food animal production are amongst the most important economic growth and development factors.

To the best of our knowledge, no study focusing on the occurrence of ABR conjointly in humans and animals has been carried out in both Cameroon and South Africa. The study contributes new knowledge on the burden, risk factors and molecular epidemiology of ESBL-PE and LA-MRSA in both countries. It reveals gaps in the current state of knowledge about antibiotic use and ABR in food animals in Cameroon and South Africa, and suggests that the debate about ABR-related consequences in the farm-to-plate continuum is neglected and should be more seriously considered in these countries. More specifically, the study has demonstrated that the food production system in Cameroon currently needs critical appraisal in terms of prevention and control measures, essential to contain ABR and ensure food safety

and security as does South Africa. The study further shows not only the active ESBL-PE and LA-MRSA dissemination in the farm-to-plate continuum but also the contamination of abattoirs, more so in Cameroon than South Africa.

The importance of Cameroonian and South African food safety is not only national but also regional. Farmers and slaughterhouses workers are not the only ones who should recognize the personal and societal impact of the emergence and spread of LA-MRSA and ESBL-producing *Enterobacteriaceae.* Awareness must be increased in the general population. The results generated in this study serve as evidence of the threat of AMR for agricultural practitioners, policy makers, scientific communities and health policies regionally and particularly in Cameroon and South Africa. The results will inform interventions for effective prevention measures and behaviour changes in the proper use of antibiotics to contain AMR in agricultural practice and in human health.

III. Limitations

The principal limitation of the study is that ESBL-PE and LA-MRSA were not investigated in pigs and humans at the farm-level nor in (ready-to-eat) food products at supermarket or restaurant-level. In fact, although the generated findings yielded an interesting overview at abattoir level, investigating ESBL-PE and LA-MRSA in farm settings would have provided further insights into the epidemiology, resistance genes, virulence factors, mobile genetic elements, risk factors and public health implications associated with these isolates in the whole farm-to-plate continuum.

In addition, the molecular analyses were only carried out on the representative and not all isolates due to financial constraints associated with the impressive number of isolated bacteria. Comprehensive molecular analysis would have certainly allowed better understanding of the molecular evolution and genetic exchanges that are likely to occur within and between bacteria in this continuum.

IV. Recommendations

- The high ESBL-PE and MRSA carriage in pigs and humans underlines the need for routine surveillance and screening of high-risk populations for better containment measures and appropriate interventions.
- The study calls for an urgent implementation of policies and activities on the rational antibiotic use in the food production industry in order to preserve the efficacy of last resort

antibiotics for future generations. Rational antibiotic use policies should also be strictly enforced in the food production industry in both countries and in human health in Cameroon.

- Multi-faceted collaboration among human, animal, and environmental health, agriculture and other sectors, good agricultural practices and effective infection control measures should be implemented to prevent the zoonotic transmission of these resistant bacteria
- Heightened awareness and education about ABR, its related consequences and effective prevention and containment measures should be provided not only to exposed populations including farmers, abattoir workers and, food handlers, but also to the general population where the consumers belong.
- Decision-makers should recognize food safety as a public health priority, to bring together all stakeholders along the whole farm-to-plate continuum, from the food producers and suppliers to the consumers, and ensure that they operate responsibly to preserve the safety of food while ensuring food security. Farmers should for instance implement effective biosecurity measures to prevent ABR emergence and on-farm contamination with resistant bacteria and genes or external dissemination when contamination does occur, while food-handlers and consumers should make use of the WHO tool "Five Keys to Safer Food" for their activities.
- Further molecular studies investigating antibiotic-resistant bacteria in general and ESBL-PE and MRSA in particular, should be carried out across the farm-to-plate continuum in order to better understanding the molecular epidemiology, evolution and transmission dynamics of these strains in Cameroon and South Africa

V. Conclusion

ABR in the food chain is a silent, pandemic threat. Its prevalence in the farm-to-plate continuum will not only endanger food safety, but also ultimately lead to the exhaustion of the available therapeutic pipeline. This represents an obstacle for sustainable development. Tackling ABR requires holistic actions and sustainable political will and commitment. It further requires rational antibiotic use in the food production system together with surveillance to monitor the transmission of resistance already present in the farm-to-plate continuum. Containing ABR throughout this continuum will substantially lessen the multi-sectorial implications associated with this issue.

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APPENDICES

I. Ethics Certificates

1. National Institute of Health (NIH)

Certificate of Completion

The National Institutes of Health (NIH) Office of Extramural Research certifies that **Luria Leslie NJOUNGANG YONTCHOUNG** successfully completed the NIH Web-based training course "Protecting Human Research Participants".

Date of completion: 01/20/2014

Certification Number: 1364263

2. Research Ethics Training Curriculum

fhi360
THE SCIENCE OF IMPROVING LIVES

FHI 360

certifies that

Luria Leslie Njoungang Yontchoung

has completed the

RESEARCH ETHICS TRAINING CURRICULUM

June 21, 2015

II. Ethical clearances

1. Animal Research Ethics Committee (AREC) Approval

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

02 February 2016

Ms Luria Leslie Njoungang Yontchoung
School of Health Sciences
Westville Campus

Dear Ms Njoungang Yontchoung,

Protocol reference number: AREC/091/015D
Project title: Molecular Epidemiology of Livestock-Associated Methicillin Resistant Staphylococcus aureus and Extended-Spectrum Beta-Lactamase producing Enterobacteriaceae in food animals, particularly pigs and related workers in Yaoundé, Cameroon

Full Approval – Research Application

With regards to your response received on 24 January 2016 to our letter of 20 October 2015. The documents submitted have been accepted by the Animal Research Ethics Committee and **FULL APPROVAL** for the protocol has been granted.

Any alteration/s to the approved research protocol, i.e Title of Project, Location of the Study, Research Approach and Methods must be reviewed and approved through the amendment/modification prior to its implementation. In case you have further queries, please quote the above reference number.

Please note: Research data should be securely stored in the discipline/department for a period of 5 years.

The ethical clearance certificate is only valid for a period of one year from the date of issue. Renewal for the study must be applied for before 02 February 2017.

I take this opportunity of wishing you everything of the best with your study.

Yours faithfully

.....

Prof S Islam, PhD
Chair: Animal Research Ethics Committee

/ms

Cc Supervisor: Professor S Essack
Cc Dean & Head of School: Dr M Soliman
Cc Registrar: Mr S Mokoena
Cc NSPCA: Ms Jessica Light

Animal Research Ethics Committee (AREC)
Ms Mariette Snyman (Administrator)
Westville Campus, Govan Mbeki Building
Postal Address: Private Bag X54001, Durban 4000
Telephone: +27 (0) 31 260 8350 **Facsimile:** +27 (0) 31 260 4609 **Email:** animalethics@ukzn.ac.za
Website: http://research.ukzn.ac.za/Research-Ethics/Animal-Ethics.aspx

2. Biomedical Research Ethics Committee (BREC) Approval

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

17 February 2016

Mrs LLN Yontchoung (215000240)
Discipline of Pharmacology
School of Health Sciences
njounganglesli@yahoo.fr

Protocol: Molecular epidemiology of livestock-associated methicillin resistant staphylococcus aureus and extended spectrum beta-lactamase producing enterobacteriaceae in food animals, particularly pigs and related workers in Yaoude, Cameroon.
Degree: PhD
BREC reference number: BE365/15

EXPEDITED APPLICATION

The Biomedical Research Ethics Committee has considered and noted your application received on 10 August 2015.

The study was provisionally approved pending appropriate responses to queries raised. Your responses dated 07 February 2016 to queries raised on 19 October 2015 have been noted and approved by a sub-committee of the Biomedical Research Ethics Committee. The conditions have now been met and the study is given **full ethics approval.**

This approval is valid for one year from **17 February 2016.** To ensure uninterrupted approval of this study beyond the approval expiry date, an application for recertification must be submitted to BREC on the appropriate BREC form 2-3 months before the expiry date.

Any amendments to this study, unless urgently required to ensure safety of participants, must be approved by BREC prior to implementation.

Your acceptance of this approval denotes your compliance with South African National Research Ethics Guidelines (2015), South African National Good Clinical Practice Guidelines (2006) (if applicable) and with UKZN BREC ethics requirements as contained in the UKZN BREC Terms of Reference and Standard Operating Procedures, all available at http://research.ukzn.ac.za/Research-Ethics/Biomedical-Research-Ethics.aspx.

BREC is registered with the South African National Health Research Ethics Council (REC-290408-009). BREC has US Office for Human Research Protections (OHRP) Federal-wide Assurance (FWA 678).

The sub-committee's decision will be **RATIFIED** by a full Committee at its meeting taking place on **08 March 2016.**

We wish you well with this study. We would appreciate receiving copies of all publications arising out of this study.

Yours sincerely

Professor J Tsoka-Gwegweni
Chair: Biomedical Research Ethics Committee

cc supervisor: essacks@ukzn.ac.za
cc postgrad: nimis1@ukzn.ac.za

Biomedical Research Ethics Committee
Professor J Tsoka-Gwegweni (Chair)
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Website: http://research.ukzn.ac.za/Research-Ethics/Biomedical-Research-Ethics.aspx

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3. National Research Ethics Committee of Cameroon (NREC) Approval

National Ethics Committee for Research in Human Health

Decree N°0977/A/MINSANTE/SESP/SG/DROS on April 18, 2012, on the establishment, organization and functioning of research ethics committees for human health within the structures of the Ministry in charge of public health

N°2016/01/684/CE/CNERSH/SP Yaoundé, 05th January 2016

Cnethique_minsante@yahoo.fr

ETHICAL CLEARANCE

The National Ethics Committee for Research in Human Health (CNERSH) in its ordinary session of December 17, 2015, examined the research proposal entitled: **"Molecular Epidemiology of Livestock-Associated Methicillin Resistant Staphylococcus aureus (LA-MRSA) and Extended Spectrum Beta-lactamase (ESBL) producing-Enterobacteriaceae in food animals, particularly pigs and related workers in Yaoundé"** submitted by **Ms Luria Leslie Njoungang Yontchoung**, Principal Investigator, of the University of KwaZulu-Natal.

The project is of great scientific and social interest. The aim of the study is to determine the resistance mechanisms and determinants of LA-MRSA and ESBL-E in pig slaughterhouses of Yaoundé. The study design is clear and well documented. The risks associated to the study are defined together with the measures to prevent and minimize them. The information leaflet and informed consent form in English and French are perfectly elaborated and easy to understand. Measures undertaken to ensure the confidentiality of data collected are presented in the document. The CVs of investigators describe them as competent and able to carry out effectively this study. For all this reasons, the National Ethics Committee for Research in Human Health (CNERSH) approve for a period of one year, the implementation of the present research proposal.

Investigators are responsible for ensuring strict compliance with the approved protocol and should not make any amendments, as minor it is, without favourable opinion of the CNERSH. Investigators are called to collaborate for activity of the CNERSH for monitoring of the implementation of the approved protocol. The final report of the project should be submitted to the CNERSH and to the Cameroonian Health Authorities.

This ethical clearance can be withdrawn in case of non-compliance with the current regulations and above-mentioned recommendations.

In witness whereof, this ethical clearance is issued and intended to serve and assert that right.

Duplicates

- MINSANTE

Chairman

Pr. Lazare KAPTUE

N.B: This ethical clearance does not dispense you from the administrative approval of research (AAR) required to carry out this study in Cameroon. The latter will be issued to you from the Ministry of Public Health.

III. Ministerial approvals

1. Ministry of Livestock, Fisheries and Animal Industries (MINEPIA) Approval

REPUBLIQUE DU CAMEROUN
PAIX – TRAVAIL - PATRIE
****
MINISTERE DE L'ELEVAGE, DES PECHES ET DES INDUSTRIES ANIMALES
****
SECRETARIAT GENERAL
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DELEGATION REGIONALE DU CENTRE
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MINISTRY OF LIVESTOCK FISHERIES AND ANIMAL INDUSTRIES
****
GENERAL SECRETARIAT
****
REGIONAL DELEGATION OF CENTER
****
PO Box 930 Yaoundé Phone 22 31 09 28

1st October 2015

The Regional Delegate of Livestock, Fisheries, and Animal Industries of Centre
To
Mrs Luria Leslie NjoungangYontchoung
PhD student UKZN.

Object:Requestofauthorization for a doctoral research project in pig slaughterhouses in Yaounde.

Mrs,

In response to your request on date of September 29, 2015, related to the object aforementioned,

I have the honour to inform you that your research project is found interesting by the Ministry of Livestock, Fisheries and Animal Industries (MINEPIA) of Cameroon and is consistent with the objectives of the program 407 entitled: "Program of improvement of sanitary surveillance and containment of zoonoses in livestock animals".

Therefore, instructions will be given to relevant district delegates in charge of the selected slaughterhouses, to ease your research and sample collection.

The Regional Delegation of Centre Region of the Ministry of Livestock, Fisheries and Animal Industries would greatly appreciate the arrangements that you would like to undertake to provide the results of your study for improvement of food safety.

Yours sincerely.

THE REGIONAL DELEGATE

Meyebe Gaston
Vétérinaire

2. Ministry of Research and Scientific Innovation (MINRESI) Research Permit

REPUBLIQUE DU CAMEROUN
Paix-Travail-Patrie

MINISTERE DE LA RECHERCHE SCIENTIFIQU
L'INNOVATION

SECRETARIAT GENERAL

DIVISION POLITIQUES SCIENTIFIQUES ET D
PLANIFICATION

CELLULE DE LA PROGRAMMATION ET DE LA
PLANIFICATION

B.P. 1457 Yaoundé – Cameroun
Tél : (237) 22 22 13 34 ou 22 22 52 02

REPUBLIC OF CAMEROON
Peace-Work-Fatherland

OF SCIENTIFIC RESEARCH AND INNOVATION

GENERAL SECRETARIAT

ON OF SCIENTIFIC POLICY AND PLANNING

PROGRAMMING AND PLANNING UNIT

PO Box 1457 Yaoundé Cameroon
Tél : (237) 22 22 13 34 or 22 22 52 02

N° 0 0 0 0 0 1 5 /MINRESI/B00/C00/C10/C14 Yaoundé, le 16 MARS 2016

AUTORISATION DE RECHERCHE
RESEARCH PERMIT

Vu la Constitution ;
Mindful constitution ;
Vu le décret n°2011/408 du 09 décembre 2011 portant organisation du Gouvernement ;
Mindful of decree n°2011/408 of 09 December 2011 organizing the Government;
Vu le décret n°2011/410 du 09 décembre 2011 portant formation du Gouvernement ;
Mindful of decree n°2011/410 of 09 December 2011 appointing the members of the Government;
Vu le décret n°2005/091 du 29 mars 2005 portant organisation du Ministère de la Recherche Scientifique et de l'Innovation ;
Mindful of decree n°2005/091 of 29 March 2005 organizing the Ministry of Scientific Research And Innovation;
Vu la demande de l'intéressé .
Considering the Applicant's request.

Noms et prénoms/ *Names* : **NJOUNGANG YONTCHOUNG Luria Leslie**

Adresses Permanentes/ *Permanent address*: **PhD Student Pharmaceutical Microbiology, School of Health Sciences, University of Kwazulu-Natal, Private Bag X54001, Durban 4000, South Africa; Email : 215000240@stu.ukzn.ac.za/ njoungangl@gmail.com.**

Adresse au Cameroun / *Address in Cameroon*: **s/c : Dr DJOKO FINYOM Cyrille Le Duc, Enseignant- Chercheur, Centre de Biotechnologie à l'Université de Yaoundé I, BP: 8094 Yaoundé/Cameroun; Tel : (+237) 6 77 81 45 90; Email : cfdjoko@gmail.com.**

Nationalité / *Nationality* : **Camerounaise**

Est autorisé (e) à effectuer des travaux de recherche en République du Cameroun dans la ou les Région(s) de: *Is hereby authorized to carry out scientific or technical research in the Republic of Cameroon in the Region of:* **Centre.**

Pour une période de / *For a period of* : **12 mois** du/from : **25/02/2016** au/to **25/02/2017**

En collaboration avec / *In collaboration with* : **Dr DJOKO FINYOM Cyrille Le Duc, Enseignant-Chercheur, Centre de Biotechnologie à l'Université de Yaoundé I, BP: 8094 Yaoundé/Cameroun; Tel : (+237) 6 77 81 45 90; Email : cfdjoko@gmail.com.**

Objet de la Recherche/*Research Title*: **The molecular epidemiology of Livestock-associated methicillin resistant staphylococcus aureus and extended-spectrum-beta-lactamase producing enterobacteriaceae in food animals, particularly pigs and exposed workers in Yaoundé, Cameroun.**

Cette autorisation de recherche n'est valable que pendant la période de recherche indiquée ci-dessus, et peut être renouvelable.
This research permit is valid only for the research period indicated above and it can be renewed.

Le Ministre de la Recherche Scientifique et de l'Innovation

Dr Madeleine Tchuinte

AMPLIATION:
- CAB/MINRESI
- SGPRC
- SGPM
- MINDEF
- DGSN
- DGRE
- CRRI - **Centre.**
- IG/MINRESI
- IG/MINRESI
- CDPSP
- Chrono/Archives

IV. Gatekeeper permissions

Njoungang Yontchoung Luria Leslie
BSc., MSc., PhD student
Principal Investigator
UKZN

Re: Support Letter for the project "**Molecular Epidemiology of Livestock-Associated Methicillin Resistant *Staphylococcus aureus* (LA-MRSA) and Extended-Spectrum Beta-Lactamase-Producing *Enterobacteriaceae* in Pigs And Exposed Workers In Yaoundé, Cameroon**"

Dear Ms. Njoungang Yontchoung,

The pig slaughter house named Hvog Ada. under my responsibility is pleased to support the attached proposal entitled "**Molecular Epidemiology of Livestock-Associated Methicillin Resistant *Staphylococcus aureus* (LA-MRSA) and Extended-Spectrum Beta-Lactamase-Producing *Enterobacteriaceae* in Pigs and Exposed Workers in Yaoundé, Cameroon**" and submitted in partial fulfillment of the requirements for the award of a PhD in Pharmaceutical Sciences at the University of Kwazulu-Natal in South Africa in collaboration with the Laboratory for Public Health-Biotechnology Centre/University of Yaoundé I and Global Viral Cameroon.

My structure fully supports the activities introduced in this proposal and the present letter is written to serve as evidence of our commitment to support the work defined in the protocol.

We look forward for a continuing and mutually successful working relationship.

Please address any contractual or administrative questions to **Mr/Mrs/Ms** NKwain Magdalene epse Nge

Tel: 67767 05.16 **Email:** NKwain@Yahoo.Com

Date 7/9/2015 **at** Yaounde **Coordinator signature**

Délégué d'Arrondissement d'Elevage
Chef Service des Industries Animales de
Ville IV

Yours sincerely.

Nkwain Magdaléne epse Nge
Inspecteur Vétérinaire Assermenté

Njoungang Yontchoung Luria Leslie
BSc., MSc., PhD student
Principal Investigator
UKZN

Re: Support Letter for the project "**Molecular Epidemiology of Livestock-Associated Methicillin Resistant *Staphylococcus aureus* (LA-MRSA) and Extended-Spectrum Beta-Lactamase-Producing *Enterobacteriaceae* in Pigs And Exposed Workers In Yaoundé, Cameroon"**

Dear Ms. Njoungang Yontchoung,

The pig slaughter house named MBAN kolo under my responsibility is pleased to support the attached proposal entitled "**Molecular Epidemiology of Livestock-Associated Methicillin Resistant *Staphylococcus aureus* (LA-MRSA) and Extended-Spectrum Beta-Lactamase-Producing *Enterobacteriaceae* in Pigs and Exposed Workers in Yaoundé, Cameroon"** and submitted in partial fulfillment of the requirements for the award of a PhD in Pharmaceutical Sciences at the University of Kwazulu-Natal in South Africa in collaboration with the Laboratory for Public Health-Biotechnology Centre/University of Yaoundé I and Global Viral Cameroon.

My structure fully supports the activities introduced in this proposal and the present letter is written to serve as evidence of our commitment to support the work defined in the protocol.

We look forward for a continuing and mutually successful working relationship.

Please address any contractual or administrative questions to

Mr/Mrs/Ms Mbouti Rita claude

Tel: 699869353 **Email:** ritaclaudemm@yahoo.fr

Date 09/10/2015 **at** YAOUNDE **Coordinator signature**

Délégué d'Arrondissement du Elevage de Yaoundé II

Yours sincerely.

Mbouti Rita Claude M.
INFIRMIER VETERINAIRE

Njoungang Yontchoung Luria Leslie
BSc., MSc., PhD student
Principal Investigator
UKZN

Re: Support Letter for the project "**Molecular Epidemiology of Livestock-Associated Methicillin Resistant *Staphylococcus aureus* (LA-MRSA) and Extended-Spectrum Beta-Lactamase-Producing *Enterobacteriaceae* in Pigs And Exposed Workers In Yaoundé, Cameroon"**

Dear Ms. Njoungang Yontchoung,

The pig slaughter house named ESSOS under my responsibility is pleased to support the attached proposal entitled "**Molecular Epidemiology of Livestock-Associated Methicillin Resistant *Staphylococcus aureus* (LA-MRSA) and Extended-Spectrum Beta-Lactamase-Producing *Enterobacteriaceae* in Pigs and Exposed Workers in Yaoundé, Cameroon"** and submitted in partial fulfillment of the requirements for the award of a PhD in Pharmaceutical Sciences at the University of Kwazulu-Natal in South Africa in collaboration with the Laboratory for Public Health-Biotechnology Centre/University of Yaoundé I and Global Viral Cameroon.

My structure fully supports the activities introduced in this proposal and the present letter is written to serve as evidence of our commitment to support the work defined in the protocol.

We look forward for a continuing and mutually successful working relationship.

Please address any contractual or administrative questions to **Mr/Mrs/Ms** ADAMA DJOUMÉ

Tel: 677535248 **Email:**

Date Yaoundé at 07/10/2017 **Coordinator signature**

Yours sincerely.

Le Délégué d'Arrondissement de l'Elevage, des Pêches et des Industries Animales

ADAMA DJOUME
Inspecteur Vétérinaire Assermenté

Luria Leslie Njoungang Yontchoung
BSc., MSc., PhD Student
Antimicrobial Research Unit
College of Health Sciences
University of KwaZulu-Natal
Private Bag X54001,
Durban, 4000,
South Africa
Telephone: +27 (0) 073 091 7990

June 2016

Dear Ms Njoungang,

Re: Permission to Conduct Sample Collection from Pigs and Personnel for a PhD Study

The abattoir named BARNESFIELD - CATO under my responsibility hereby agrees to support the research project described in the doctoral proposal entitled "Molecular Epidemiology of Livestock-Associated Methicillin Resistant Staphylococcus aureus (LA-MRSA) and Extended-Spectrum Beta-Lactamase-Producing *Enterobacteriaceae* in Pigs and Exposed Workers in Cameroon and South Africa" being undertaken at the University of Kwazulu-Natal in South Africa.

We do so on the understanding that the identity of the abattoir will not be divulged and that all data collected and generated will be treated with utmost confidentiality and anonymity on the explicit and express consent of the participants.

Please address any logistical questions to Mr/~~Mrs/Ms~~ P. MAHARAJ

Tel 0825007678 Email parasmefreys.co.39

Yours sincerely.

Maharaj

Name & Signature

P. MAHARAJ.

Luria Leslie Njoungang Yontchoung
BSc., MSc., PhD Student
Antimicrobial Research Unit
College of Health Sciences
University of KwaZulu-Natal
Private Bag X54001,
Durban, 4000,
South Africa
Telephone: +27 (0) 073 091 7990

June 2016

Dear Ms Njoungang,

Re: Permission to Conduct Sample Collection from Pigs and Personnel for a PhD Study

The abattoir named DARNALL ABATTOIR under my responsibility hereby agrees to support the research project described in the doctoral proposal entitled "Molecular Epidemiology of Livestock-Associated Methicillin Resistant Staphylococcus aureus (LA-MRSA) and Extended-Spectrum Beta-Lactamase-Producing *Enterobacteriaceae* in Pigs and Exposed Workers in Cameroon and South Africa" being undertaken at the University of Kwazulu-Natal in South Africa.

We do so on the understanding that the identity of the abattoir will not be divulged and that all data collected and generated will be treated with utmost confidentiality and anonymity on the explicit and express consent of the participants.

Please address any logistical questions to Mr/~~Mrs/Ms~~ STEVEN APPALSAMY

Tel 0824960198 Email STEVENAPPALSAMY@GMAIL.COM

Yours sincerely.

Name & Signature

V. Material Transfer Agreement

MATERIAL TRANSFER AGREEMENT

This **MATERIAL TRANSFER AGREEMENT** "**AGREEMENT**" IS ENTERED INTO as of July 07, 2015 between Dr. Cyrille F. Djeko of the Laboratory for Public Health Biotechnology/The Biotechnology Center (LPHB/BTC), University of Yaounde I "THE PROVIDER" and Prof. Sabiha Yusuf Essack of the University of KwaZulu-Natal (UKZN) College of Health Sciences, "THE RECIPIENT", for the transfer of material, with accompanying data, for research purposes. This research is carried out in partial fulfilment for the award of a PhD in Pharmaceutical Sciences to the student named Lucie Laurie Njoungang Yontchoung "THE STUDENT".

1. **Background**:

   A. Recipient Investigator is willing to work with Provider in a collaborative manner to jointly assist the PhD student in the analysis the material (defined below) for scientific research purposes.

   B. The parties wish to work cooperatively in accordance with the terms of this agreement. Now therefore, Recipient and Provider, intending to be legally bound hereby agree as follows:

2. **Materials**: THE PROVIDER, will transfer the Materials to THE RECIPIENT upon receipt of this signed Agreement provided that THE PROVIDER has an adequate supply of the Materials. THE PROVIDER has no obligation to provide specific quantities of the Materials or to make additional quantities if the supply is depleted. "Materials" means the biological, chemical, or other materials (including associated data) or any materials consisting in whole or in part of samples derived from human subjects or food animals, together with all progeny, mutations, formulations, and derivatives, derivatives or parts thereof.

3. **THE PROVIDER Investigator**. Materials will be provided by:
   Dr Cyrille F. Djeko, Co- Investigator
   Canefour Intersurance PO Box 7089 Yaounde, Cameroon
   cfdjeko@gmail.com

4. **THE RECIPIENT Investigator**. Materials will be transferred to:
   Prof. Sabiha Yusuf Essack, Co-Investigator
   School Of Health Sciences
   College Of Health Sciences
   University Of KwaZulu-Natal
   Private Bag X54001
   Durban 4000
   South Africa
   essacks@ukzn.ac.za

5. **THE PhD STUDENT investigator**.
   Materials will be collected and analysed by Ms. Lucie Laurie Njoungang Yontchoung.

6. **Use of Materials**. THE RECIPIENT agrees that the Materials and Confidential Information will solely be used for the Authorized Purpose. "Authorized Purpose" is set forth in Attachment A. THE RECIPIENT shall not use the Material: (i) for any commercial purposes, including selling, commercial screening, or transferring Material to a third party for commercial purposes; (ii) in any product or for the purpose of producing any product; (iii) for providing any service in which a product or service is sold or otherwise made commercially available; and (iv) for use in humans or animals or for any clinical, diagnostic, prognostic or therapeutic purposes.

7. **Transfer of Materials**. THE RECIPIENT shall not transfer the Materials to any other person or entity without the prior express written consent of THE PROVIDER and the PhD STUDENT. Approval will be subject to such person or entity entering into an agreement with THE PROVIDER substantially similar to this Agreement.

8. **Supervision**. THE RECIPIENT shall ensure that all persons who are authorized to use the Materials are informed of and abide by the terms of this Agreement.

9. **Term and Termination**. The term of this Agreement shall, unless earlier terminated by either party, be Ten (10) years from the Effective Date ("Term"). Either party may terminate this Agreement for convenience upon sixty (60) days prior written notice to the other party. Either party may terminate this Agreement upon written notice if the other party is in breach of any provision of this Agreement, and such party fails to cure such breach with five (5) days after receipt of the notice. Upon termination of this Agreement before expiration, THE RECIPIENT shall, at the PROVIDER's option: (a) return to THE PROVIDER any remaining Materials and any copies of Confidential Information of THE PROVIDER that are in the possession or under the control of THE RECIPIENT, or (b) dispose of the Confidential Information and remaining Materials in accordance with the PROVIDER's instructions, subject to any applicable law. Any terms of this Agreement that by their nature extend beyond the Term or expiry of this Agreement shall survive the termination or expiry of this Agreement. This includes, without limitation, confidentiality, the disclaimer of warranty, limitation of liability, indemnification, and this survival provision.

10. **Confidentiality**. Except as otherwise provided for in this Agreement, during the Term and for a period of five (5) years following the expiration or earlier termination hereof, THE RECIPIENT shall exercise reasonable care to maintain in confidence all Confidential Information disclosed by THE PROVIDER pursuant to this Agreement. THE RECIPIENT shall only disclose Confidential Information to those of its directors, officers, employees, or agents having a need-to-know, as reasonably necessary to facilitate the Authorized Purpose, provided that such persons are obligated in writing or otherwise bound by fiduciary obligations to THE RECIPIENT to maintain in confidence and not use the Confidential Information except as permitted by this Agreement. The limitations on use and the nondisclosure obligations contained in this Agreement shall not apply to Confidential Information that THE RECIPIENT can demonstrate by its written records is: (a) required to be disclosed by applicable law or regulation, provided that THE RECIPIENT shall give THE PROVIDER prompt written notice and sufficient opportunity to object to such use or disclosure, or to request confidential treatment of the Confidential Information; (b) public knowledge at the time of disclosure by THE PROVIDER, or becomes public knowledge through no wrongful act by THE RECIPIENT; (c) rightfully known by THE RECIPIENT prior to the date of disclosure by THE PROVIDER; (d) disclosed to THE RECIPIENT on an unrestricted basis from a third party not under a duty of confidentiality to THE PROVIDER; or (e) independently developed by employees or agents of THE RECIPIENT without access to or use of Confidential Information."Confidential Information" means, collectively, the Materials and all technical, financial, and business information of any kind whatsoever, and all tangible and intangible embodiments thereof of any kind whatsoever, disclosed by THE PROVIDER to THE RECIPIENT or obtained by THE RECIPIENT through observation or examination of the foregoing or the Materials, but only to the extent such information or embodiment is maintained as confidential by THE PROVIDER.

11. **No Use of Name**. Except as otherwise required by applicable law, regulation, or order of a governmental agency or court of competent jurisdiction, neither party shall use the name of the other party or the other party's directors, officers, or employees in any advertising, news release or other publication, without the prior express written consent of the other party.

12. **Written Report and Test Results**. THE RECIPIENT shall keep THE PROVIDER informed of all uses made of the Materials and the Confidential Information and, at the conclusion of its research, shall provide THE PROVIDER, with a complete, detailed written report describing such research, the results of such research, and the data generated thereby. The RECIPIENT shall own and retain all right, title, and interest in and to the results of the research and data generated thereby. THE PROVIDER shall have the right to use and to disclose the results of such research and the data generated thereby.

13. **Ownership and Inventions**. The PROVIDER and the RECIPIENT shall retain the exclusive right, title, and interest in and to the Materials, including but not limited to, all right, title, and interest in any patents and patent applications and other intellectual property ("IP") rights relating to the Materials. The PROVIDER and the RECIPIENT shall jointly own all Resulting IP. Results associated with human and animal samplings, acquisition and discoveries are referred to as "Core Results". Ownership of all specimens used under this Agreement shall be governed by the laws of the given Country of Origin and any written

1

Agreement between THE PROVIDER and Country of Origin. THE RECIPIENT shall request permission to use, disclose, reproduce, and prepare derivative works, in any manner and for any purpose, and to have or permit others to do so.

14. **Publication**. Notwithstanding the confidentiality obligations set forth in this Agreement, THE RECIPIENT may publish results obtained using the Materials if THE RECIPIENT(a) publishes the material jointly with THE PROVIDER, and acknowledges the scientific contributions of all those involved in acquisition and analysis of the materials (b) responsibilities of authorship are agreed upon by both parties prior to publication (c) provides THE PROVIDER, with copies of any such material at least 30 days prior to publication, and (d) THE RECIPIENT acknowledges THE PROVIDER, as the source of the Materials in any such publication or presentation. The parties agree that all publications connected with Attachment A will be joint publications and authorship on all resulting publications will acknowledge the scientific contributions of all those involved in acquisition and analysis of the Material.

15. **Disclaimer of Warranty**. THE RECIPIENT acknowledges that the Materials are experimental in nature and may have unknown characteristics, may carry infectious agents, or may be otherwise hazardous. The Materials and Confidential Information are provided **"AS IS" AND WITHOUT ANY REPRESENTATIONS OR WARRANTIES. THE RECIPIENT'S USE OF THE MATERIALS AND CONFIDENTIAL INFORMATION SHALL BE AT THE RECIPIENT'S OWN RISK, AND THE PROVIDER, ASSUMES NO LIABILITY OR OBLIGATION TO THE RECIPIENT AS A RESULT THEREOF.**

16. **Dispute Resolution**. Should there be a conflict or disagreement between THE PROVIDER, THE RECIPIENT and/or the PhD STUDENT which cannot be resolved by the parties involved, then either party can approach the Academic Leader Research or Dean and Head of UKZN School of Health Sciences (or the College Dean of Research if the Dean and Head of School is one of the conflicting parties) about the conflict. The Dean and Head of School (or College Dean of Research) will then either arbitrate or choose a senior academic of the School not involved in the conflict to arbitrate. The arbitrator's decision is final and cannot be appealed.

17. **Export Restrictions**. In performing this Agreement, THE RECIPIENT may gain access to information, technology or items that are export-controlled by Cameroon. Without limitation, the parties shall comply with all such applicable export laws and regulations.

18. **Compliance**. Both parties shall comply with all laws and governmental rules, regulations, and guidelines that are applicable to the Materials or the use thereof, and with any safety precautions accompanying the Materials. If the Materials involve human tissue samples, both parties shall also comply with all laws, rules, regulations, guidelines, and safety precautions relating to the protection of human subjects and the security and confidentiality of Protected Health Information. As laws relating to data security and privacy rapidly evolve, amendment of this Agreement may be required to provide for procedures to ensure compliance with such developments. If current or future laws, rules, or regulations adversely impact a party's performance under this Agreement, the parties will negotiate in good faith to amend the Agreement as necessary to be consistent with the requirements of applicable law. If the parties are unable to modify the Agreement to fully comply with such laws, rules, and regulations, either may immediately terminate this Agreement.

19. **No Other Rights**. This Agreement does not grant THE RECIPIENT any license or other rights except as expressly provided in this Agreement.

20. **No Assignment**. THE RECIPIENT may not assign this Agreement, whether by operation of law or otherwise, without the prior express written consent of THE PROVIDER. Any purported assignment in violation of this section is void.

21. **No Waiver**. This Agreement shall be binding upon and for the benefit of the undersigned parties, their successors, and assigns. Failure to enforce any provision of this Agreement is not a waiver of any term hereof.

22. **No Agency**. Nothing in this Agreement establishes a relationship of principal and agent, employer and employee, a partnership, or joint venture between THE PROVIDER, and the RECIPIENT.

23. **Entire Agreement**. This Agreement embodies the entire understanding between the parties and supersedes any prior understanding and agreements regarding the subject matter hereof. No change, modification, extension, termination, or waiver of this Agreement is valid unless made in writing and signed by duly authorized representatives of the parties.

AGREED:

THE PROVIDER

The Laboratory for Public Health

Biotechnology, the Bioethnology

Center

University of Yaoundé I

Po Box 7019

Yaoundé, Cameroon

Tel: +237 677 81 45 90

Signature:

Name: Dr. Cyrille F. Djoko

Title: Co-Investigator Cameroon

AGREED:

THE RECIPIENT

University of KwaZulu-Natal

Office of the Registrar

University Road

Chiltern Hills

Westville, 3629

Durban

South Africa

Signature:

Name: Prof. Urmilla Bob

Title: Dean of Research University of KwaZulu-Natal

ATTACHMENT A

Materials:

Aliquots of interesting bacterial isolates collected through the study entitled: "**Molecular Epidemiology of Livestock-Associated Methicillin Resistant *Staphylococcus aureus* (LA-MRSA) And Extended-Spectrum Beta-Lactamase-Producing *Enterobacteriaceae* in Pigs and Related Workers in Yaoundé and Surrounding Areas – Cameroon**".

Collaboration Research Project

The overall purpose of the study is set to determine the prevalence, phenotypic and genotypic characteristics including but not limited to the virulence factors and resistance mechanisms of circulating LA-MRSA and ESBL-producing *Enterobacteriaceae*; and furthermore, to improve knowledge about risks factors, spread, resistance mechanisms, resistance genes and resistant determinants of these resistant pathogens in pig production industry and occupationally exposed humans in Yaoundé and surrounding -Cameroon.

VI. Import Permit

health

Department:
Health
REPUBLIC OF SOUTH AFRICA

Private Bag X828, PRETORIA, 0001. 27th Floor, Room 2710, Civitas, Cnr Thabo Sehume & Struben Street, PRETORIA, 0001
Tel: +27 (0) 12 395 8000, Fax: +27 (0) 12 395 8422

(012) 395 8366/8965 Ms Lineo Motopi

importexportpermit@health.gov.za J1/2/4/14 No 3/15

IMPORT PERMIT

In terms of Regulation 178 of RG No 9699 of 02 March 2012 –

Professor Sabiha Yusuf Essack
Professor: Pharmaceutical Sciences
University of Kwazulu Natal
Room E5-05-012
E Block, Level 5
Westville Campus, University of Kwazulu Natal
University Road, Chiltern Hills
Westville
3629
Tel. No.: (031) 260 4946 Fax. No.: (031) 260 7872

is hereby authorised to import into the Republic of South Africa –

5 ml cryovials x 100	Live stock associated methicillin - resistant *Staphylococcus aures*
5 ml cryovials x 100	Extended – spectrum beta lactamase producing *Enterobacteriaceae*

from –

Dr Cyrille F. Djoko
LPHB, The biotechnology Centre
University of Yaounde I
P.O.Box 7039
Yaounde
Cameroon
Tel. No: +237 677 814 590 Fax. No:

for – Research

This export permit is subject to the following conditions:

1. The substance shall be imported into the country specified above, within the legal requirements of that country.
2. The material shall be exported from South Africa and handled in accordance with the provisions of Regulation 178 of RG No 9699 of 02 March 2012.
3. The export permit shall not be used for any trade or advertising purposes.
4. **This export permit shall expire on 31 October 2016.**

DIRECTOR-GENERAL: HEALTH
Date: 31/10/2014
Ms P Netshidzivhani

VII. Information leaflet

CNERSH Approval No.: *216/01/684/CE/CNERSH/SP*
BREC Approval No.:*BE365/15*
AREC Approval No.: *AREC/091/015D*
Ministry of Livestock, Fisheries and Animal Industries of Cameroon Authorization No.:*061/L/MINEPIA/SG/DREPIA/CE*
Ministry of Scientific Research and Innovation No.:*015/MINRESI/B00/C00/C10/C14*

Principal Investigator: Luria Leslie Njoungang Yontchoung
BSc., MSc., PhD Student
Department of Pharmaceutical Sciences
School of Health Sciences
College of Health Sciences
University of KwaZulu-Natal
Durban 4000
South Africa
Tel:
Email: *njoungangl@gmail.com*

Supervisor: Professor Sabiha Yusuf Essack
B. Pharm., M. Pharm., PhD
Dean: Teaching & Learning,
College of Health Sciences
University of KwaZulu-Natal
Private Bag X54001
Durban 4000
South Africa
Tel: +27 (0) 31 2604946
Email: *essacks@ukzn.ac.za*

Co-supervisor: Doctor Cyrille Leduc Djoko Finyom
BSc., MSc., PhD Biochemistry
The Biotechnology Center
University of Yaounde I
Po Box 7039
Yaoundé, Cameroon
Tel: +237 677814590
Email: *cdjoko@gmail.com*

Promoter: The University of KwaZulu-Natal
School of Health Sciences
College of Health Sciences
Private Bag X54001
Durban 4000
South Africa
Email: khumalot8@ukzn.ac.za
Web site: http://www.ukzn.ac.za

INTRODUCTION

Date:…….(dd)/..…. (mm)/…….(year)

Dear volunteers

I am Luria Leslie Njoungang Yontchoung a Doctoral student of the University of KwaZulu-Natal (UKZN) School of Health Sciences, College of Health Sciences in South Africa. I am responsible of a Doctoral Research Project entitled "**Molecular Epidemiology of Livestock Associated Methicillin Resistant *Staphylococcus aureus* (LA-MRSA) and Extended-Spectrum Beta-Lactamase-Producing *Enterobacteriaceae* in Food Animals, Particularly Pigs and Related Workers in Yaoundé-Cameroon**" under the supervision of Professor Sabiha Yusuf Essack from the College of Health Sciences of University of KwaZulu-Natal and with the support of the University. The information concerning the different ethical approvals obtained in South Africa and in Cameroon are detailed above.

This study which will take 3 years tries to examine the resistance to antibiotics of bacteria. We are examining some specific bacteria named *S. aureus* and *Enterobacteriaceae* and normally present on the skin of animal and human vertebrates as well as in the environment. These commensal bacteria have been recently identified to be non-susceptible at the activity of numerous antimicrobial agents. This means that despite the presence or consumption of antibiotic drugs, they are able to persist in your organism and to create disease. Because there is a limitation in the creation of new antibiotics, we arriving at the point where a simple injury could once again kill due to the inactivity of antibiotics on pathogens. The study aims to identify and determine the specific resistant strains *S. aureus* and *Enterobacteriaceae* family present in the pig slaughterhouses. By doing this, the impact of these resistant pathogens in human and animal health will be evaluated in order to implement effective prevention measures, strategies and policies which will concur to the amelioration of human and animal health.

Before taking part to this study, it is indispensable for you to read the following information concerning the project. In fact, the reason of this document is to explain you the objectives, procedures, potential risks, benefits and inconvenience of the study. If there is anything unclear or incomprehensible in this document, please do not hesitate to ask for clarification or more information from the study personnel for more explanation about that part of study. If you would like to participate to this study, please sign as appropriate below.

GENERAL AIM AND OBJECTIVES OF THE STUDY

The general aim of this research is to determine the elements involved in the resistance of *Staphylococcus aureus* and *Enterobacteriaceae* to the antimicrobial agents, in order to suggest adequate measures to improve health. The study considers the different risk factors, the frequency and characteristics of these bacteria found in pigs of slaughterhouses and in pig workers in Yaoundé. Male and female workers in the profession-related to pig's slaughterhouses (such as pig's slaughterer, pig's carriers, pig's seller, etc.) aged of 18 years and older are eligible to participate. I am soliciting your volunteer participation in this research because you are member of this category of persons. Please, be sure that your participation is completely voluntary. If you decline your participation in this study, there will not have any negative consequences for you. On the other hand, if you decide to take part, please, you also need to be conscious of the fact that you may stop participating at any time and you may decide not to answer any specific question.

If you volunteer to participate in this study, the following will happen:

- You will be assigned a unique participant identification number, which will be used to link your survey responses to results of your activities and provide confidentiality. Your name will not be asked or recorded on any study materials.
- You will receive a questionnaire and I am going to ask you questions about your socioeconomics conditions (age, sex, education, incomes, etc.), your medical record (recent hospitalization, recent surgery, etc.), the utilization of antimicrobial and slaughterhouse practices; some of them might affect your privacy or in other words, your personal behaviour.
- I will collect your anterior nares and hands to perform laboratories analysis.
- I will collect anterior nares and rectum of pigs processing at the slaughter houses.

TIME REQUIRED FOR PARTICIPATION

Your participation will take about 30 minutes -answering questionnaire- and will require more time for pig's sample collection in abattoir.

POTENTIAL RISKS OF THE STUDY

The researchers consider that the risks or discomforts to you and your animal are minimal. You can skip questions that make you uncomfortable or stop taking the survey at any time.

BENEFITS

You will not receive any payment for your participation in this study. Your participation will help to understand the resistance of antibiotics in Cameroon; will improve the current available data reports on

the different types of resistance existing and finally, will serve to proof to notify policy makers about the danger poses by these resistant pathogens in the Cameroon and around the world.

CONFIDENTIALITY

If you decide to leave the study, the material required for your identification including questionnaire, informed consent, data recorded will be destroyed except if you give authorization to the researcher to use it after your withdrawal. In case you decide to participate, all your responses will be confidential and stored appropriately with restrained access to authorized person only. Neither your name nor your address will be recorded and no other person will know your answers. Your data will be conserved as described by the following measures which are applied for all applicants.

During the research:

- Your name and any other person named during the study will be replaced by a code
- Only the researcher and authorized persons will have access to the data containing names and codes which will be stored separately from the material of research, from the data and informed consent
- All the research material including informed consent and data will be stored in a barred-workbook and in a local locked
- Numerical data will be protected in files with access granted by a password detained by the researcher

During the dissemination of the results:

- The names of the participant will never appear in a report
- The results will be presented in a global form in order to avoid the communication of individual results
- The results of the study will be published in scientific journals and nobody will be recognized
- A brief résumé will be sent to participants who will have demonstrated the desire to have their results by indicating their Email or Postal address in the appropriate space provided below.

THE ELECTRONIC/POSTAL ADDRESS WHERE I WOULD LIKE TO RECEIVE A SHORT RÉSUMÉ OF THE RESULTS IS THE FOLLOWING:

_____ _____

At the end of the study:

- All the material and data may be used later for other research
- All the material and data will be destroyed at later in 10 years.
- Measures have been done for persons desirous to know their results.
    - A short résumé of the results will be sent to participants who will have done the request by indicating their address at the space provided.
    - The results will not be available before the end of the year 2016.

- If this address changes by this date, I encourage you to inform us of your new address.

VOLUNTARY CONSENT AND PARTICIPATION IN THE STUDY

You have read the information above concerning the study; an opportunity has been given to you to ask questions about the process and risks of the study as well as any other doubt regarding this information sheet. You understand what the study is about and how and why it is being conducted. You voluntarily consent to participate in this study by signing the informed consent form below. A copy of this document will be dispatched to you for your record.

THANKS

Your collaboration is indispensable and precious for the accomplishment of this study. That's why we would like to express you our profound gratitude for the time and attention that you have accepted to bring to your participation and to our study.

In the event of any interrogation, please do not hesitate to contact the researcher or the Biomedical Research Ethics Committee as following detailed:

Principal Investigator: Luria Leslie Njoungang Yontchoung
BSc., MSc., PhD Student
Department of Pharmaceutical Sciences
School of Health Sciences
College of Health Sciences
University of KwaZulu-Natal
Durban 4000
South Africa
Tel: +27 (0)63 276 8472
Email: *njoungangl@gmail.com*

Supervisor: Professor Sabiha Yusuf Essack
B. Pharm., M. Pharm., PhD
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University of KwaZulu-Natal
Private Bag X54001
Durban 4000
South Africa
Tel: +27 (0) 31 2604946
Email: *essacks@ukzn.ac*

BIOMEDICAL RESEARCH ETHICS ADMINISTRATION

Research Office, Westville Campus

Govan Mbeki Building

University of KwaZulu-Natal
Private Bag X 54001, Durban, 4000
KwaZulu-Natal, SOUTH AFRICA
Tel: +27(0) 31 2602486
Fax: +27 (0) 31 2604609
Email: BREC@ukzn.ac.za

VIII. Informed consent

I the undersigned…….. certify that I have been invited to participate in a study entitled "**Molecular Epidemiology of Livestock Associated Methicillin Resistant *Staphylococcus Aureus* (MRSA) And Extended-Spectrum Beta-Lactamase-Producing *Enterobacteriaceae* In Food Animals, Particularly Pigs And Related Workers In Yaoundé-Cameroon**" with Ms Luria Leslie Njoungang Yontchoung from the School of Health Sciences, College of Health Sciences of the University of KwaZulu-Natal in Durban-South Africa as Principal Investigator.

- I confirm that I have read and understood the contents of the information sheet.
- I have well understood the aim and objectives of the research as well as the potential risks and benefits.
- I confirm that the occasion has been given to me to ask any questions and I certify that I have received proper answers to any of my questions.
- I understand that my personal information will be strictly confidential with limited access and that I have the right to withdraw from the study at any time, for any reason, without any consequence, and without any influence to my legal rights.
- I understand that for further interrogation about the study I am free to contact the researcher Luria Leslie Njoungang Yontchoung at the address below mentioned:

  Principal Investigator: Luria Leslie Njoungang Yontchoung
  BSc., MSc., PhD Student
  Department of Pharmaceutical Sciences
  School of Health Sciences
  College of Health Sciences
  University of KwaZulu-Natal
  Durban 4000
  South Africa
  Tel: +27 (0)63 276 8472
  Email: *njoungangl@gmail.com*
- I Also understand that for concerns related to my rights as participant or to the researcher, I can freely contact the Biomedical Research Ethics Committee at the below address:

  BIOMEDICAL RESEARCH ETHICS ADMINISTRATION
  Research Office, Westville Campus
  Govan Mbeki Building
  University of KwaZulu-Natal
  Private Bag X 54001, Durban, 4000
  KwaZulu-Natal, SOUTH AFRICA
  Tel: +27(0) 31 2602486
  Fax: +27 (0) 31 2604609

By signing this document, I freely accept to take part to this study and give my permission under the conditions specified by the information document to the student researcher Ms Luria Leslie Njoungang Yontchoung from the University of KwaZulu-Natal to use my nasal and hands swabs, as well as nasal and rectal swabs of the pigs present in slaughterhouse (if applicable) for current and if useful, future studies involving antimicrobial resistance.

I additionally consent to:

Use any of my photographs for research **Yes** ☐ **No** ☐

Signature of the participant: …….

Date: …… (dd) /…… (mm)/……. (yyyy)

Subject ID code: _____

Site/Location/Region: _____

Gender: ☐ Male ☐ Female

I have explained the aim, objectives, benefits, potential risks and drawback of the study to the participant. I have answered with the best of my knowledge the questions of the participant and I have verified his/her understanding.

Signature of the researcher or study personnel (Names and signature)	**Date**
_____	_____

Copy of the participant

IX. Questionnaire

N° _____

Subject ID

Date: …… (Day) /…… (Month)/……. (Year)

This questionnaire is specific for slaughterhouse workers and should take approximately 30 minutes to complete. Please, understand that questions are personal to you and do not forget that your answers will be confidential. We need your honesty and complete response to each question.

Instructions to all participants: Check ☑ the appropriate box or write your answer in the space provided.

Section 1: Demographic Information

These questions are about socio-demographic information. Remember, your answers will remain strictly confidential.

1. What is your gender?
   - ❑ Male
   - ❑ Female
2. In what range is your age?
   - ❑ 18-24
   - ❑ 25-31
   - ❑ 32-38
   - ❑ 39-45
   - ❑ 46-52
   - ❑ Above 52
3. What is your highest educational level?
   - ❑ Never been to school
   - ❑ Attended some primary school but not completed
   - ❑ Graduate primary school
   - ❑ Graduate secondary school
   - ❑ Graduate high school/ university
   - ❑ Other (specify)_____
4. In what range is your average monthly income?
   - ❑ Below 55 US $
   - ❑ 55-110 US $
   - ❑ 110-165 US $
   - ❑ 165-220 US $
   - ❑ 220-275 US $
   - ❑ Above 275 US $
5. Where is your current residence location?
   District/Region _____
6. How many persons with you live in your house?
   - □Below 2
   - □Between 2-4
   - □Between 4-6
   - □More than 6 (specify number…….)

7. How many rooms possess your house?
   - □1
   - □2
   - □3-5
   - □More than 5
8. What is your principal profession?
   - □ Wholesaler of living pigs (consist to buy from the farm and provide it at market for merchandizing)
   - □Intermediary seller of living pigs (seller of pig inside market)
   - □Pig transporter (consist to handling living pigs to the slaughter house and from the slaughter house to the butcher place)
   - □Pig slaughterer (consist to kill and eviscerate pig)
   - □Butcher (consist to sell pork per kilogram to the population)
   - □Retailer of viscera (person who collects viscera of pigs, clean them and conserve them for other activities)
   - □Retailer of grilled pork (is a person who buys a pig for another activity related to pig, the merchandizing of grilled pork in street)
   - □Other (specify……..)
9. Since how many years have you been in this profession?
   - ❑ 0-4
   - ❑ 5-9
   - ❑ 10-14
   - ❑ Above 15
10. Do you have another occupation related to hospital?

    □Yes □No

11. Have any of your family members worked with animal or at a hospital (clinic)?

    □Yes □No

12. If yes, who is it for you?
    - □Brother/sister
    - □Father/mother
    - □Aunt/uncle
    - □Wife/husband
    - □Other (specify……..)
13. What of these professions does he practice? Check ☑all that apply
    - □Pig's slaughterer
    - □Pig-breeder
    - □Wholesaler of living pigs
    - □Intermediary seller of living pigs
    - □Retailer of viscera
    - □Butcher (seller of pork/ kg)
    - □Retailer of grilled pork
    - □Farmer (crop production and livestock)
    - □Medical practitioner
    - □Paramedical practitioner (nurse, laboratory technician, pharmacist)
    - □Others……..

Section 2: Clinical History

The next questions are about medical history. Please answer honestly. Remember, your answers will remain strictly confidential.

14. In the past 12 months have you or a family member been hospitalized? [If no, skip to question 16]
    □Yes □No
15. How many times last year?
    □1-2 times
    □3-4 times
    □4-5 times
    □6 or more
16. Approximately how many days have you stayed at hospital?

    □0-7
    □8-14
    □15-22
    □23-30
    □More than 30
    □Don't know
17. Have you or a family member recently (within one month) used an antibiotic?

    □Yes □No
18. If yes, please specify check ☑all that apply. If no, please skip to question 20.

    □ Cotrimoxazole
    □ Oflocet/Levomax/ Ciprofloxacin
    □ Ceftriaxone/Cefpodoxim/Imipenem
    □Penicillin/Amoxicillin
    □Other please specify……..
19. What might be the reason of your consumption?
    □Malaria
    □Typhoid fever
    □Sexual transmissible infection
    □Flu/cough
    □Fever
    □Other please specify……..
20. Where have you purchased these drugs?

    □Public pharmacies (public Hospital, dispensaries, Health Centres, etc.)
    □Private Pharmacies
    □Market/Street seller
    □Leftovers of relatives or past prescriptions
    □Unknown
21. Have you presented symptoms of or have you been diagnosed with any of the following? Check ☑all that apply.
    □Malaria
    □Diabetes mellitus

□Hypertension
□Skin problem
□Nasal problem
□Typhoid fever
□Hepatitis
□HIV/AIDS
□Tuberculosis
□Asthma
□Stomach pain

Section 3: Slaughter house practices, accessibility and Implementation measures

The next questions concern activities and practices in slaughterhouse. Please answer honestly. Remember, your answers will remain strictly confidential.

22. Is the slaughter house close to your house?
    □Yes □No
23. On the average how many times are you in contact with pigs per week?

    □Always (100% of the time)
    □Almost always (75-99% of the time)
    □Sometimes (25-74% of the time)
    □Rarely/Never (0-24% of the time)
24. Are you in contact with other type of animal through your work or personal living?
    □Yes □No
25. If yes, what type of animal?
    □Poultry
    □Oxen
    □Sheep
    □Calves
    □Pets (cats, dogs)
    □Other (Specify……..)
26. On the average how many times are you in contact with other animals per week?
    □Always (100% of the time)
    □Almost always (75-99% of the time)
    □Sometimes (25-74% of the time)
    □Rarely/Never (0-24% of the time)
27. What safety precaution do you used for slaughterhouse activities? Check ☑all that apply.
    □Nothing□
    □Gloves
    □Mask
    □Work clothes
    □Apron
    □Skullcap
    □Boot
    □Hand washing without soap or bleach
    □Convenient hand washing (means with soap or bleach during 30-45 s)
    □Wheelbarrows for transport
    □Other (specify) _____
28. How frequently do you clean your slaughterhouse?

□Never [Skip to next questions]
□2-4 times daily
□One time every day
□2-3 times a week
□Monthly
□2-4 times monthly
□4 or more times monthly

29. What type of method do you use to clean or uncontaminated your slaughterhouse? Check ☑all that apply.
    □Nothing
    □Bleach
    □Soap
    □Antibiotics
    □Sweeping
    □Water
    □Waste clearance
    □Other (specify) _____
30. How do you handle waste disposal including blood, organ unfit for consumption and contaminated thing? Check ☑all that apply
    □Thrown in environment (river, lake, natural dustbin)
    □Use as natural fertilizer for crop production
    □Use as industrial waste
    □Use for feeding of pigs and other animals
    □Other (specify)_____