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MOLECULAR EPIDEMIOLOGY OF ANTIBIOTIC RESISTANT *CAMPYLOBACTER* SPP. FROM FARM-TO- FORK IN AN INTENSIVE PIG PRODUCTION SYSTEM IN KWAZULU-NATAL, SOUTH AFRICA.

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A dissertation submitted in fulfilment of the academic requirements for the degree of Master of Medical Science (Medical Microbiology) in the School of Laboratory Medicine and Medical Sciences, University of KwaZulu-Natal.

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A dissertation submitted to the School of Laboratory Medicine and Medical Sciences, College of Health Science, University of KwaZulu-Natal, Westville Campus, for the degree of Master of Medical Science (Medical Microbiology).

This is a dissertation in which the second chapter has been written as manuscript with an overall introduction as chapter 1 and final summary of conclusions, limitations, and recommendations as chapter 3.

This is to certify that the content of this dissertation is the original research work of Miss Viwe Sithole, supervised by;

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DECLARATION

I, Miss Viwe Sithole, declare as follows:

- That the work described in this dissertation has not been submitted to UKZN or any other tertiary institution for purposes of obtaining an academic qualification, whether by myself or any other party.

- That my contribution to the project was as follows:

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    - This dissertation does not contain other person's data, pictures, graphs or other information unless specifically acknowledged as being sourced from other persons.

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Signed: 

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DEDICATION

To my dearest late brother

Olwethu “Odz” Sithole

This research is dedicated to you my brother. Your life was taken away from us too soon. You may not have been given a chance to complete your masters as you had hoped. The thought of your hard work, positive spirit and dedication motivated me to complete mine.

This one is for you Odz

I miss you dearly

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LIST OF ABBREVIATIONS AND ACRONYMS

ABR	Antibiotic resistance
AGISAR	Advisory Group on the Integrated Surveillance of Antimicrobial Resistance
AME	Aminoglycoside modifying enzyme
AMR	Antimicrobial resistance
asp	Aspartokinase
AST	Antibiotic susceptibility testing
ATLASS	Assessment Tool for Laboratories and AMR Surveillance Systems
CDC	Centres for Disease Control
CLSI	Clinical and Laboratory Standards Institute
COAG	Committee on Agriculture
DMT	Drug metabolite transporters
EFSA-ECDC	European Food Safety Authority-European Centre for Disease Prevention and Control
ERIC-PCR	Enterobacterial repetitive intergenic consensus polymerase chain reaction
ESBL	Extended spectrum β-lactamase
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FAO	Food and Agriculture Organization of the United Nations
GAP	Global Action Plan
GBS	Guillain-Barre syndrome
hipO	Hippuricase
IMP	Inner membrane protein
MATE	Multi antimicrobial extrusion

mCCDA	Modified charcoal cefoperazone deoxycholate
MDR	Multidrug resistance
MDRGIs	Multidrug resistance genomic islands
MFP	Membrane fusion protein
MFS	Major facilitator superfamily
MOMP	Major outer membrane protein
MOU	Memorandum of understanding
MRSA	Methicillin-resistant *Staphylococcus aureus*
NDoH	National Department of Health
OIE	World Organisation for Animal Health
OMP	Outer membrane protein
PBP	Penicillin binding protein
PCR	Polymerase chain reaction
PMQR	Plasmid-mediated quinolone resistance
PVS	Performance of Veterinary Services
QRDR	Quinolone resistance determining region
RND	Resistance-nodulation-cell division
ROS	Reactive oxygen species
RT-PCR	Real-time polymerase chain reaction
SADC	South African Development Community
SAVA	South African Veterinary Association
SLS	*N*-lauryl sulfate
SPE	Swine proliferative enteritis

TMQR	Transferable mechanisms of quinolone resistance
UPGMA	Unweighted pair group with arithmetic averages
VBNC	Viable but non-culturable
WAAW	World Antimicrobial Awareness Week
WAHIS	World Animal Health Information System
WHO	World Health Organization
XDR	Extremely drug resistant

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ABSTRACT

Background: *Campylobacter* spp. are among the leading foodborne pathogens, causing Campylobacteriosis, a zoonotic infection that results in bacterial gastroenteritis and diarrheal disease in animals and humans. The emergence and transmission of antibiotic resistance and virulence in *Campylobacter* spp. is increasingly reported. We investigated the molecular epidemiology of antibiotic resistant *Campylobacter* spp. isolated across the farm-to-fork-continuum in an intensive pig production system in the uMgungundlovu District, Kwazulu-Natal, South Africa.

Methodology: Following ethical approval, samples were collected over a period of sixteen weeks from selected critical points (farm, transport, abattoir and retail) using a farm-to-fork sampling approach according to WHO-AGISAR guidelines. Overall, 520 samples were investigated for the presence of *Campylobacter* spp. which were putatively identified using selective media with identity and speciation confirmed by polymerase chain reaction (PCR) of specific genes. Resistance profiles were ascertained by the Kirby-Bauer disk diffusion method according to EUCAST and/or CLSI guidelines. Selected antibiotic resistance and virulence genes were identified using PCR and DNA sequencing. Clonal relatedness among the isolates was determined using enterobacterial repetitive intergenic consensus polymerase chain reaction (ERIC-PCR).

Results: Altogether, 378/520 (72.7%) samples were positive for *Campylobacter* spp. with *C. coli* as the most predominant (73.3%), followed by *C. jejuni* (17.7%) with 9.0% classified as "other". Relatively high levels of resistance were observed in *C. coli* and *C. jejuni* to erythromycin (89% and 99%), streptomycin (87% and 93%), tetracycline (82% and 96%), ampicillin (69% and 85%), and ciprofloxacin (53% and 67%) respectively. The lowest percentage resistance observed was for gentamicin (12%) for both *C. coli* and *C. jejuni*, and nalidixic acid (28% and 27%) for *C. coli* and *C. jejuni* respectively. Multi-drug resistance (MDR) was noted among 330/378 (87.3%) isolates. The antibiotic resistance genes observed were the *tetO* (74.6%), the $bla_{OXA\text{-}61}$ (2.9%) and *cmeB* (11.1%) accounting for the resistance to tetracycline and ampicillin while the membrane efflux pump could confer resistance to ampicillin, tetracycline, ciprofloxacin, and erythromycin. All *C. coli* and *C. jejuni* isolates (21) with the *gyrA* gene exhibited mutation at the Thr-86-Ile region in the quinolone-resistance-determining region (QRDR) and all *C. coli* and *C. jejuni* isolates (18) exhibiting erythromycin resistance showed common transitional mutations A2075G and A2074C in the *23S rRNA* gene. Of the virulence genes tested, *ciaB*, *dnaJ*, *pldA*, *cdtA*, *cdtB*, *cdtC* and *cadF* were detected in 48.6%, 61.1 %, 17.4%, 67.4%, 19.3%, 51% and 5% of all *Campylobacter* isolates respectively. The ERIC-PCR banding

patterns revealed that isolates along the continuum were highly diverse with isolates from the same sampling points belonging to the same major ERIC-types.

Conclusion: We showed relatively high levels of resistance to antibiotics commonly used in intensive pig production in South Africa with some evidence, albeit minimal, of transmission across the farm-to-fork continuum. This together with the virulence profiles present in *Campylobacter* spp. presents a challenge to food safety and a potential risk to human health. This is further exacerbated by the reduction in antibiotic treatment options necessitating routine surveillance and monitoring together with antibiotic stewardship, comprehensive biosecurity, and good animal husbandry in intensive pig production.

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Antimicrobial resistance (AMR) according to the World Health Organisation's (WHO) definition is the ability of a bacterial, viral, parasitic or fungal microorganisms to resist the action of one or more antimicrobial agents (drugs) that were previously effective against it resulting in the ineffectiveness of the drug and thus resulting in infection persistence and transmission (WHO, 2014).

Overuse of antimicrobials in humans, animals and crops has increased the emergence of AMR which is now a major threat to human and animal health. This has led to various stakeholders and organisations worldwide taking measures to address this issue. In South Africa, various governmental departments, associations, statutory boards, and stakeholders both in human and animal health play important roles to provide framework for management of AMR (Naidoo, 2017). The National Department of Health (NDoH) has drafted and developed a comprehensive approach to AMR containment by way of the "National AMR Strategy Framework" policy document that complements the international efforts to manage and tackle AMR. It is an approach that will be implemented at all levels of the health and agricultural systems. This AMR National Strategy Framework outlines the key strategic objectives and pillars critical in AMR prevention which are the surveillance and early detection of AMR, infection prevention and control and proper use of antimicrobials through antimicrobial stewardship. The key enablers of these strategic objectives are the legislative and policy reform for health systems strengthening, education, communication to educate and create awareness and research (National Department of Health, 2018).

A WHO report entitled "*Prioritization of pathogens to guide discovery, research and development of new antibiotics for drug resistant bacterial infections, including tuberculosis*" confirms the serious threat of antimicrobial resistance, particularly, drug resistant tuberculosis and 12 other classes of priority pathogens for which new antibiotic treatments are urgently required. Pathogens of critical priority include carbapenem resistant *Acinetobacter baumannii*, carbapenem resistant *Pseudomonas aeruginosa*, and carbapenem and 3rd generation cephalosporin resistant- *Enterobacteriaceae*. Pathogens of high priority encompass vancomycin resistant *Enterococcus faecium*, vancomycin and methicillin resistant *Staphylococcus aureus*, fluoroquinolone resistant *Campylobacter* spp. and fluoroquinolone resistant *Salmonella* spp. (WHO, 2017b).

The WHO and OIE list of critically important antibiotics for human and veterinary medicine respectively, were also developed to encourage the prudent use of shared class antibiotics, i.e., antibiotics that were equally important for human and animal health. Antibiotics used in food-producing animals were frequently the same, or closely related to those used in human medicine and resistance emerging in animals could be transmitted to humans, thereby limiting therapeutic options (WHO, 2019).

Table 1: Critically important antibiotics for human medicine (WHO, 2019).

Antibiotic class	**Examples of antibiotics**
Aminoglycosides	gentamicin
Ansamycins	rifampicin
Carbapenems and other penems	meropenem
Cephalosporins (3rd,4th and 5th generation)	ceftriaxone, cefepime, ceftaroline, ceftobiprole
Glycopeptides	vancomycin
Glycylcyclines	tigecycline
Lipopeptides	daptomycin
Macrolides and ketolides	azithromycin, erythromycin, telithromycin
Monobactams	aztreonam
Oxazolidinones	linezolid
Penicillins (antipseudomonal)	piperacillin
Penicillins (aminopenicillins)	ampicillin
Penicillins (aminopenicillin with β-lactamase inhibitors)	amoxicillin-clavulanic-acid
Phosphonic acid derivatives	fosfomycin
Polymyxins	colistin
Quinolones	ciprofloxacin
Drugs used solely to treat tuberculosis or other mycobacterial diseases	isoniazid

Table 2: Antibiotics of veterinary importance for food-producing animals (OIE, 2019).

Antibiotic class	Examples of antibiotics
Aminocyclitol	Spectinomycin
Aminoglycosides	Dihydrostreptomycin, streptomycin, amikacin, apramycin, fortimycin, framycetin, gentamicin, kanamycin, neomycin, paromomycin, tobramycin.
Amphenicols	Florfenicol, thiamphenicol.
Cephalosporins (3rd and 4th generation)	Cefoperazone, Ceftiofur, Ceftriaxone, Cefquinome.
Macrolides	Erythromycin, Gamithromycin, Tulathromycin, Carbomycin, Josamycin, Kitasamycin, Mirosamycin.
Penicillins: aminopenicillins, aminopenicillins with β-lactamase inhibitor,	Amoxicillin, Ampicillin, Hetacillin, Amoxicillin + Clavulanic Acid, Ampicillin + Sulbactam.
Quinolones second generation (fluoroquinolones)	Ciprofloxacin, Danofloxacin, Difloxacin, Enrofloxacin, Marbofloxacin, Norfloxacin, Ofloxacin, Orbifloxacin, Sarafloxacin.
Sulfonamides	Sulfadoxine, Sulfafurazole, Sulfaguanidine, Sulfachlorpyridazine, Sulfadiazine, Sulfadimethoxazole.
Tetracyclines	Chlortetracycline, Doxycycline, Oxytetracycline, Tetracycline.

Antibiotic resistance results in the reduced effectiveness of antibiotics resulting in "difficult to treat" or untreatable infections and diseases. This threatens global health and food safety as animal wastes are contaminated with resistant microorganisms leading to cross contamination in the food chain, further contributing to the emergence and spread of AMR. Antibiotic resistant *Campylobacter* spp. are prevalent in food animals including pigs, poultry, cattle, goats, and sheep (Karikari et al., 2017; Igwaran and Okoh, 2020; Pillay et al., 2020). There are reduced treatment options for resistant bacteria which are often costly (FAO, 2016). There is therefore a need for research to be conducted in the South African pig farms to address the ABR and increase the understanding and knowledge of the emerging *Campylobacter* pathogen on South African farms. This will assist in the implementation of measures for prevention, containment, and control of the antibiotic resistance burden.

1.2 Literature Review

AMR has become a major public health concern and crisis globally. It can be spread through food systems by movement of livestock and agricultural produce within and between countries in addition to human travel (Robinson et al., 2016). One example is that of the emergence of the transferable plasmid mediated colistin resistant gene (*mcr-1*) in animals and humans (Coetzee et al., 2016; Robinson et al., 2016; Kieffer et al., 2017). The gene has spread to other members of *Enterobacteriaceae* in human, animals, food, and environmental samples (Coetzee et al., 2016; McGann et al., 2016; Zeng et al., 2016). It was also identified in *E.coli* isolates from patients with urinary tract infection in South Africa and the United States (Coetzee et al., 2016; Zeng et al., 2016). Colistin is a polymyxin and an effective last resort antibiotic for the treatment of infections caused by multidrug resistant and extremely drug resistant (XDR) Gram-negative pathogens (Carroll et al., 2019). The drug has been declared by WHO as the "highest priority critically important antibiotic for human medicine" (WHO, 2019).

1.2.1 Antimicrobial Resistance as a One Health Issue

One Health is a collaborative effort of multiple disciplines working globally, nationally and/or locally on optimal health at the human-animal-environmental interface. One Health recognizes the connection between the health of people, the health of animals and environment and that this can be ensured through the research efforts of scientists and practitioners in a variety of disciplines (Robinson et al., 2016). AMR is described as a One Health issue due to the emergence and dissemination of resistant microorganisms within and between humans, animals and the environment that is occurring worldwide. This in turn leads to the ineffectiveness of antimicrobials and the subsequent increase in mortality (Robinson et al., 2016).

Resistance to antibiotics in animals, and particularly food animals, is attributed to overuse and misuse of antibiotics, incorrectly prescribed antibiotics and long exposure periods. The extensive use and long exposure periods create ways for bacteria to fix genes conferring resistance to the antibiotics and this resistance can be transferred to human gut microbiota through ingestion during consumption of the contaminated food, direct contact with animals and via environment (Ventola, 2015; Robinson et al., 2016) (**Figure 1**).

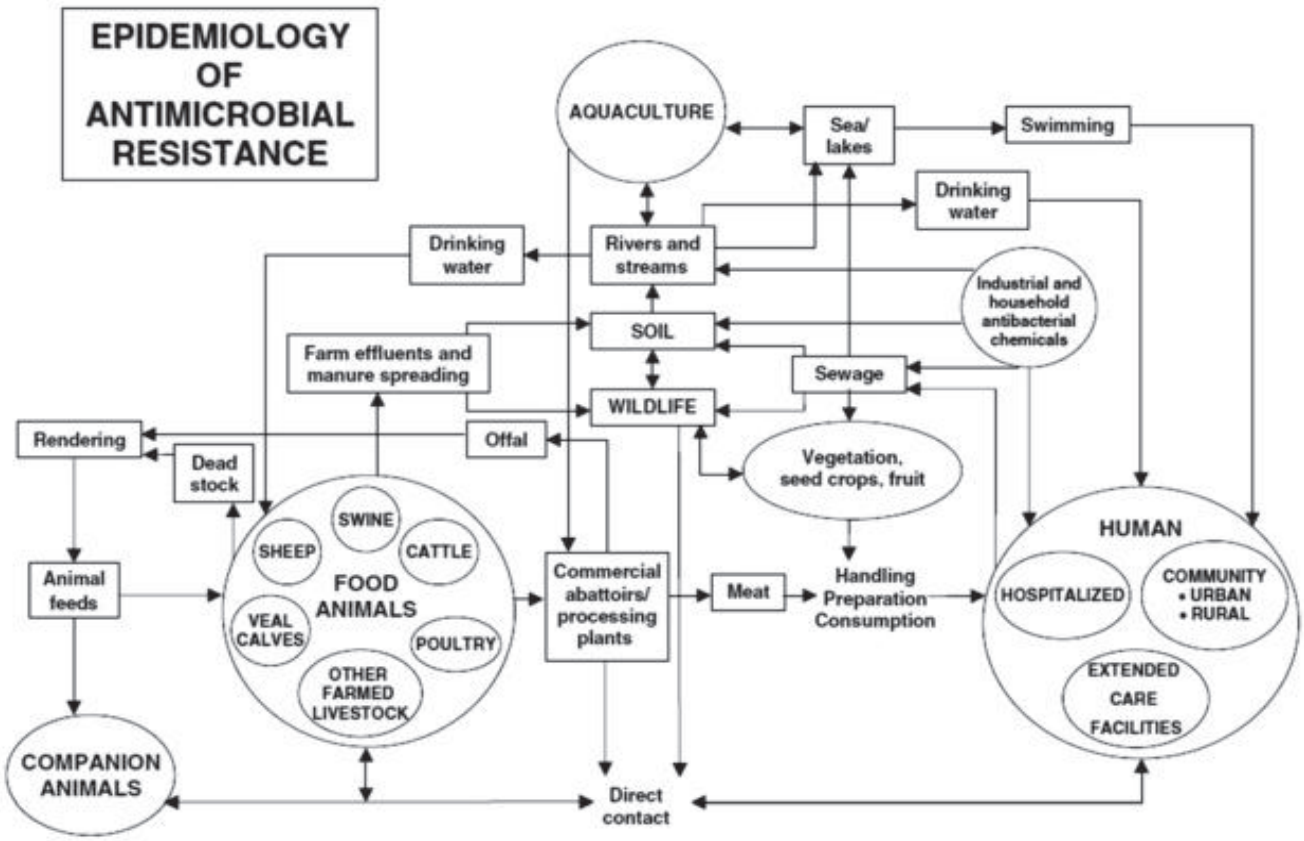

Figure 1. AMR transmission and circulation between humans, animals and the environment. The figure depicts potential pathways by which resistant organisms can spread among populations of humans, animals by direct contact or via secondary sources including, water sources, food, manure or fomites. The figure indicates different areas where antimicrobials are used and include human medicine, food animals, companion animals, aquaculture, horticulture, and disinfectants used in consumer products (Landers et al., 2012).

A study was conducted by Grøntved et al. (2016) on methicillin-resistant *Staphylococcus aureus* (MRSA) CC398 in humans and pigs with the aim to describe the importance of the human introduction and transmission of MRSA CC398 in pig herds and the subsequent spread in Norway. Their results revealed that primary introductions of MRSA likely occurred by human transmission to the three sow farms investigated with secondary transmission to other pig farms, mainly through animal trade and to a lesser extent via humans or livestock transport vehicles (Grøntvedt et al., 2016).

Another study in The Netherlands by Dohmen et al. (2015) determined the prevalence of carriage of extended-spectrum β-lactamases producing enterobacteriaceae (ESBL-PE) in pig farmers, family members and employees, and its association with carriage in pigs. The study revealed that daily

exposure to pigs carrying ESBL-PE is associated with ESBL carriage in humans. Their investigation noted that human and pig isolates within the same farm harboured similar ESBL gene types and had identical sequence and plasmid types, suggesting clonal transmission from pigs to humans. Human carriage of ESBLs was positively associated with average number of hours working on farm per week (OR = 1.04, 95% CI 1.02–1.06, p 0.0008), and presence of ESBLs in pigs (OR = 12.5, 95% CI 1.4–111.7, p 0.02) The prevalence was 13% (95% CI 4–23%) among people living/working on a farm with ESBL-PE-carrying pigs, and 27% (95% CI 10–44%) among humans with daily exposure to ESBL-PE-carrying pigs(Dohmen et al., 2015).

1.2.2 WHO, FAO AND OIE Initiatives in Addressing AMR

Global bodies such as the WHO, World Organisation for Animal Health (OIE) and the Food and Agriculture Organisation of the United Nations (FAO) speak in unison in addressing the emergence and spread of AMR by way of the Global Action Plan (GAP) to contain AMR. The goal is to ensure that antimicrobial agents continue to be effective and successful to cure and prevent diseases in humans and animals, promote prudent and responsible use of antimicrobial agents and ensure global access to medicines of good quality to all who need them (WHO, 2015a). In order to achieve this goal, the GAP sets out five objectives:

- "To improve awareness and understanding of antimicrobial resistance through effective communication, education and training.
- To strengthen the knowledge and evidence base through surveillance and research.
- To reduce the incidence of infection through effective sanitation, hygiene and infection prevention measures.
- To optimize the use of antimicrobial medicines in human and animal health.
- To develop the economic case for sustainable investment that takes account of the needs of all countries and to increase investment in new medicines, diagnostic tools, vaccines and other interventions" (World Health Organization, 2015).

Antimicrobials play a very important role in agricultural sectors. They are used in terrestrial and aquatic animals as well as plant production for treatment and non-therapeutic purposes. To support the implementation of the requests made at the 39th session of FAO conference resolution 4/2015 on AMR, FAO drafted a five-year action plan on AMR to support the GAP developed by WHO in addressing and combating AMR. Four main focus action areas of work on AMR have been identified by FAO in relation to the objective of the GAP on AMR as shown in **Figure 2** (FAO, 2016).

Figure 2: FAO focus areas in relation to objectives of GAP on AMR. The figure outlines the four main focus areas of the FAO Action Plan on AMR supporting the Global Action Plan's objectives (FAO, 2016).

Focus area 1: "Raising awareness" on AMR and related threats by developing communication and advocacy products that will help countries to disseminate key messages and understand challenges and risks faced from AMR. This focus area also advocates for consideration of AMR integration into policy-level discussions on food and agriculture at global and national levels in partnership with other organisations concerned (FAO, 2016).

Focus area 2: "Surveillance and monitoring of AMR and antimicrobial use in food and agriculture". This focus area aims to support integrated systems of surveillance and monitoring and promote extensive data generation and sharing across sectors at local and global levels. This focus area also aims to make information relevant to food and agriculture AMR widely available. The organisation plans to achieve these by developing training materials on antimicrobial use, AMR and related surveillance and monitoring, supporting the inclusion of antimicrobial use and AMR as core components of professional education, and, promoting and contributing to research to improve knowledge on antimicrobial use and AMR in the food and agriculture sector (FAO, 2016).

Focus area 3: “Strengthen governance related to antimicrobial use and AMR in food and agriculture”. This focus area aims to support countries to address AMR by ensuring there’s information generated in support of improved policy and decision making. It aims to support national level work by setting international standards relevant to AMR by revising or developing legislations that meet international standards together with the timely review and revision of these international standards (FAO, 2016).

Focus area 4: “Promoting good practices in food and agriculture systems and the prudent use of antimicrobials”. This focus area aims to develop and support practical measures to be taken in food and agricultural sector to reduce the need of antimicrobials focusing on improved biosecurity in order to reduce infections, reduction of antimicrobial usage through good practices in therapeutic use, identifying alternatives to antimicrobial use, and, prevention of the spread of antimicrobial resistance by practising good hygiene (FAO, 2016).

The OIE also published its official strategy detailing the scope of its activities against AMR that supports the objectives established by the WHO-GAP. The OIE Strategy on AMR and the prudent use of antimicrobials outlines the goals and tactics in place to support member countries (OIE, 2016). The strategy outlines the following four main objectives and work plans:

Objective 1: “Improve awareness and understanding” of the role of antibiotics and global threat of AMR among veterinarians, farmers and citizens in member countries to support the responsible and ethical use of antimicrobial products in animals. This will be achieved through development and implementation of tools and policies including targeted communication measures and advocacy materials, to understand the risks of AMR, increasing awareness and understanding of AMR through veterinary statutory bodies and veterinary education establishments, to encourage a way of life that supports responsible use of antimicrobials, conducting workshops, conferences and symposia that addresses the issue of AMR and promotes prudent use of antibiotics at global, regional and national levels, and expansion of its portfolio of guidance, education and scientific reference materials (OIE, 2016).

Objective 2: “Strengthen knowledge through surveillance and research” The evaluation missions of OIE performance of veterinary services (PVS) have established that in many countries antimicrobial drugs are greatly available and their distribution and use is loosely controlled and poorly monitored. The OIE will thus support member countries in their development, implementation, and their monitoring and surveillance systems to report on antimicrobial use and emergence of resistant organisms. The development of a global database on the use of antimicrobials in animals is a priority of the OIE. This will establish baseline information to analyse trends over time and assess the success

of actions taken to ensure responsible use of antimicrobial agents. This global database will in time be linked to the OIE World Animal Health Information System (WAHIS) which is the OIE's web-based online reporting system for information on animal populations and disease, thus OIE will increase the development, use and functionality of the WAHIS and the linkage of these databases will allow data analysis on antimicrobial use putting into consideration populations by country and region. The OIE will also guide and support research into other alternatives to use of antibiotics to encourage new developments and technologies that will reduce animal dependence on antimicrobials (OIE, 2016).

Objective 3: "Support good governance and capacity building". With regard to this objective, the OIE's plan is to assist member countries 'in developing and implementing their national action plans and policies governing the use of antimicrobials in animals to control AMR and promote prudent use. The OIE is also committed to support and work alongside veterinary services of member countries ensuring that they have the capacity to implement OIE standards so there would be continued improvement in countries' veterinary services. OIE also wants to ensure that member countries are engaged through regular trainings of focal points on veterinary products, so government officials are informed of the most up-to date standards for regulation of veterinary products establishing direct links and support processes. OIE will ensure that highly trained and competent veterinarians and veterinary paraprofessionals are at the forefront of national and regional efforts to improve animal health and welfare and stewardship of antimicrobial products. Member countries will also get assistance from OIE will also provide tools and guidance to assist member countries in their initiatives on AMR risk assessment associated with antimicrobial agents and their use in animals. Additionally, OIE will support member countries in developing legislations governing the manufacture, marketing authorisation, importation, distribution and use of veterinary products (OIE, 2016).

Objective 4: "Encourage implementation of international standards" The OIE aims to encourage and support member countries to adopt and implement the OIE standards for combating AMR in animals through prudent use of antimicrobials, and, in so doing will help in improving the biosecurity and support animal health and welfare and public health. OIE also encourages adoption of the recommendations in the OIE list of antimicrobials of veterinary importance by member countries thus engendering harmonisation between sectors, countries and regions achieved through shared standards ensuring generation of comparable data (OIE, 2016).

The OIE annual report on antimicrobial agents intended for use in animals, provide data voluntarily submitted by countries through collaborative process to better understand global antimicrobial consumption (OIE, 2020). According to the recent analysis of the fourth round of data collection on the use of antimicrobials in animals by countries for the period September 2018 to May 2019, there is

significant global progress on the monitoring and control of antimicrobial use in animals. Of the 153 countries that participated, Africa and the Americas, were the highest countries that showed progress in the more detailed reporting of their quantitative data demonstrating growth and ommitment in development of monitoring systems for veterinary antimicrobial agents. The report showed that the use of antimicrobials for growth promotion has declined evident by the lowest proportion of countries (35 out of 153 countries, 23%) using growth promoters from the beginning of the OIE global data collection records in 2015. Of note is that 25 out of the 35 countries using growth promoters do not have a regulatory framework (OIE, 2020).

The same report also had data analysis of globally reported quantitative data on antimicrobial agents intended for use in food animals in 2016. This was undertaken with an understanding that a lot of countries contributing to the OIE database are in their first stages of development of national monitoring systems on antimicrobial use in animals. Poultry was mentioned by 91 of the countries that provided data as the food producing animal species in which antimicrobials were used followed by bovines, sheep and goats and pigs. Most countries were thus using antibiotics in these species. The most commonly reported antimicrobials used in food animals by 93 countries were the tetracyclines (35.3%), penicillins (16.4%), macrolides (10.9%) and polypeptides (10.5%) (OIE, 2020).

The FAO conducts regional conferences hosted in turn by a different member state from the region, attended by ministers of agriculture from the regions and high officials of member states on a biennial basis in Africa, Asia and the Pacific, Europe, Latina America and the Caribbean to elaborate on challenges of the region and priority matters related to food and agriculture as well as discussion on global policies and regulatory matters, technology and innovations helping to improve food security. Observers from countries/nations outside the region, international and regional organizations are invited to the conference, including but not limited to Arab ones. (FAO, 2020b).

The greatest burden of AMR is projected to occur in continents like Asia and Africa where there are weak health systems and infrastructures, inadequate legislation and regulatory surveillance systems on use of antimicrobials and high prevalence of infectious diseases (FAO, 2020b).

The WHO, FAO and OIE launched a global tripartite database to view country progress on the development and implementation of national action plan on AMR as of 29 May 2017. The survey shows that there is already a wide range of activities ongoing in countries. In Africa 20% of countries have national AMR action plan under development or plans involve only one sector or ministry. According to the survey, it was reported that South Africa has developed and publishes its AMR action plan on human health, animal health and food safety (WHO, 2018).

The global campaign now termed "World Antimicrobial Awareness Week" (WAAW) as from second quarter of 2020 (previously World Antibiotic Awareness Week) launched by WHO in collaboration with FAO and OIE is a campaign conducted in November every year to raise global awareness and understanding of AMR and to promote prudent us of antimicrobials such as antibiotics to avoid the further emergence and spread of drug-resistant infections (WHO, 2020). In support of this campaign, the Africa Centres for Disease Control and Prevention (Africa CDC) organised a virtual launch of the WAAW Africa 2020 on 18–24th November 2020, including a panel discussion with the aim to address the understanding and experiences of AMR in Africa (Africa CDC, 2020).

The FAO's update and report on AMR, indicated that the Southern Africa region is making substantial strides towards reversing the threat of AMR. In fighting the spread of AMR, the regional economic body, South African Development Community (SADC) validated a regional AMR control strategy premised on the One Health approach and aligned to the global AMR action plan in a meeting held in Johannesburg, South Africa on 10 and 11 December 2019 (FAO, 2020b).

In the focus area of surveillance, the FAO's main technical advisory committee on agriculture (COAG) issued a 27th session report that outlines the progress on the implementation of FAO action plan on AMR 2016-2020. In the report, FAO has enhanced laboratory capacity on AMR and residue monitoring by development of FAO Assessment Tool for Laboratories and AMR Surveillance Systems (ATLASS) which will improve national AMR surveillance systems in the food and agriculture sectors (FAO, 2020a).

1.2.3 *Campylobacter* spp.

Campylobacter spp. are a group of Gram-negative, spirally curved and rod shaped bacteria that have polar, bipolar or no flagella (Man, 2011; Nadeem O. Kaakoush et al., 2015). *Campylobacter* spp. are fastidious obligate microphillic bacteria that require concentrations of oxygen between 2-10% (O'Kane and Connerton, 2017) and a temperature range between 37 and 42°C for optimal growth. Specific gas packs are required for rapid growth of the organism in the laboratory (Davis and DiRita, 2008). The genus *Campylobacter* belongs to the family *Campylobacteraceae* with three genera, *Campylobacter*, *Arcobacter* and *Helicobacter*. The genus *Campylobacter* consists of 25 species two provisional species and eight subspecies (as of August 2013) (Nadeem O Kaakoush, Mitchell, and Man, 2015) with *Campylobacter jejuni* subsp. *Jejuni* (herein referred to as *C. jejuni*) and *Campylobacter coli* (*C. coli*) as the most common species responsible for a bacterial foodborne infectious disease named Campylobacteriosis causing gastroenteritis by the colonisation of the gastrointestinal tracts in animals and humans resulting in diarrhoea (Nicolle and SHEA Long-Term-Care-Committee, 2001; Man, 2011; Facciolà et al., 2017; Heredia and García, 2018). Other species such as *C. ureolyticus* and *C.*

hyointestinalis have also been associated with gastroenteritis. In humans, they are associated with gastrointestinal conditions such as inflammatory bowel disease, colorectal cancer, bloody or watery diarrhoea and cramps. They are also reported to involve extra gastrointestinal manifestations including but not limited to lung infections, bacteraemia, meningitis, and reactive arthritis in immunocompromised individuals. The major and main recognised complications of *Campylobacter* spp. infection are the Guillain Barre Syndrome (GBS) and Miller-Fischer syndrome which is a clinical variant of GBS. GBS is a neurologic condition triggering an autoimmune response causing progressive weakness in limbs which also affects respiratory and cranial nerve-innervated muscles (Man, 2011; Bolton, 2015; Harrison, 2015; Nadeem O. Kaakoush et al., 2015; Facciolà et al., 2017). The most known clinical manifestations of campylobacteriosis in foodborne animals are abortion and gastroenteritis. The most recognised cause of abortions in late pregnancy and stillbirths in sheep and goats is as a result of colonisation by *C. jejuni*. *C. fetus* subsp *fetus* is known to localize in the pregnant uterus and results in abortions during late pregnancy in cattle and sheep, stillbirth, or birth of weak lambs, which eventually die soon after birth. *C. jejuni* and *C. coli* can cause enteritis and diarrhoea with occasional blood in sheep, pigs and cattle, with similar symptoms also noticeable with *C. fetus*, *C. laridis* and *C. upsaliensis* which are also notable causes of enteritis and diarrhoea in cattle. *C. jejuni* has also been isolated in cows with mastitis. Lesions have also been observed as a result of *C. jejuni* infestation which consist of mild cellular infiltration of the mucosa, severe lesions in the small intestine and occasional ulceration and haemorrhage (CABI, 2018).

The swine proliferative enteritis (SPE) known as the disease of the lower small intestine and occasionally caecum and colon which includes porcine intestinal adenomatosis, proliferative ileitis, terminal ileitis, necrotic enteritis and haemorrhagic enteritis have been noted in pigs colonised by *C. mucosalis* (formerly *C. sputorum* subsp *mucosalis*) and *C. hyointestinalis* (CABI, 2018).

Campylobacter spp. also causes diarrheal disease in piglets with clinical symptoms and histopathological lesions similar to those observed in humans infected with *C. jejuni*. Increased rectal temperatures and acute watery diarrhoea with presence of occult blood, minimal inflammatory enterocolitis with presence of edema, hyperaemia, and mucus were noted. Damage to surface epithelial cells with the presence of intracellular bacteria, mainly in the large intestine was also observed (De Vries et al., 2017).

Campylobacter spp. have been proven to be most prevalent in food animals including pigs, poultry, cattle, goats, and sheeps (Karikari et al., 2017; Igwaran and Okoh, 2020; Pillay et al., 2020). The most encountered thermotolerant *Campylobacter* spp. in food borne and waterborne infections are *C. jejuni*, *C. coli* and some *C. lari* (Frasao, Marin, and Conte-Junior, 2017). The main route of transmission of

Campylobacter is oral route, via consumption of undercooked meat, contaminated dairy products and contaminated water. Other factors and routes of transmission involved include, direct contact with the farm animals, companion animals as well as animal product contamination including human related animal handling (slaughtering, processing practices and storage procedures (Facciolà et al., 2017; Reddy and Zishiri, 2018). Among the antibiotics indicated for the treatment of food animal infections including swine are some of the critically important antibiotics listed by OIE and include streptomycin, gentamicin, erythromycin, ampicillin, ciprofloxacin and tetracycline. The use of these drugs is essential in the animal sector to protect animal health and welfare, contribute to food safety, and protect public health thus preventing transmission of the infectious animal diseases to humans (OIE, 2019). However, there is high resistance observed particularly to *Campylobacter* isolates to these drugs due to the fact that there is excessive use and misuse of these antibiotics in the animal sector environments and this has become a major threat to human and animal health (Ma et al., 2020).

1.2.4 Prevalence and Susceptibility Profiles of *Campylobacter* spp. in Pigs

High prevalence of *Campylobacter* spp. in pigs was shown in a number of studies with *C. coli*, and *C. jejuni* most commonly observed compared with *C. lari*, *C. upsaliensis* and *C. hyointestinalis* (Gwimi et al., 2015; Matthew-Belmar, Amadi, and Stone, 2015; Karikari et al., 2017). Gwimi et al. (2015) conducted a study on the prevalence of *Campylobacter* spp. in faecal samples of pigs and humans in Zuru Kebi in Nigeria. The study revealed a high percentage of *Campylobacter* spp. in pigs 92.66% with *C. coli* as the most encountered species 78.71% followed by *C. jejuni* at 14.03%, *C. upsaliensis* at 5.40% and *C. hyointestinalis* as the least encountered at 1.80%. Percentage isolation in humans was at 60.63%, 24.50%, 7.45%, 5.32%, and 2.13% for *C. coli*, *C. jejuni*, *C. upsaliensis*, *C. hyointestinalis* and *C. lari* respectively (Gwimi et al., 2015).

Another study by Matthew-Belmar et al. (2015) in Grenada on the antimicrobial resistance profiles of *C. jejuni* and *C. coli* recovered from faecal samples of young healthy domestic pigs showed an overall prevalence of 95.6% made up of 53.5% *C. jejuni* and 46.5% *C. coli*. High resistance rate was observed to tetracycline with 58.5% and 61.1% for *C. jejuni* and *C. coli* respectively, followed by ampicillin at 18.5% and 14.8% in *C. jejuni* and *C. coli* respectively (Matthew-Belmar et al., 2015).

A study in Ghana, on antibiotic resistance of *Campylobacter* recovered from faeces and carcasses of healthy livestock observed a higher prevalence of *Campylobacter* spp. in pig faecal samples at 28.7% compared to other food animals tested, e.g., cattle (13.2%), sheep (18.6%), and goats 18.5%. There was also a higher prevalence in carcasses of pigs at 36.3%. Species identified in pig faeces were *C. coli, C. jejuni*, C. *lari and C. jejuni* subsp. *doylei* at 48.2%, 48.2%, 3.4% and 0% respectively. Species

discovered in pig carcasses were *C. jejuni* 28.4%, *C. coli* 10.8%, *C. lari* 10.8% and *C. jejuni* subsp. *doylei* 2.7%. The isolates showed resistance rates of 100% each to erythromycin, 86% and 60% to tetracycline, 83% and 97% to ampicillin, 10% and 51% to nalidixic acid, 24% and 35% to ciprofloxacin and 10% and 30% to gentamicin in pig faecal and carcass samples respectively. The species-specific resistance profiles revealed resistance to nalidixic acid at 23%, 2.9% and 100%, to ciprofloxacin at 40.2%, 37.7% and 93.3%, to gentamicin at 21.1%, 1.4% and 26.7%, to erythromycin at 99.5%, 100% and 100%, to ampicillin at 96.5%, 84% and 96.7%, and, to tetracycline at 69.8%, 89.8% and 96.7% in *C. jejuni*, *C. coli* and *C. lari* respectively (Karikari et al., 2017).

Jonker and Picard (2010) conducted a study to investigate the antimicrobial susceptibility patterns of thermophilic *Campylobacter* spp. isolated from intestinal tracts of pigs and chickens in South Africa. The prevalence of *Campylobacter* in this study was however very low at 7.88% with *C. coli* predominating in pigs (5 out of 6 isolates) and *C. jejuni* predominating in poultry (23 out of 32 isolates from chicken). *C. jejuni*. isolated from the poultry farms in Gauteng showed resistance to the β-lactams at (82.4 % and 94.1 %) to amoxicillin and ceftiofur respectively. Porcine *Campylobacter* isolates were more susceptible to tetracyclines at 34.4 % and 33.3 % to chlortetracycline and doxycycline, respectively compared to the poultry isolates that showed 70 % and 60 % resistance to chlortetracycline and doxycycline, respectively. Resistance to erythromycin was detected in 46.35% of isolates from the Western Cape with higher resistance levels observed in *C. coli* 72.73% than *C. jejuni* 20%. *C. coli* was highly resistant to both erythromycin (72.7 %) and lincomycin (72.6 %), a lincosamide similar to clindamycin. A higher percentage of porcine isolates were resistant to the lincosamides at 83.3 % and to erythromycin at 66.7 % (Jonker and Picard, 2010).

Another study in South Africa conducted by Uaboi-Egbenni et al. (2011) investigated the prevalence, haemolytic activities and antibiotic susceptibility profiles of *Campylobacter* spp. isolated from pigs in three farms in the Venda region, Limpopo province. The study confirmed the high incidence of *Campylobacter* spp. in faecal samples of pigs where 84.6% of the samples were positive. *C. coli* and *C. jejuni* were the most prevalent species. *C. coli* had a higher incidence at 55.3% from farm X, 50% farm Y and 76.9% farm Z compared to *C. jejuni* with 5.3%, 7.9% and 12.8% farm X, Y and Z respectively. Most *Campylobacter* spp. in all farms were resistant to the antibiotics tested. *Campylobacter* species in Farm X showed resistance of 54.5%, 100%, 54.5%, 63.3%, 100% to tetracycline, vancomycin, ampicillin, kanamycin and methicillin respectively and less than 50% resistance to ciprofloxacin, cefixime, and erythromycin. Greater than or equal to 50% of the *Campylobacter* spp. in Farm Y were resistant to ciprofloxacin (57.1%), tetracycline (50%), cefixime

(53.6%), vancomycin (67.9%), gentamicin (53.6%), methicillin (75%), and erythromycin (53.6%). In farm Z, > 50% but < 100% of the isolates were resistant to tetracycline, gentamicin and erythromycin, while 40.7% were resistant to ciprofloxacin. Approximately 100% of *Campylobacter* spp. in this farm were resistant to cefixime, vancomycin, kanamycin, and methicillin. The study concluded that *Campylobacter* antibiotic resistance in food producing animals is indeed a global issue even in the pig production industry. The study recommended that more surveillance on the prevalence and susceptibility profiles of bacteria of concern to human health and monitoring of antibiotic usage is needed so to combat this problem (Uaboi-Egbenni et al., 2011).

Di Donato et al. (2020) investigated the prevalence, antimicrobial resistance patterns of *C. coli* isolated in swine at slaughterhouse in Italy and the genetic diversity between profiles obtained from swine and human strains. The high percentage of contaminated pig carcasses (50.4%) and faeces (32.9%) in the study proved that the pig animal species is indeed one of the main animal reservoirs of *C. coli*. Resistance of *C. coli* isolated from pigs showed high resistance against quinolones and fluoroquinolones (74.66% and 70.13%) to specifically nalidixic acid and ciprofloxacin respectively, and a 90.95%, 90.95%, 37.55%, and 25.79% to streptomycin, tetracycline, erythromycin and gentamicin respectively. In this study, almost all human isolates (90.90%) and swine isolates (64.19%) were assigned to the ST-828 clonal complex (Di Donato et al., 2020).

The results obtained from the literature on prevalence and susceptibility profiles of *Campylobacter* species provides evidence of the prevalence of resistant *Campylobacter* spp. in food animal production systems that pose a risk of campylobacteriosis in food animals and humans.

1.2.5 Virulence Factors and Pathogenesis of *Campylobacter* spp.

The major virulence factors contributing to the pathogenesis of *Campylobacter* infection include flagella-mediated motility, adherence to intestinal mucosa, invasion and colonisation, survival in host cells and the ability to produce cytotoxins as summarised in **Figure 3**.

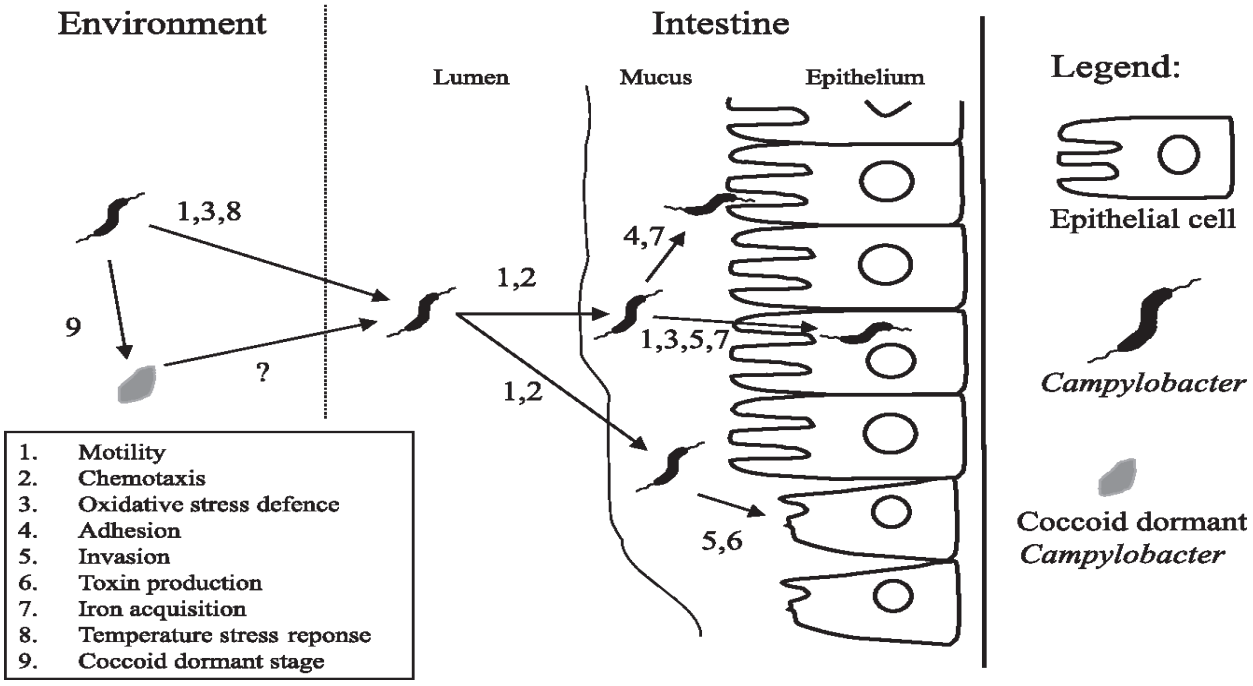

Figure 3: **Virulence factors and colonisation phase(s) of *Campylobacter* spp. in the intestine.** The figure describes several *Campylobacter* virulence factors together with the colonization phases in which they are predicted to be expressed when colonising the intestine (Van Vliet and Ketley, 2001).

1.2.5.1 Motility and Chemotaxis

For colonisation to take place, *Campylobacter* spp. requires motility under chemotactic conditions, i.e., the ability to move into the mucus layer that covers intestinal cells. Motility in *Campylobacter* spp. is achieved by polar flagella which allows penetration of the mucus barrier. *Campylobacter* flagella consists of the O-linked glycosylated flagellin sub-units encoded by *flaA* and *flaB* genes involved in the regulation of the *Campylobacter* flagellum and it also consists of the two regulator component systems, the sensor *FlgS* and the response regulator *FlgR* involved in the regulation of the flagellum. The *flaA* gene is thought to be responsible for the expression of adherence, gastrointestinal tract colonisation and host cells invasion. The *flaA* and *flaB* genes are expressed independently from their own promoters, with *flaA* gene expressed from the σ^{28} promoter and *flaB* gene from σ^{54} with *flaA* expressed at higher levels than *flab* (Van Vliet and Ketley, 2001).

Chemotaxis is the process mediating movement of a motile organism up or down a chemical gradient in response to a chemical stimulus. This system controls bacterial motility towards a favourable environment or away from unfavourable environment. Bacterial chemotaxis is a signal transduction

system that facilitates sensing and responding to a stimulus and relies on coupling proteins to establish connections between receptors and histidine kinases (Zautner et al., 2012).

In *C. jejuni* chemotaxis is a two-component signal transduction system consisting of a membrane-associated histidine autokinase/sensor CheA and a cytoplasmic response regulator protein CheY (Zautner et al., 2012). The genes of the *Che* proteins known to be involved with chemotactic responses in *C. jejuni* are the *CheA*, *CheB*, *CheR*, *CheV*, *CheW* and the *CheY* genes. The genome of the *C. jejuni* harbors genes encoding methyl accepting chemotaxis proteins (chemoreceptors) called transducer like proteins (Tlps) that sense extracellular stimuli in the form of ligands and transmit these signals to the cytoplasmic core chemotaxis signal transduction proteins network (Che) (Chandrashekhar, Kassem, and Rajashekara, 2017). The change in the conformation of the chemoreceptors (Tlps) directs auto-phosphorylation of a histidine kinase core signal transduction protein, CheA, which in turn phosphorylates CheY which acts a cytoplasmic response regulator and interacts with flagellar motor switch proteins FliM and FliN in the phosphorylated state to influence changes in the bacterial cell flagella rotation in response to a stimulus (Chandrashekhar et al., 2017; Reuter et al., 2018). The two enzymes, methyl-esterase CheB and methyltransferase CheR are the crucial methylation adaptation system proteins that mediate manipulation and modification of the methylation status of methyl accepting chemotactic domains in the Tlp chemoreceptors to accommodate adaptation. CheB has a de-methylation activity, it demethylates the Tlp-signalling domains during adaptation to repelling stimuli and the CheR methylates the designated sites. The CheW protein is required to interact with the signalling domains of CheA-Tlps to form the final ternary signalling complex Tlp-CheW-CheA. CheV is a commonly used coupling scaffold protein of *C. jejuni* parallel to the CheW. CheV is a CheW-like linking-protein with an additional response regulator-domain that may play a role in mediating adaptation to attractant that it attaches the histidine autokinase (CheAY) to. These processes thus facilitate sensing and response to a stimulus playing a critical and important role in pathogenesis of this enteric pathogen (Van Vliet and Ketley, 2001; Silva et al., 2011; Zautner et al., 2012; Chandrashekhar et al., 2017).

1.2.5.2 Adherence, Invasion and Colonisation by *Campylobacter* spp.

Adherence and invasion into epithelial cells are critical steps essential for disease development in order for bacteria to produce toxins and the toxic activity eventually leads to creation of cell infection. Through the mobility of the bacteria that is achieved by flagella, *Campylobacter* spp. can pass through the mucus, attach and penetrate the epithelial cells. There are three distinct processes *C. jejuni* has to establish to interact with host epithelial cells: 1) adherence to host cells 2) invasion into the intestinal

epithelium cells and 3) survival inside the host cells (Backert and Hofreuter, 2013) as detailed in **Figure 4**.

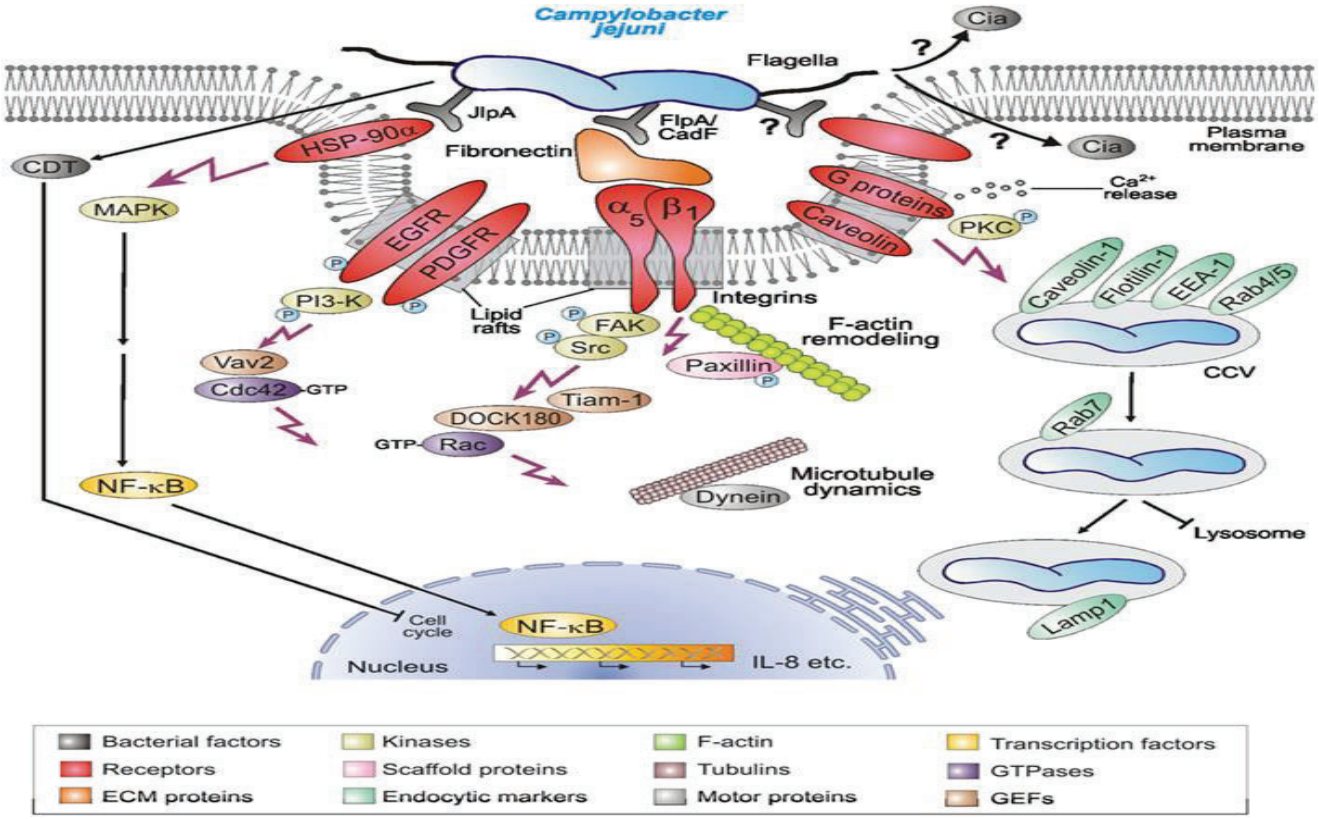

Figure 4: Hypothetical model for the establishment of infection by *C. jejuni*. There are numerous reported and unknown factors by which C. jejuni adheres to host cells. The figure shows that the uptake of the bacteria is accomplished through several host cell receptors and several signalling molecules and pathways. This results in microtuble/ F-actin rearrangement at the entry site of the bacterium and eventually, engulfment (Ó Cróinín and Backert, 2012).

The adhesion process of *C. jejuni* is a multifactorial event that involves co-operative action of several factors. *C. jejuni* adheres to host cell via several bacterial factors and the uptake of the bacteria is achieved through numerous host cell receptors. This results in re-arrangement of the localized F-actin and/or microtubule at the bacterial entry site resulting in bacterial engulfment and uptake (Ó Cróinín and Backert, 2012).

For host cell invasion and colonisation to take place, binding of the bacterial surface adhesins to host cell receptors or extracellular matrix surrounding cells is required. Multiple bacterial cell structures contribute to *Campylobacter* interaction with the host cell. Adhesin proteins on the bacterial surface in

C. jejuni spp. have been suggested to play a role in adhesion and invasion. These include, outer membrane proteins specific to fibronectin (Fn): CadF and FlpA proteins, the *Campylobacter* adhesin autotransporter protein CapA; the phospholipase A (pldA); the surface exposed lipoprotein specific to heat shock protein JlpA, the periplasmic binding proteins PEB1-4 and some other bacterial factors whose precise role in the invasion mechanisms in *C. jejuni* is not clear, these include, the sialylation of the lipo-oligosaccharides (LOS) outer core, *cia* invasive antigens and capsular polysaccharides (CPS). CadF and FlpA are the two most well characterised surface exposed proteins that have been demonstrated to contribute to *C. jejuni* colonisation and pathogenesis by recognising and binding to the host extracellular matrix glycoprotein fibronectin thus stimulating epithelial cell signalling pathways enabling rearrangement of the host cell actin cytoskeleton, cell invasion and intracellular multiplication causing acute disease (Ó Cróinín and Backert, 2012; Konkel et al., 2020).

The capA and capB were the first two autotransporter proteins to be identified from genome sequence of *C. jejuni* NCTC 11168. These are implicated as possible adhesins. They are known to adhere to the Caco-2 cells and result in colonisation and persistence (Young, Davis, and DiRita, 2007). The capC protein is a relatively new virulence factor of *C. jejuni* that has been revealed as an outer membrane protein that has molecular features consistent with capA and capB previously characterised autotransporter proteins and it has also been proven to play a role in adhesion to host epithelial cells contributing to infection process (Mehat et al., 2018).

The surface exposed lipoprotein JlpA is required for the efficient adherence and binding of the bacterial cell to the HEp-2 host epithelial cells. JlpA binds to the surface localised host cells Hsp90α and initiates the activation of NF-κB and p38 mitogen-activated protein MAP kinase contributing to proinflammatory host cell responses upon *C. jejuni* infection (Young et al., 2007).

The periplasmic binding proteins PEB1-4 are the known adhesions of the *C. jejuni* crucial for adherence to HeLa cells. The *ciaB* gene *(Campylobacter* invasive antigen B*)* know to be responsible for *Campylobacter* invasion, is a gene known to be involved in *Campylobacter* translocation into host cells for the purpose of host cell invasion thus contributing to caecal colonization in chicken The invasion-associated marker (*iam*) gene is responsible for *Campylobacter* invasion of host cell, the *pldA* gene is also related to cell invasion and is responsible for the synthesis of an outer membrane phospholipase that is important for caecal colonization (Wafaa, Mekky, and Enany, 2019).

1.2.5.3 Secretion Systems and Toxin Production

Cytolethal distending toxin (CDT), is the best known and characterised toxin produced by *Campylobacter* spp. It is composed of three protein subunits CdtA, CdtB, CdtC encoded by *cdtA*, *cdtB*, *cdtC* genes responsible for the expression of the toxins. These prevent the eukaryotic cells from entering mitosis thus leading to cell death. All these three *Cdt* genes are needed for the CDT holotoxin to be functionally active. In order to ensure binding of the CDT holotoxin into the cell membrane and for the *CdtB* gene to be successfully internalised and delivered into the nucleus of the host cell, both *CdtA* and *CdtC* are thought to be essential. *CdtA* and *CdtC* are mainly responsible for host-cell recognition. The DNaseI-like activity of the *CdtB* gene then breaks the double strand of the DNA and cell-cycle arrest thus inducing host DNA damage **Figure 5** (Biswas et al., 2011; Silva et al., 2011; Reddy and Zishiri, 2018).

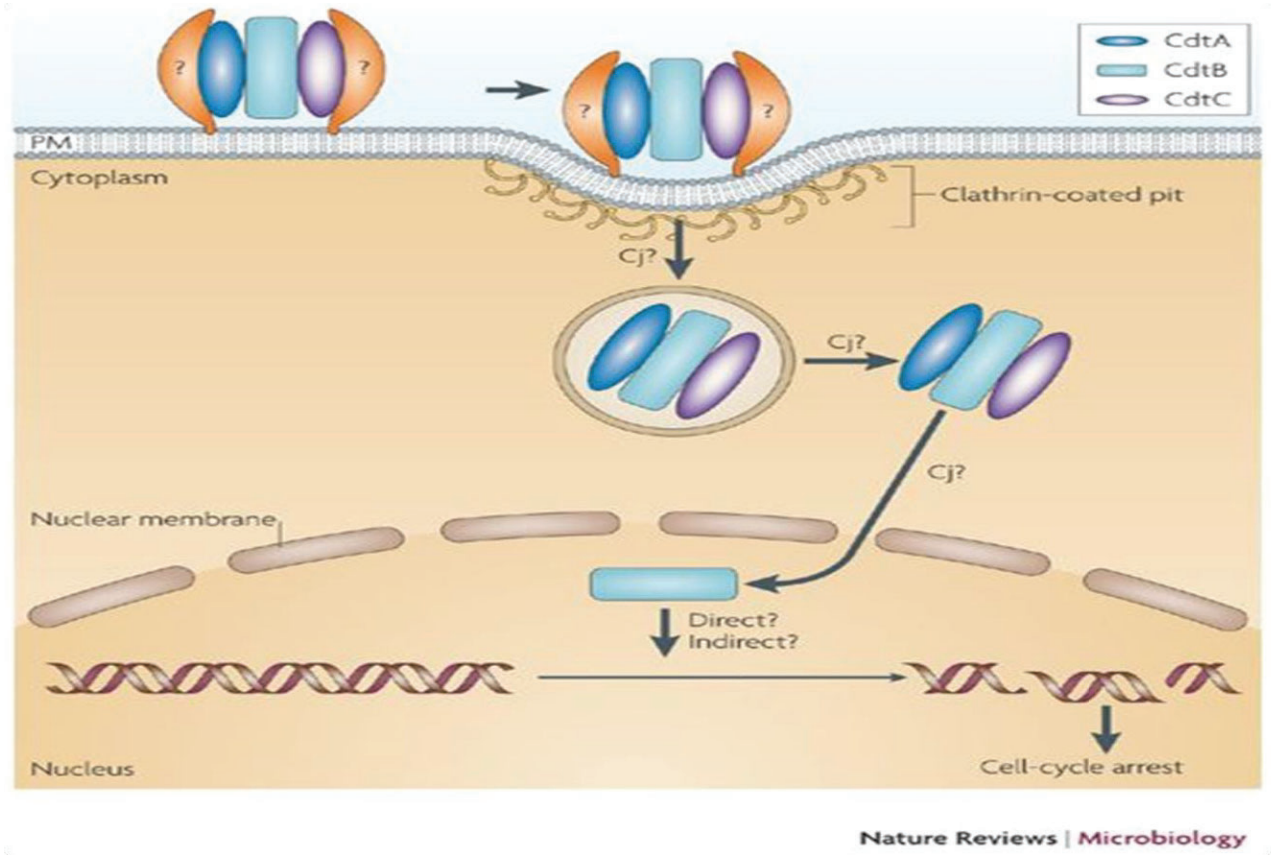

Figure 5: **Cytolethal distending toxin (CDT) mechanism observed in *Campylobacter jejuni***. The cytolethal distending toxin (CDT) holotoxin subunits, CdtA, CdtB and CdtC bind to an unknown receptor on the host cell surface. Following internalization, CdtB gets transport into the nucleus. Once in the nucleus, the toxin leads to double-strand DNA breaks and cell-cycle arrest. (Young et al., 2007) .

In both the pig and poultry environments, studies have shown that *Campylobacter* isolates most likely carried all the three cytotoxicity (*cdt*) genes although there is different distribution of the *cdtA*, *cdtB*, and *cdtC* genes among the isolates and samples (Denis et al., 2017; Reddy and Zishiri, 2018; Ngobese, Zishiri, and El Zowalaty, 2020; Pillay et al., 2020).

1.2.5.4 Survival in the Host Cell

In order to survive natural environments, *Campylobacter* spp. needs to cope with a number of stresses. *Campylobacter* must be able to defend itself against toxic compounds such as atmospheric levels of oxygen, must survive nutrient limitations, and temperature stress conditions (Bronowski, James, and Winstanley, 2014).

i. Temperature Stress Response

Campylobacter spp. encounter a wide range of temperatures in the food chain. They are exposed to temperatures of chicken in the intestine at 42ºC, human intestine at 37ºC, refrigerated foods at 4ºC and a variety of other temperatures. Temperature controls bacterial gene regulatory circuits and in order for *Campylobacter* spp. to be able to adapt and survive the different temperatures, thermoregulation has to occur, and this is possible through gene expression regulation. Thermoregulation in bacteria is achieved by three mechanisms: DNA supercoiling; changes in mRNA conformation and in protein conformation with supercoiling being the central factor in many temperature-regulated virulence regulons (Aroori, Cogan, and Humphrey, 2013). At the DNA level, the extent of DNA supercoiling is influenced by temperature thus affecting the rate of transcription and eventually thermal regulation of gene expression takes place. Regulation at the RNA level is achieved through RNA thermo-sensors which are known to offer a particularly rapid response to a change in temperature and control translation of a specific message resulting in mRNA to assume a variety of secondary and tertiary structures which have consequences on protein translation by altering interactions of the mRNA with ribosomes (Lam, Wheeler, and Tang, 2014).

The thermal stress response of bacteria consists of well-coordinated responses and processes mostly carried out by the regulated production and expression of various heat shock proteins (HSPs). These HSPs also have thermotolerance as well as other stress response functions by acting as chaperones to promote the folding of most cellular proteins and proteolysis of potentially deleterious, misfolded proteins. Several HSPs have been identified in *C. jejuni* and these include the GroESL, DnaJ, DnaK and ClpB proteins, however, dnaJ has been the most commonly identified protein in *C. jejuni* pathogenesis (Wafaa et al., 2019). A study in South Africa by Ngobese et.al (2020) detected the *dnaJ* gene in *Campylobacter* spp. isolated from different livestock production systems with percentage

isolation rate of 80% of pig faecal samples 81% in goats, 70% in chicken and 16% in cattle faeces (Ngobese et al., 2020). Pillay et al. (2020) also detected the *dnaJ* gene in a study on the characterisation of *Campylobacter* spp. isolated from poultry in KwaZulu-Natal, South Africa where she also evaluated virulence profiles of *Campylobacter* spp. isolated from the poultry farming system. The gene was detected in 70% in both *C. jejuni* and *C. coli* isolates (Pillay et al., 2020).

ii. Oxidative Stress Defence

Microaerophiles and anaerobes, unlike aerobes, live in habitats where there's favourable growth conditions e.g. in the gastrointestinal tracts of livestock where oxygen levels are slow, where there are high nutrients and optimal growth temperatures i.e. 42 ºC (Ugarte-Ruiz et al., 2018). *C. jejuni* and *C. coli* are microaerophilic foodborne pathogens that must survive and overcome harsh environmental stresses such as high oxygen tension in the atmosphere especially during the processing, transport and storage of meat products. In the presence of oxygen, bacterial growth produces reactive oxygen species (ROS) and needs to neutralise or defend itself against these compounds (high levels of oxygen and ROS) which may damage intracellular macromolecules thus leading to protein, nucleic acid, and membrane damage (Bronowski et al., 2014). To survive the increased oxidative stress conditions, *C. jejuni* has developed specific defence mechanisms including expression of a repertoire of proteins involved in the breakdown of ROS, catalase (KatA), an enzyme which decomposes hydrogen peroxide to water and oxygen, superoxide dismutase *sodB* which dismutates superoxide to hydrogen peroxide and oxygen, alkyl hydroperoxide reductase *ahpC* which converts organic peroxides to alcohols and detoxifies hydrogen peroxide and an expression of proteins that regulate the response to ROS, the PerR and MarR type transcriptional regulator proteins RrpA and RrpB (Bronowski et al., 2014; Oh, McMullen, and Jeon, 2015; Ugarte-Ruiz et al., 2018). Re-annotation and re-analysis of the *C. jejuni* NCTC11168 genome sequence revealed the presence of two MarR type family transcriptional regulators Cj1546 and Cj1556, originally annotated designated as RrpA and RrpB. An investigation demonstrated that these have been implicated in the responses to both oxidative and aerobic (O_2) stress responses enhancing bacterial survival in the environment (Gundogdu et al., 2011, 2015). *C. jejuni* possesses the peroxide-sensing regulator PerR playing an important role in oxidative stress defence, regulating the oxidative stress defence genes in *C. jejuni*. The *C. jejuni* PerR regulator is presumed to be a zinc-containing metalloprotein that exhibited differential metal ion dependency. So, PerR and iron play important roles in oxidative stress resistance (Palyada et al., 2009; Kim et al., 2011).

A study conducted by Ugarte-Ruiz et al. (2018) investigated the presence of the oxidative and aerobic stress regulators as well as the prevalence of transcriptional factors that have been implicated in controlling both oxidative and aerobic stress responses amongst all *C. jejuni* and *C. coli* wild-type

isolates. These included RrpA, RrpB PerR, Fur, CosR, CsrA, CprRS and RacRS. All *C. jejuni* strains contained the encoding genes *PerR*, *Fur*, *CosR*, *CsrA*, *CprRS* and *RacRS* that play a role in response to oxidative or aerobic stress. The majority of *C. jejuni* strains contained the oxidative and aerobic stress regulators RrpA whereas the presence of RrpB appeared more limited (Ugarte-Ruiz et al., 2018).

iii. Iron Acquisition

Micronutrients like iron and sulfur are the key essential elements for many metabolic enzymes and are essential for colonisation and infection of the host. *Campylobacter* spp. access sulfur from the host intestine by releasing cysteine containing peptide from host epithelial cells. Iron sources are usually not available as free iron but mostly bound in complexes such as sulfur-iron complex. *Campylobacter* spp. obtains the nutrient iron needed for its growth from a range of sources in the host body fluids and tissues to enable its survival and multiplication. Unlike other microorganisms that produce and release siderophores in the environment to capture and bind iron in the form of Fe^{3+}, *Campylobacter* spp. are unable to produce their own siderophores but instead they take up and use several exogenous siderophores as sources of ferric iron i.e., ferric complexes released by other bacteria. Expression and repression of genes responsible for iron uptake by cells needs to be regulated, allowing cells to acquire enough iron uptake for survival and avoid accumulation of excess iron that may lead to damaging ROS. The regulation of the genes involved in the iron uptake is controlled by the regulatory protein Fur (Miller, Williams, and Ketley, 2009; Indikova, Humphrey, and Hilbert, 2015). **Figure 6** below illustrates the different *Campylobacter* iron uptake systems, components, and sources.

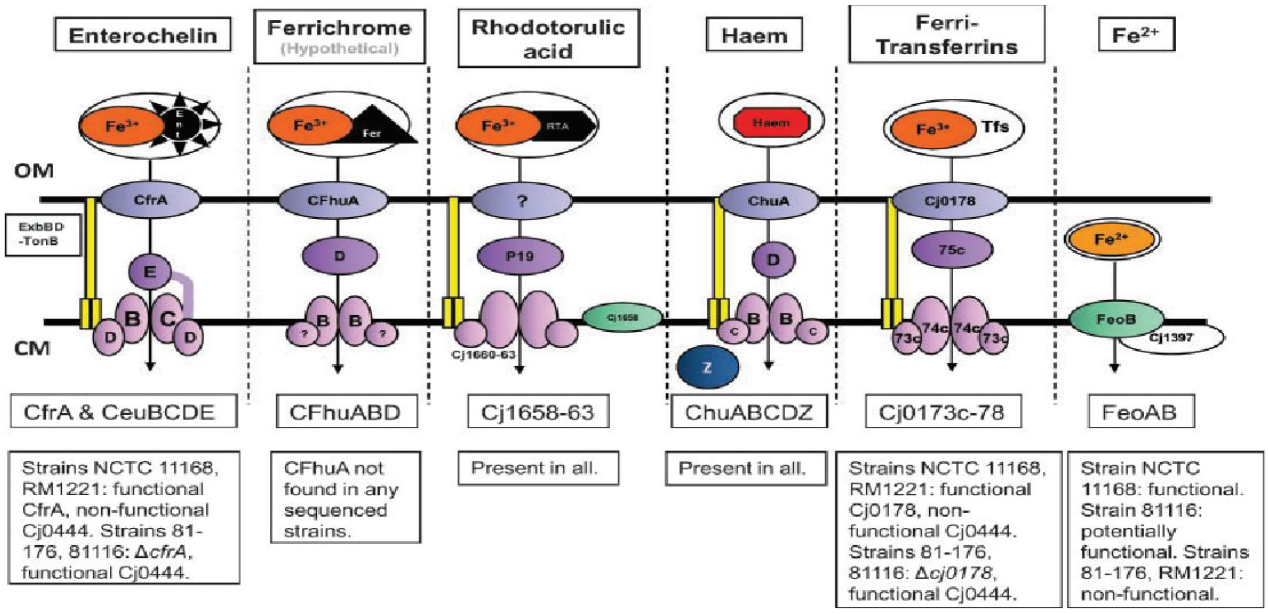

Figure 6: ***Campylobacter jejuni*** **iron uptake systems** (Miller et al., 2009). In this figure, ferric iron sources are typically bound by a surface ligand-gated porin. They then gets transported into the periplasm from the cytoplasmic membrane (CM) to the outer membrane (OM) via energy derived from proton-motive force, the energy transduction system ExbBD-TonB. Once within the periplasm, iron sources are delivered to the CM ABC transporter systems by the periplasmic binding proteins.

Ferric iron sources are bound by surface ligand-gated-porin receptors and transported into periplasm by energy transduction system ExbBD-TonB. Iron is then delivered into the cytoplasmic membrane ABC transporter systems by periplasmic binding proteins (Miller et al., 2009).

iv. Viable but non-culturable (VBNC) state: Coccoid form

Studies have shown that *C. jejuni* can respond to unfavourable conditions including aerobic conditions and low nutrient environments by entering a viable but non-culturable state (VBNC) state. The VBNC state is a state where bacteria reduce their metabolic activity but are still viable exhibiting decreased intracellular ATP levels but have the potential to recover and cause infections. In the VBNC state, bacterial cells undergo morphological changes from spiral rods and develop a coccoid morphology. Therefore, the ability of the bacteria to enter into a VBNC state is considered to be a strategy to survive under stress conditions (Bronowski et al., 2014; Oh et al., 2015).

1.2.6 Antibiotics Mechanisms of Action and Resistance in *Campylobacter* spp.

Antibiotics are regularly used in animal food production systems for the well-being of animals and to promote growth with an estimated 50-80% use in animal health, agriculture and aquaculture compared to human health globally. They are used for prophylaxis (prevention of infection), metaphylaxis (treatment of sick and healthy animals when there is an outbreak of disease), or for growth promotion as feed additives (Van et al., 2020). The European Union banned the use of antibiotics for growth promotion purposes in 2006. In the USA, the new regulations preclude the use of medically important antibiotics for growth promotion and permit only the use of these drugs for therapeutic or preventive purposes under the supervision of a veterinarian (Van et al., 2020). In South Africa, growth promoter antibiotics are still currently used in animal feed as they play an important role in preventing imbalances of gut flora that may lead to enteritis and dysbacteriosis that adversely affect gut health resulting in less efficient digestion and absorption of nutrients thus affecting animal welfare. A limited range of these growth promoter antimicrobials that are not of shared use in human medicine are used (SAVA, 2016; National Department of Health, 2018).

Table 3: Antimicrobial growth promoters (AGP) currently registered in South Africa (SAVA, 2016).

Active	**Antimicrobial group**
Zinc Bacitracin	Cyclic peptide
Bacitracin methylenedisalicylate	Cyclic peptide
Flavophospholipol	Glycolipid
Olaquindox	Quinoxaline
Avilamycin	Orthosomycin
Virginiamycin	Streptogrammin

Antibiotics works by binding to specific bacterial targets thereby inhibiting the targets normal homeostatic activity resulting in either bacteriostatic (cessation of replication) or bactericidal (killing) effects on the bacteria (Yang et al., 2019).

There are three main categories of antibiotic resistance: acquired, intrinsic and adaptive. Acquired resistance is through incorporation of exogenous genetic material for example plasmid/s carrying resistance genes. Bacteria also have their internal intrinsic resistance that they possess naturally, an example is that of the lack of bacterial target, to prevent binding. Adaptive resistance is due to an environmental trigger for example sub-inhibitory concentrations of antimicrobial agents causing changes in gene/s that will enable antibiotic tolerance (Garneau-Tsodikova and Labby, 2016).

In *Campylobacter* spp., just like in most bacterial taxa, antibiotic resistance is a result of spontaneous mutations in the genome or acquisition of antibiotic resistance genes via conjugation, transduction or transformation (Elhadidy et al., 2020). Conjugation is a mechanism of horizontal gene transfer that involves transfer of DNA through cell-to-cell contact (from donor cell to the recipient cell) via cell surface adhesins or pili. Transformation is the process of uptake of 'free' DNA directly from the environment and incorporation into bacterial genomes. Transduction is the transfer of DNA from one cell to another by viruses called bacteriophages that specifically infect bacteria (Von Wintersdorff et al., 2016).

In response to antibiotic selection pressure, bacteria have developed resistance mechanisms to survive and the mechanisms of resistance vary according to antibiotic class. *Campylobacter* spp. have developed resistance mechanisms towards antibiotics including fluoroquinolones, macrolides, β-lactams, aminoglycosides and tetracyclines (Economou and Gousia, 2015). The mechanisms that are associated with antibiotic resistance are:

i. Alteration, modification or protection of the target site.
ii. Inactivation or modification of antibiotics.
iii. Low membrane permeability and expression of antibiotic efflux pumps (Yang et al., 2019).

1.2.6.1 Fluoroquinolones

Fluoroquinolones are broad spectrum agents indicated for a range of resistant Gram-positive and Gram-negative infections including Campylobacteriosis. They are derived from the quinolone antibiotics family and are synthetic constructs. In fluoroquinolones, the eighth carbon atom of the backbone is replaced by a nitrogen atom and there is a fluorine atom addition at the sixth or seventh position giving them a broader spectrum activity (Redgrave et al., 2014; Olkkola, 2016).

The targets of fluoroquinolones are the DNA gyrase and topoisomerase IV enzymes which are encoded by two pairs of genes (*gyrA* and *gyrB*) and (*parC* and *parE*), respectively. These enzymes are involved in the modulation of DNA supercoiling required for DNA synthesis, transcription and cell division. These form covalent bonds at the DNA break forming enzyme cleaved DNA complexes called cleavage complexes. Fluoroquinolone acts by binding to these cleavage complexes in the cleavage ligation site thus blocking DNA strand re-ligation resulting in DNA synthesis inhibition leading to bacterio-stasis until cell death at high and lethal concentrations of the drug (Iovine, 2013). DNA gyrase specifically subunit A is the sole target of quinolones in *Campylobacter* spp. and therefore mutations associated with fluoroquinolone resistance in *C. jejuni* and *C. coli* mainly occur in the *gyrA* gene. Studies have shown that no mutations in *gyrB* have been linked with fluoroquinolone resistance in *Campylobacter* since the complete genome sequence of *C. jejuni* and *C. coli* revealed lack of *parC* and *parE* genes (Tang et al., 2017; Abdeltawab et al., 2019).

The main mechanism of resistance to quinolones in *Campylobacter* spp. is achieved by inactivation and modification of the fluoroquinolone target to avoid being recognised by the antibiotic and also through efflux of fluoroquinolone. The most high-level resistance is achieved by mutations within the quinolone resistance determining region (QRDR) of the gene that encode the targets of these drugs, *gyrA.* Mutations in the gene result in substitution of amino acids resulting in the alteration of the target protein structure and change in fluoroquinolone binding affinity of the enzyme. Several most common specific point mutations in *gyrA* observed in quinolone resistant *Campylobacter* spp. are: Thr-86-Ile, Asp-90-Asn, Thr-86-Lys, Thr-86-Ala, Thr-86-Val, Ala-70-Thr and Asp-90-Tyr and Ala-70-Thr (Tang et al., 2017; Abdeltawab et al., 2019). The most reported amino acid substitution in *Campylobacter* spp. affects position 86 and 90 of *gyrA* with Threonine86Isoleucine (Thr86-Ile) as the most widely described amino acid change in fluoroquinolone resistance that leads to increased resistance to nalidixic acid and

ciprofloxacin (Olkkola, 2016; Correia et al., 2017; Lluque et al., 2017). A study by Zirnstein et al. (2000) on the characterization of *gyrA* mutations associated with fluoroquinolone resistance in *C. coli* by PCR and DNA sequence analysis showed that *C. coli* isolates that were resistant to nalidixic acid and ciprofloxacin had the mutations Thr-86 to Ile (ACA to ATA) and Thr-86 to Ile (ACT to ATT) respectively in the QRDR region of the *gyrA* gene that were thought to be responsible for the increased resistance to these antibiotics (Zirnstein, 2000).

Transferable mechanisms of quinolone resistance (TMQR), alterations in cytoplasmic quinolone uptake and increased efflux by the *CmeABC* efflux pump also play a role in quinolone resistance in Gram-negative bacteria. There are six distinct families of plasmid-mediated quinolone resistance (PMQR) genes to date involved in fluoroquinolone resistance identified in other Gram-negative bacteria. The Qnr proteins coded by the plasmid genes *qnrA, qnrB, qnrC, qnrD, qnrS,* and *qnrVC* belong to the pentapeptide-repeat protein family and confer quinolone resistance by physically protecting DNA gyrase and topoisomerase IV from quinolone inhibition. These *qnr* genes are associated with transposable elements on plasmids and appear to have been acquired from chromosomal genes in aquatic bacteria. Another plasmid mediated quinolone resistance mechanism is the acetylation of quinolones with amino nitrogen substitution by a variant of aminoglycoside acetyltransferase AAC (6′)-Ib (Jacoby, Strahilevitz, and Hooper, 2014). A study conducted by Marin et al. (2020) in Eastern Spain on the molecular characterization of antimicrobial resistance in thermophilic *Campylobacter* isolated from poultry breeders and their progeny studied the presence and distribution of antibiotic resistance genes in poultry production. The qnrS gene detected at 69.6% (in,16 of 23 isolates) was thought to confer the quinolone resistance observed (Marin et al., 2020).

Quinolones are also known to use lipid mediated pathways and porins to enter into the cell. Gram-negative bacteria confer quinolone resistance through reduction of the intracellular drug concentration by either decreased uptake of the drugs, increased efflux or by both. For hydrophilic drugs that cannot diffuse into the cell due to the outer membranes' lipopolysaccharide permeability barrier, these drugs enter the cell through outer membrane porin channels. Mutations occur in these porins either through porin loss, modification of the size or porin down regulation. Loss of the expression of *OmpF*, *OmpD*, *OmpA* and *OmpC* has been associated to antibiotic resistance including quinolones. Unlike other Gram-negative organisms, *Campylobacter* however, consist of one major outer membrane protein MOMP chromosomally encoded by the *porA* gene through which nutrients and antibiotics transit. The expression of the MOMP mediates altered membrane permeability. It forms a smaller cation-selective pore that limits the entry of most antibiotics with a molecular weight greater than 360 (Iovine, 2013) and contributes to intrinsic resistance through efflux of antibiotics (Correia et al., 2017).

Efflux pump are membrane proteins located in the bacterial cell membrane that function as exporters expelling drugs form the intracellular compartments thus lowering their concentration inside the bacterial cell to sub-toxic levels (Santajit and Indrawattana, 2016). The genomic sequence of *C. jejuni* revealed four families of efflux pumps in which the membrane transporter proteins belong to: Drug/ metabolite transporters (DMT), multi antimicrobial extrusion (MATE), major facilitator superfamily (MFS), and resistance-nodulation-cell division (RND) with the RND as the most abundant pump identified in both *C. jejuni* and *C. coli*. This pump relies on a tripartite protein complex "CmeABC" that is formed from an inner membrane protein (IMP) an outer membrane protein (OMP), and a periplasmic membrane fusion protein (MFP) (**Figure 7**) (Abdeltawab et al., 2019).

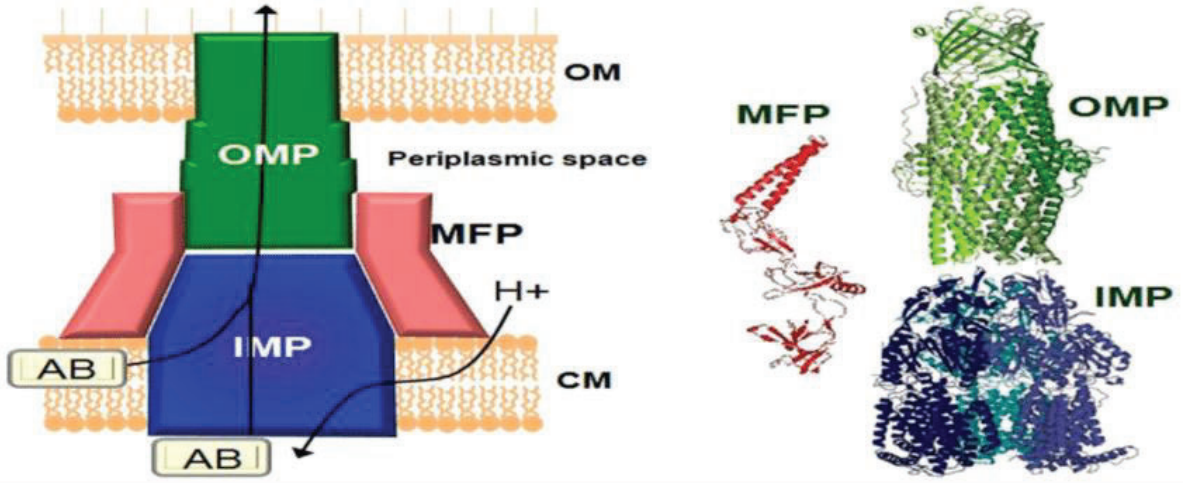

Figure 7: Structure of the resistance nodulation division (RND) superfamily which shows it's dependents on the tripartite protein complex formed from an inner membrane protein (IMP) an outer membrane protein (OMP), and a periplasmic membrane fusion protein (MFP) which connect IMP and OMP (Abdeltawab et al., 2019).

Efflux of fluoroquinolones occurs via this chromosomally encoded *Cme*ABC multidrug efflux pump resulting in decreased intracellular concentration of fluoroquinolones and other antibiotics and is the key player in antibiotic resistance (Santajit and Indrawattana, 2016). The tripartite *Cme*ABC constitute of CmeB, which is an inner membrane transporter protein, CmeA, a periplasmic membrane fusion protein, CmeC, which is an outer membrane protein, and they assemble into a tripartite efflux complex and function to expel antimicrobials out of bacterial cell. The expression of *cme*ABC is modulated by two transcriptional regulators named *cme*R and *cos*R. The *cme*R regulator controls the expression of both RND and MFS types of efflux pumps in *Campylobacters.* In RND, the *cme*R is located

immediately upstream of the *cme*ABC operon and acts as a repressor for *cme*ABC. Mutations in *cme*R or its binding site leads to overexpression of *cme*ABC and enhances resistance to drugs (Abdeltawab et al., 2019). The second transcriptional regulator which is the regulator for the oxidative stress response in *Campylobacter* regulating *cme*ABC, and *cos*R binds to a site upstream of the *cme*R binding site in the promoter region of *cme*ABC and represses the efflux operon. The *cme*R also modulates the expression of a periplasmic fusion protein (Cj0561c) and a MFS transporter (*cme*G Cj0035c) in *C. jejuni*. The *Cme*G is the only functionally characterized MFS transporter in *Campylobacters* found in all strains of *C. jejuni* and plays a role as a multidrug efflux pump inducing intrinsic resistance to different antibiotics in *Campylobacter* spp. It also plays a role of resistance to oxidative stress in *Campylobacter*. Inactivation of *cme*G significantly decreased the resistance to several classes of antimicrobials including ciprofloxacin, gentamicin, tetracycline, erythromycin, ethidium bromide, and cholic acid, while, overexpression of *cme*G enhanced the resistance to various fluoroquinolones, including ciprofloxac in, enrofloxacin, norfloxacin, and moxifloxacin (Abdeltawab et al., 2019).

In a study by Di Donato et al. (2020) that investigated the "prevalence, population diversity and AMR of *C. coli* isolated in an italian swine at slaughterhouse", several resistance genes were identified including: *gyr*A, *tet*O, *cme*A, *cme*B, *cme*C and *cme*R. Sequencing of the QRDR region of the *gyr*A gene showed presence of mutations in the genome of all human isolates and in 64.81% of the pig isolates. *Tet*O gene was identified in 72.72% and in 88.88% of human and pig isolates, respectively. The *Cme*ABC multidrug efflux pump with its *cme*R regulator gene was identified in every human and pig strain analysed, covering a range between 99.38% and 100% (Di Donato et al., 2020). Yao et al. (2016) identified a more resistance enhancing *Cme*ABC variant (RE-*Cme*ABC) that confers enhanced resistance to multiple antimicrobials. In the study the RE-*Cme*ABC variant was shown to be especially prevalent in *C. jejuni* in chickens and swine in China. The study also showed that RE-*Cme*ABC is easily transferred between *Campylobacter* isolates by natural transformation and so, it is highly expected that RE-*CmeABC* will increase in prevalence from antimicrobial use (Yao et al., 2016).

1.2.6.2 Macrolides

Macrolides are products produced by Streptomyces bacteria. They are widely used antibiotic agents that are effective against Gram-positive and Gram-negative bacteria including *Campylobacter* spp. In *Campylobacter* infection, macrolides such as erythromycin are often considered the drug of choice for the clinical therapy. Macrolides act by inhibiting protein elongation and protein synthesis through interaction with the 50s subunit of bacterial ribosome by binding to 23S rRNA nucleotides which are stabilised by ribosomal proteins L3, L4, L22 and L34 thus resulting in the change in the ribosome and termination of elongation of peptide chain (Iovine, 2013; Wieczorek and Osek, 2013).

The main mechanisms of resistance to this class of antibiotics in *Campylobacter* spp. are: target site modification through mutations in the *23SrRNA* genes, mutations in the ribosomal proteins L4 and L22 encoded by *rpID* and *rpIV* genes, ribosomal methylation encoded by an acquired ribosomal RNA methylase gene (*erm*B); antibiotic efflux from bacterial cell and altered membrane permeability mediated by expression of the major outer membrane porin (MOMP) chromosomally encoded by *porA* (Tang et al., 2017; Abdeltawab et al., 2019). Point mutations in the peptidyl encoding region in domain V of the *23S rRNA* gene at positions 2074 and 2075 confer high macrolide resistance levels with the 2075 substitution being more common (Iovine, 2013; Bolinger and Kathariou, 2017). Suk-Kyung et al. (2016) conducted a study on macrolide resistant mechanisms focusing on erythromycin-resistant *Campylobacter* spp. in Korea where they investigated the molecular resistance mechanisms focusing on region V of the *23S rRNA* gene, the *rplD* (L4) and *rplV* (L22) genes as well as the presence of virulence factors in erythromycin resistant *C. jejuni* and *C. coli* strains isolated from animals and carcasses. Sequence analysis of the 316bp amplicon of the *23S rRNA* gene showed a single point mutation A2075G transition in all highly erythromycin resistant isolates in the *23SrRNA* gene. The study also reported several amino acid substitutions in the *rplD* and *rplV* genes encoding the L4 and L22 ribosomal proteins in *C. jejuni* and *C. coli* strains (Suk-Kyung et al., 2016). Mutations in the 50S ribosomal sub unit L4 protein encoded by *rplD* gene also confer high-level macrolide resistance in other bacterial species (Corcoran et al., 2006).

Liu et al. (2019) analyzed the prevalence of *erm*(B)-harboring multidrug resistance genomic islands (MDRGIs) in *Campylobacter* strains isolated from pigs and chickens in Guangzhou, Shanghai, and Shandong; China, for better understanding of macrolide resistance in *Campylobacter.* Seventy-four *erm*(B)-positive isolates (72 *C. coli* and 2 *C. jejuni*), were isolated from chicken samples, and no *erm*(B)-positive isolates were obtained from swine samples (Liu et al., 2019).

A *CmeABC* and *CmeG* efflux pump also contributes to macrolide resistance notably erythromycin in *Campylobacter* spp. A study by Elhadidy et al. (2018) conducted a study on a set of *C. jejuni* strains isolated from broiler carcasses in Belgium. In the study, characterisation of the clonal population structure, analysis of antimicrobial resistance profiles and occurrence of antimicrobial resistance determinants was studied. The study was done to understand the molecular and phenotypic basis of antibiotic resistance in *C. jejuni* including antimicrobial resistance mechanisms and the identification of particular clones associated with the resistance genes carriage and spread. The locus mutations *23S rRNA*, *rplD, rplV* and *cmeRABC* previously associated with erythromycin resistance in *Campylobacter*

were studied among the erythromycin-resistant strains in the study. Mutations were identified in the cmeR-cmeA intergenic region and also observed in *CmeR* (Elhadidy et al., 2018). Hao et al. (2013) conducted a study on the "mutational and transcriptomic changes involved in the development of macrolide resistance in *C. jejuni"* to get an understanding of the molecular processes leading to the generation of high-level resistance in *Campylobacter*. In this study, the dynamic changes in target gene mutations, expression of antibiotic efflux genes, and the global transcriptome were analysed in the context of macrolide resistance. Multiple mutations were observed in the target genes, *23S rRNA* gene, ribosomal proteins L4 and L22 genes, and *cmeR*. Moreover, the overexpression of *cmeABC* in the mutant selection process was partly explained by the point mutations H174N and a deletion (del490A) in CmeR. The del490A nucleotide deletion would cause a frameshift and truncation of the CmeR open reading frame, which may explain the overexpression of *cmeB.* The H174N substitution in CmeR also occurred concomitantly with the overexpression of *cmeB*, suggesting that the H174N mutation likely affects the function of CmeR. The enhanced expression of the efflux genes is required to facilitate the development of highly resistant mutants, in which mutations in the *23S rRNA* gene or other changes stabilize the resistance phenotype (Hao et al., 2013).

The contribution of *CmeG* to antibiotic resistance including erythromycin resistance in *Campylobacter* was also studied by Jeon et al. (2010) where the contribution of *CmeG* to antibiotic and oxidative stress resistance in *C. jejuni* was investigated. It was observed that *CmeG* mutations rendered *C. jejuni* more susceptible to various antibiotics including erythromycin and that this efflux pump functions as a multidrug efflux transporter contributing to MDR in *Campylobacter* spp. (Jeon et al., 2010).

1.2.6.3 β-Lactams

β-Lactams are a class of antibiotics including penicillins, carbapenems, cephalosporins, cephamycins and monobactams that all have a β-lactam ring required for antibiotic activity (Iovine, 2013). Their primary targets are the penicillin binding proteins (PBPs). They interact with PBPs which are responsible for the cross-linking of the peptidoglycan chains in the cell wall making them unavailable for the synthesis of new peptidoglycan and this thus inactivates bacterial peptidoglycan trans-peptidases required to make the bacterial cell wall structure. The cell wall thus lacks structural integrity which results in osmotic swelling and lysis until cell death (Iovine, 2013; Kapoor, Saigal, and Elongavan, 2017).

Mechanisms of resistance to these drugs include:

i. Enzymatic inactivation by β-lactamases through presence of class D β-lactamase *OXA-61* which was identified in *Campylobacter*. These enzymes modify and inactivate the antibiotics by hydrolysing the β-lactam ring (Santajit and Indrawattana, 2016).

ii. Alteration of the OMPs for reduced uptake. The cation-selective OMPs exclude most β-lactams that are anionic and with molecular weight higher than 360 (Stones, 2011; Iovine, 2013).
iii. Efflux (*CmeABC*) being the potent efflux pump to preclude β-lactams entry into the bacterial cell (Iovine, 2013).

β-lactamases are enzymes with structural similarity to PBPs with higher affinity for β-lactam antibiotics. They hydrolyse β-lactams with an ester and amide bond and the functionality of the drug is lost. β-lactamases are classified using two classification systems, i.e., the Ambler (structural) and Bush–Jacoby–Medeiros (functional) classification systems (Kapoor et al., 2017). The structural classification is based on the protein sequence that classifies β -lactamases into class A, C, and D enzymes which utilize serine for β -lactam hydrolysis and class B metallo-enzymes which require divalent zinc ions for substrate hydrolysis. The functional classification scheme takes into account substrate and inhibitor profiles in an attempt to group the enzymes in ways that can be correlated with their phenotypes in clinical isolates (Bush and Jacoby, 2010).

C. jejuni can produce more than one type of β-lactamase. Four distinct molecular β-lactamases classes have been identified (Ambler, 1980). The most prevalent β-lactamases of *C. jejuni* are class A β-lactamases, also known as penicillinase-type of β-lactamases. They are expressed in *Campylobacter* spp. and confer resistance to ampicillin, penicillin, amoxicillin, carbenicillin and ticarcillin but can be overcome by β-lactamase inhibitors clavulanic acid, tazobactam and sulbactam (Stones, 2011; Iovine, 2013).

Class B β-lactamases also called metallo β-lactamases are unique group of β-lactamases that differ structurally from the other β -lactamases by their requirement for a zinc ion at the active site. They are the class of enzymes with the ability to hydrolyse carbapenems but however, are not inhibited by clavulanic acid or tazobactam and instead are inhibited by metal ion chelators such as EDTA, dipicolinic acid, or 1,10-o-phenanthroline (Bush and Jacoby, 2010). Class C β-lactamases also called cephalosporinases hydrolyse cephalosporins including broad spectrum cephalosporins and confer resistance to all β-lactams with the exception of carbapenems and are usually also resistant to inhibition by clavulanic acid or tazobactam (Bush and Jacoby, 2010).

Class D β-lactamases are oxacillin-hydrolysing enzymes conferring resistance to penicillin, cloxacillin, oxacillin, and methicillin. They are weakly inhibited by clavulanic acid (Kapoor et al., 2017). OXA-61 is a class D β-lactamase that is known to contribute to resistance to β-lactams in *C. jejuni* as previously

noted by Alfredson and Korolik (2005) in their study on the "Isolation and expression of a novel molecular class D-lactamase, OXA-61, from *C. jejuni*" in Australia (Alfredson and Korolik, 2005).

A study by Stones (2011) on "β-lactam resistance in *Campylobacter*" showed that β-lactam resistance is a multi-faceted mechanism integrating various chromosomally encoded β-lactamases, putative transferable β-lactamases and efflux. The study showed that upon inactivation of the efflux pump gene *cmeB* in the reference strain NCTC11168, an increased susceptibility to a number of β-lactams including the cephalosporins by which *Campylobacter* are reported to be resistant to was noted. Data from the study suggested that the efflux pump *CmeABC* confers the innate resistance of *Campylobacter* to this class of drugs. The product of the *CjBla2* gene identified in the study was observed to encode oxacillinase (Stones, 2011).

1.2.6.4 Tetracyclines

Tetracyclines are inhibitors of protein synthesis and they gain access to bacterial ribosome through outer membrane OmpF, OmpC porin channels of Gram-negative enteric bacteria using the hydrophobic pathway (Iovine, 2013). Tetracycline binds to Mg2+ positively charged cations in order to pass through the porins in the outer membrane and once in the periplasmic space the tetracycline molecules dissociate from magnesium into the cytoplasm where they bind to the ribosomal 30s subunit site inhibiting protein synthesis necessary for growth and survival of bacteria by preventing aminoacyl-*tRNA* from attaching to ribosomal A site thus hindering the elongation phase of protein synthesis (Wieczorek and Osek, 2013). They have activity against both Gram-positive and Gram-negative microorganisms (Iovine, 2013).

Resistance to tetracycline is attributed to acquisition and distribution of genetically mobile tetracycline resistance (*tet*) genes. Mechanisms of resistance to tetracycline reported in *Campylobacter* spp. include protection of the ribosomal binding site by ribosomal protection protein encoded by *tetO* gene that recognises an open A site in the ribosome and binds to it causing a conformational change allowing the release of the bound tetracycline molecule (Tang et al., 2017). The efflux protein genes, *tetA* and *tetB*, that code for membrane-associated proteins that function to export tetracycline from the cell also confer tetracycline resistance (Sheykhsaran et al., 2019). A number of studies have evaluated the presence of these resistance determinants in *Campylobacter* spp. Tetracycline resistance genes in *C. jejuni* and *C. coli* isolated from poultry carcasses were investigated in a study by Abdi Hachesoo et al. (2014) in Shiraz, Iran. The most common *tet* gene identified was *tet* (O), found in 83.1% of all the isolates, with *tet* (O) seen in 92.5% of *C. coli* isolates and 74.4% of *C. jejuni* isolates. *tet* (A) was found in 18% of *Campylobacter* spp. (Abdi Hachesoo et al., 2014). A study in the Eastern Cape, South Africa that

evaluated resistance gene determinants in *Campylobacter* spp. isolated from retailed meat carcasses detected the *tetA* (68.82%) *tetB* (12.90%), *tetM* (38.71%) tetracycline resistance genes (Igwaran and Okoh, 2020).

Efflux mediated by the multi drug efflux pump (*CmeABC*) is also another tetracycline resistance mechanism used by *Campylobacter* spp. (Tang et al., 2017). A study conducted by Pumbwe and Piddock (2002) demonstrated that *CmeB* contributes to intrinsic resistance in *C. jejuni*. The results of the study suggested that inactivation of the *CmeABC* efflux pump by disruption of the *cmeB* gene by insertional mutagenesis led to a resultant knockout strain (NCTC 11168-*cmeB: kanr*) displaying a 2-4-fold increase in susceptibility to a range of antibiotics that included β-lactams, fluoroquinolones, macrolides, chloramphenicol, tetracycline and to ethidium bromide, acridine orange and *N*-lauryl sulfate (SLS). The studies also suggested that when both *CmeABC* and *tetO* are functional, the impact on tetracycline resistance is synergistic (Pumbwe and Piddock, 2002).

1.2.6.5 Aminoglycosides

Aminoglycosides are positively charged protein synthesis inhibitors of Gram-negative and positive organisms. They initially bind to negatively charged bacterial membranes and also bind to the bacterial A-site located on the *16S rRNA* of the 30s segment of ribosomes for transfer across the cytoplasmic membrane. Aminoglycosides are bactericidal and disturb translocation of the nascent peptide chain from A site to P site of the ribosome resulting to premature termination of protein synthesis. They also inhibit protein synthesis by interfering with proof-reading leading to incorporation of incorrect amino acids and dysfunctional protein (Iovine, 2013). Aminoglycosides are broad spectrum antibiotics that are used in the treatment of Gram-negative and some Gram-positive infections including *Campylobacter* bacteraemia (Olkkola, 2016).

The most common mechanisms of resistance to aminoglycosides are:

i. Mutations at the ribosomal target sites, mainly in the *rrs* gene that codes for *16S rRNA* to hinder aminoglycoside binding (Olkkola, 2016).
ii. Enzymatic modification of the aminoglycosides binding site (A-site on *16S rRNA*) by *16SrRNA* methyl transferases. These enzymes are produced by bacteria to methylate their *16S rRNA* (Olkkola, 2016).
iii. Inactivation of the aminoglycosides by aminoglycoside modifying enzymes (AMEs) which are divided into three sub-classes based on the modification they apply, i.e., aminoglycoside phosphotransferases (*APH*) types I (*aphA-1*), III (*aphA-3*), and VII (*aphA-7*), aminoglycoside acetyl transferases (*AAC*), and 6-aminoglycoside nucleotidyl/adenyl transferases (*ANT*). They

function by chemical modification of the aminoglycoside at specific positions either by phosphorylation, acetylation or adenylation of the amino group of the antibiotic molecule leading to inactivation and reduced aminoglycoside affinity for rRNA A-site (Fabre et al., 2018).

iv. The bacterial cell wall also serves as an intrinsic barrier through its impermeability causing repulsion of the of aminoglycoside and even if the aminoglycoside were to enter the cell, these would be expelled out of the cell by *CmeABC* efflux pump (Garneau-Tsodikova and Labby, 2016) .

The widely spread *ant (6)-I* genes encoding aminoglycoside O-nucleotidyltransferases among streptomycin resistant strains of *C. jejuni* and *C. coli* of human and animal origin were studied by Hormeño, Ugarte-Ruiz, Palomo, *et al.*(2018) in Spain. Genes encoding ANT (6)-I enzymes were detected in both human and animal species with *C. coli* being the most prevalent species among streptomycin resistant isolates to have these genes. Another study was conducted in Lisbon, Portugal on whole genome sequence analysis to identify the molecular determinants associated with to several antibiotics in multidrug-resistant *C. jejuni* and *C. coli* isolates, with a focus on aminoglycoside resistance determinants. The *Campylobacter* isolates had MDR profiles and were particularly resistance to gentamicin, kanamycin, tobramycin, spectinomycin and streptomycin. The putative aminoglycoside resistance genes were *aph*(*2″*) found in all except one gentamicin resistant isolate, *aph*(*3′*) found in 75.0% kanamycin resistant isolates, *ant*(*6*) found in 58.3% streptomycin resistant isolates and *ant*(*9*) found in 58.3% spectinomycin resistant isolates (Fabre et al., 2018).

1.2.7 Control Measures and Treatment Challenges

Antibiotic use for prevention and treatment of disease is a common practice in pig farms in periods where there is stressful periods of umbilical cord cutting canine trimming, castration of males, overcrowding, high temperatures and inadequate aeration (Economou and Gousia, 2015).

Currently, treatment and control of *Campylobacter* spp. and Campylobacteriosis in food animals including swine is traditionally through use of the veterinary antibiotics including streptomycin, gentamicin, erythromycin, ampicillin, fluoroquinolones; ciprofloxacin, tetracyclines and (clindamycin) (OIE, 2019). However, overuse of antibiotics has led to antibiotic-resistant strains and resistance to some of these drugs have been documented in *Campylobacter* spp. as discussed above. This has been a challenge in treatment thus far. The WHO published a global priority list of most important antibiotic resistant bacteria including *Campylobacter* for which there is an urgent need for new treatments. Pathogens were stratified into three priority tiers: critical, high, and medium.

Campylobacter spp. is included on the prioritization list under priority 2 tier (high) for which innovative new treatments are urgently needed. *Campylobacter* spp. were noted as responsible for foodborne diseases and the major cause of acute diarrohea. Increasing resistance to antibiotics was observed in both high-income and low-and middle-income countries (WHO, 2017b).

In addition to treatment of *Campylobacter*, preventive approaches in all stages of the food chain through good hygiene practices, enhanced biosecurity, and bactericidal treatment such as heating and prospective faecal testing to ascertain shedding status are other important control measures. There are also proposed treatments that have been pursued in agriculture to prevent transmission and persistence which include:

i. Development of small molecule *Campylobacter* growth inhibitor compounds that can be directed against specific processes known to contribute to colonization or they can be developed as narrow-spectrum growth inhibitors.
ii. Use of probiotics and prebiotics - live organisms that are administered and confer beneficial effect on the health of its host.
iii. Use of bacteriophages which are viruses that infect bacteria and have a lytic activity. They use protein receptors to bind and penetrate bacterial cells and multiply within cytoplasm till bacterial death.
iv. Use of vaccines that will be directed against the bacteria which involve administration of a product that can induce immunity and confer immune memory thus enabling rapid defences in case of contamination by bacteria.
v. Anti-*Campylobacter* bacteriocins which are antimicrobial compounds synthesised by other bacteria that are administered in animal feed and have the potential to reduce *Campylobacter* colonisation in animals. They work by targeting and reducing the viability of closely related bacteria (Federighi, 2017; Johnson, Shank, and Johnson, 2017; Hansson et al., 2018).

The poultry and the pig production environments from different countries have been implicated in the emergence and spread of resistant and virulent *Campylobacter* spp. Reservoirs of *Campylobacter* spp. in the food production present a risk for human infection through consumption of contaminated animal food products and contaminated environments. No studies have been conducted to investigate the burden of *Campylobacter* along the farm-to-fork continuum advocated by the World Health Organization Advisory Group on Integrated Surveillance of Antimicrobial Resistance (WHO-AGISAR) as one of the appropriate methods in monitoring foodborne pathogens. Thus, this study will

provide data collected along the farm-to-fork continuum in the pig production system that can be used to inform prevention and control strategies for *Campylobacter* infections as well as identify gaps for further in-depth research in the country.

The FAO, OIE and WHO global reports have identified that AMR is a global public health concern that is influenced by the use of antibiotics in both human and animals (OIE, 2019). Objective 2 (*Strengthen the knowledge and evidence base through surveillance and research*) of the WHO global report on "antimicrobial resistance surveillance" points out the gaps that exist in surveillance and data sharing related to antibiotic resistance in foodborne bacteria and its impact on both animal and human health (WHO, 2015b). The literature has proven that the swine food production systems are contaminated with resistant strains of *Campylobacter* organism. This study will also contribute data on prevalence and resistance profiles of *Campylobacter* spp. in the KZN food intensively produced pigs across the farm to fork continuum. This study ascertained the prevalence, antibiotic resistance profiles, selected resistance and virulence genes as well as clonal relatedness within the *Campylobacter* species from intensively reared pigs in the KZN region.

1.3 Study Rationale

Studies have revealed that *Campylobacter* is a leading foodborne pathogen which causes gastroenteritis in humans. Knowledge of the prevalence and associated risk factors caused by these bacteria in South Africa and their antimicrobial susceptibility patterns is thus necessary. This study investigated antibiotic resistance and prevalence of *Campylobacter* spp. in swine isolates from a pig production farm in KwaZulu-Natal, South Africa.

1.4 Aim

The aim of the study was to determine the molecular epidemiology of antibiotic resistant *Campylobacter* spp. from farm-to-fork in intensively produced pigs in the uMgungundlovu district in KwaZulu-Natal, South Africa.

1.5 Objectives

i. To collect samples from different sampling points along the farm-to-fork continuum, i.e., the farm, transport, abattoir and retailed meat as well as the hands/nares of farm workers on explicit voluntary informed consent.

ii. To identify and confirm *Campylobacter* spp. using:

- Modified charcoal cefoperazone deoxycholate (mCCDA) blood-free agar (Oxoid, England) containing *Campylobacter* selective supplement SRO155.

- Tryptose agar plates supplemented with 5% defibrinated sheep blood for further purification of the isolates.
- Real time Polymerase chain reaction (RT-PCR) to confirm *Campylobacter* to genus and species level using genus and species-specific genes *16SrRNA*, hippuricase gene (*hipO*) and the aspartokinase gene *(asp*) *genes* for *C. jejuni* and *C. coli* respectively.

iii. To assess susceptibility profiles of *Campylobacter* spp. using the Kirby-Bauer disk diffusion method against the WHO-AGISAR recommended panel of antibiotics for *Campylobacter* spp. according to European Committee for Antimicrobial Susceptibility Testing (EUCAST) and Clinical and Laboratory Standards Institute guidelines (CLSI).

iv. To detect the presence of selected resistance genes (*gyrA, TetO, 23SrRNA, Bla*$_{OXA-61,}$ and *CmeB*) and virulence factors (*cadF*, *ciaB, pldA, dnaJ*), cdtA, *cdtB*, and *cdtC* by PCR.

v. To detect mutations, if any in the *gyrA* and *23SrRNA* genes by DNA sequencing of the resistance genes identified by PCR.

vi. To ascertain clonal relatedness between isolates through strain typing by ERIC-PCR.

1.6 Overview of Methodology

- ***Ethical considerations***

Ethical approval was obtained from the Animal Research Ethics Committee (Reference: AREC 007/018) and the Biomedical Research Ethics Committee (Reference: BCA444/16) of the University of KwaZulu-Natal. Permission to conduct the study was also obtained from the South African National Department of Agriculture, Forestry and Fisheries (Reference: 12/11/1/5 (879).

Human samples were obtained from participants 18 years or older upon explicit, voluntary, verbal informed consent as per the participant information leaflet (Appendix 4). All information disclosed by the farm was treated as confidential as per the memorandum of understanding (MOU) between the Antimicrobial Research Unit (ARU) and the farm.

This study investigated the molecular epidemiology of antibiotic-resistant *Campylobacter* spp. from farm-to-fork in intensive pig production system in uMgungundlovu district municipality in KwaZulu-Natal, South Africa. The experimental part of the study was conducted at the University of Kwazulu-Natal (UKZN) at the ARU, in the School of Health Sciences. Samples along the pig farm-to-fork continuum (farm: litter, faeces, slurry and farm workers; transport: holding crates; abattoir: carcass swab, carcass rinsate and caeca; and retail meat: head, body and thigh) were evaluated for the presence of *Campylobacter* spp. Molecular confirmation by PCR, targeting the genus *Campylobacter* spp.

(*16SrRNA*) and species-specific *C. coli* and *C. jejuni* (*asp* and *hipO*) genes respectively was undertaken. Susceptibility profiles were assessed using the Kirby-Bauer disk diffusion method against the WHO-AGISAR recommended panel of antibiotics for *Campylobacter* spp. Selected antibiotic resistance and virulence genes were detected using real-time PCR. Genetic relatedness between isolates across the continuum was evaluated by ERIC-PCR.

1.7 Dissertation Structure

The study is presented in three chapters as follows:

Chapter 1 provides the introduction, literature review, rationale for the study as well as the aims and objectives.

Chapter 2 provides information about the investigations undertaken and the findings that are presented in the form of a manuscript prepared for Science of the Total Environment journal.

Chapter 3 presents the conclusions, limitations, recommendation and significance of the study.

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CHAPTER TWO

This dissertation is in a manuscript format as follows:

Viwe Sithole, Daniel G. Amoako, Akebe Luther King Abia, Linda A. Bester, Sabiha Y. Essack; Molecular epidemiology of antibiotic-resistant *Campylobacter* spp. from farm-to-fork in an intensive pig production system in KwaZulu-Natal, South Africa. To be submitted to Science of the Total Environment.

AUTHOR CONTRIBUTIONS

Viwe Sithole, as the principal investigator, co-conceptualized the study, undertook the laboratory work and data collection, analysed the data and drafted the manuscript.

Daniel G. Amoako, as principal supervisor, co-conceptualized the study, guided the literature review, supervised the laboratory work, facilitated data collection and analysis, vetted the results and undertook critical revision of the manuscript.

Akebe L. K. Abia, as co-supervisor, co-conceptualized the study, supervised the laboratory work, facilitated data collection and analysis, vetted the results and undertook critical revision of the manuscript.

Linda A. Bester, as the co-supervisor, co-conceptualized the study, supervised the laboratory work and undertook a critical revision of the manuscript.

Sabiha Y. Essack, as co-supervisor, co-conceptualized the study, guided the literature review and ethical clearance application, facilitated data collection and analysis and undertook critical revision of the manuscript.

Molecular Epidemiology of Antibiotic Resistant *Campylobacter* spp. from Farm to Fork in an Intensive Pig Production System in KwaZulu-Natal, South Africa

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ABSTRACT

Background: *Campylobacter* spp. are among the leading foodborne pathogens, causing Campylobacteriosis, a zoonotic infection that results in bacterial gastroenteritis and diarrheal disease in animals and humans. The emergence and transmission of antibiotic resistance and virulence in *Campylobacter* spp. is increasingly reported. We investigated the molecular epidemiology of antibiotic resistant *Campylobacter* spp. isolated across the farm-to-fork-continuum in an intensive pig production system in the uMgungundlovu District, Kwazulu-Natal, South Africa.

Methodology: Following ethical approval, samples were collected over a period of sixteen weeks from selected critical points (farm, transport, abattoir and retail) using a farm-to-fork sampling approach according to WHO-AGISAR guidelines. Overall, 520 samples were investigated for the presence of *Campylobacter* spp. which were putatively identified using selective media with identity and speciation confirmed by polymerase chain reaction (PCR) of specific genes. Resistance profiles were ascertained by the Kirby-Bauer disk diffusion method according to EUCAST and/or CLSI guidelines. Selected antibiotic resistance and virulence genes were identified using PCR and DNA sequencing. Clonal relatedness among the isolates was determined using enterobacterial repetitive intergenic consensus polymerase chain reaction (ERIC-PCR).

Results: Altogether, 378/520 (72.7%) samples were positive for *Campylobacter* spp. with *C. coli* as the most predominant (73.3%), followed by *C. jejuni* (17.7%) with 9.0% classified as "other". Relatively high levels of resistance were observed in *C. coli* and *C. jejuni* to erythromycin (89% and 99%), streptomycin (87% and 93%), tetracycline (82% and 96%), ampicillin (69% and 85%), and ciprofloxacin (53% and 67%) respectively. The lowest percentage resistance observed was for gentamicin (12%) for both *C. coli* and *C. jejuni*, and nalidixic acid (28% and 27%) for *C. coli* and *C. jejuni* respectively. Multi-drug resistance (MDR) was noted among 330/378 (87.3%) isolates. The antibiotic resistance genes observed were the *tetO* (74.6%), the $bla_{OXA\text{-}61}$ (2.9%) and *cmeB* (11.1%) accounting for the resistance to tetracycline and ampicillin while the membrane efflux pump could confer resistance to ampicillin, tetracycline, ciprofloxacin, and erythromycin. All *C. coli* and *C. jejuni* isolates (21) with the *gyrA* gene exhibited mutation at the Thr-86-Ile region in the quinolone-resistance-determining region (QRDR) and all *C. coli* and *C. jejuni* isolates (18) exhibiting erythromycin resistance showed common transitional mutations A2075G and A2074C in the *23S rRNA* gene. Of the virulence genes tested, *ciaB*, *dnaJ*, *pldA*, *cdtA*, *cdtB*, *cdtC* and *cadF* were detected in 48.6%, 61.1 %, 17.4%, 67.4%, 19.3%, 51% and 5% of all *Campylobacter* isolates respectively. The ERIC-PCR banding patterns revealed that isolates along the continuum were highly diverse with isolates from the same sampling points belonging to the same major ERIC-types.

Conclusion: We showed relatively high levels of resistance to antibiotics commonly used in intensive pig production in South Africa with some evidence, albeit minimal, of transmission across the farm-to-fork continuum. This together with the virulence profiles present in *Campylobacter* spp. presents a challenge to food safety and a potential risk to human health. This is further exacerbated by the reduction in antibiotic treatment options necessitating routine surveillance and monitoring together with antibiotic stewardship, comprehensive biosecurity, and good animal husbandry in intensive pig production.

Keywords: Antibiotic-resistance; *Campylobacter* spp.; multidrug resistance; farm-to-fork; intensive pig production.

1. Introduction

Antibiotic resistance (ABR) is a global public health crisis. It can be spread through food systems by movement of livestock and agricultural produce within and between countries together with human travel (Robinson et al., 2016). Antibiotic resistance is attributed to overuse and misuse of antibiotics, incorrectly prescribed antibiotics, and long exposure periods, particularly in food animals. Antibiotics are widely used as growth promotors, for prophylaxis, metaphylaxis and treatment in food animal production systems. This extensive use and long exposure periods creates ways for bacteria to entrench genes that confer resistance to the drugs, providing opportunities for the transfer of this resistance to human gut microbiota through consumption of contaminated food, direct contact with animals, via the environment and via occupationally-exposed workers (Ventola, 2015; Robinson et al., 2016).

Campylobacter spp. are prevalent in food animals including pigs, poultry, cattle, goats, and sheep (Karikari et al., 2017; Igwaran and Okoh, 2020; Pillay et al., 2020). The gastrointestinal tract of food producing animals serves as a reservoir of *Campylobacter* spp. which are shed through faeces. They are enteric pathogens that are the leading cause of gastroenteritis, specifically Campylobacteriosis, a bacterial foodborne diarrheal disease in man and animals. The most frequently encountered thermotolerant *Campylobacter* spp. in foodborne and waterborne infections are *C. jejuni*, *C. coli* and some *C. lari* (Frasao et al., 2017; Heredia and García, 2018).

Streptomycin, gentamicin, erythromycin, ampicillin, ciprofloxacin, and tetracycline are classified as critically important drugs for food animals by OIE to protect animal health and welfare, contribute to food safety, and ensure public health (OIE, 2019). However, there is an associated risk of the development and/or escalation of antibiotic resistance to these antibiotics should they be excessively used and/or misused.

Sampling considerations at different points along the food animal production continuum is of utmost importance as it provides data on both antimicrobial use and resistance and identifies transmission (if any) along the food chain. Sampling at the production site e.g., farms will give indication of resistance arising from on farm antimicrobial use. Transport and holding pens reflect what is expected to contaminate retail meats and includes cross contamination with strains persisting in the environment. Post slaughter addresses the contamination of meat during in plant processing while retail meat products may reflect cross contamination during handling (WHO, 2017a).

The purpose of the study was to describe the molecular epidemiology of antibiotic resistant *Campylobacter* spp. from farm-to-fork in intensively produced pigs in the uMgungundlovu district in KwaZulu-Natal, South Africa.

2. Methodology

2.1 Ethical Considerations

Ethical approval for this study was received from the Animal Research Ethics Committee (Reference: AREC 007/018) and the Biomedical Research Ethics Committee (Reference: BCA444/16) of the University of KwaZulu-Natal. We also obtained Section 20A permission to conduct the study from the South African National Department of Agriculture, Forestry and Fisheries (Reference: 12/11/1/5) All information obtained from the farm was kept confidential as part of the memorandum of understanding (MOU) between the Antimicrobial Research Unit (ARU) and the farm.

2.2 Study site

The study was carried out at an intensive pig production facility in the uMgungundlovu district municipality in KwaZulu-Natal (KZN), South Africa.

2.3 Participants

Hand and nasal samples were obtained from occupationally exposed adult (>18 years old) workers on the farm upon explicit, voluntary, written informed consent.

2.4 Sampling

The "farm to fork approach" recommended by WHO-AGISAR (Advisory Group on Integrated Surveillance of Antimicrobial Resistance) was implemented in this study (WHO, 2017a).

Samples were collected across the farm-to-fork continuum as follows:

i. Production (animals on farm) samples collected included faeces, litter (the bedding), and slurry and were collected every two weeks from birth to slaughter.
   The block sampling method was used to ensure an even representation of the entire herd within the pig pen house.
ii. Hand and nares samples from the farm workers were collected on weeks 3, 5-7 & 9 over a period of four months based on individual voluntary consent.
iii. Holding and transport samples consisted of swab samples from the holding pens and truck floor and were collected before and after transportation of the pigs to the abattoir.
iv. Post slaughter (abattoir) samples collected included carcass swabs, caecal samples and carcass rinsate.
v. Swab samples of meat products including whole cuts (head, body and thigh) sold to consumers were collected from the farm at the retail point.

The final sample size across all sources was 520 represented in a flow diagram (**Figure S1).**

2.5 Isolation of *Campylobacter* spp.

Samples were putatively identified as previously described (Shobo et al., 2016; Reddy and Zishiri, 2018) with slight modifications. Briefly, 1g of each faecal and litter sample was suspended in 4ml of an enrichment blood-free *Campylobacter* charcoal broth (Himedia Laboratories Pvt., Ltd, Mumbai, India) supplemented with modified charcoal cefoperazone deoxycholate (mCCD) *Campylobacter* selective supplements. Ten swab samples were pooled into 1ml sterile distilled water and into 4ml of *Campylobacter* charcoal broth. Following incubation at 42°C for 24hrs under microaerophilic conditions , approximately 6 drops (100µl) of the enrichment charcoal broth was filtered through over a 0.45µm pore size cellulose nitrate filter (Sartorius Stedim Biotech, Gottingen, Germany) onto mCCDA *Campylobacter* blood-free selective agar base (Oxoid LTD, Basingstoke, United Kingdom) containing CCDA selective supplement SRO 155E (Oxoid LTD, Basingstoke, Hampshire, England) and incubated at 42°C for 24hrs under microaerophilic conditions (Reddy and Zishiri, 2018). Ten single colonies showing typical morphology of *Campylobacter* spp. with smooth, colourless translucent to grey appearance were randomly selected from the mCCDA *Campylobacter* blood-free selective agar base (Oxoid LTD, Basingstoke, United Kingdom) and sub-cultured onto Tryptose blood agar base plates (Biolab, Longmeadow Business Estate South, Modderfontein, South Africa) supplemented with 5% defibrinated sheep blood. Single colonies were stored in tryptone soya broth (Oxoid LTD, Basingtoke, Hampshire, England) with 20% glycerol at -80°C for further investigations (Shobo et al., 2016). Quality control strains *C. jejuni* ATCC 33560 and *C. coli* ATCC 33559 were used in the bacterial identification process.

2.6 DNA Extraction

DNA was extracted using the conventional boiling method as previously described (Shobo et al., 2016; Reddy and Zishiri, 2018) with slight modifications. Briefly, colonies were suspended in 200µl sterile distilled water, vortexed to homogenise cells, boiled at 100°C for 15 minutes then cooled on ice for 5 minutes. The suspension was then centrifuged at 13000rpm for 5 minutes and supernatant collected and stored at -25°C for further use. The *C. jejuni* ATCC 33560 and *C. coli* ATCC 33559 controls were subjected to the same DNA isolation process. The concentration and purity of the DNA was ascertained using Nanodrop 2000, UV-Vis Spectrophotometer (Thermo-Fisher Scientific, Waltham, MA, USA).

2.7 Molecular confirmation of Isolates

Campylobacter isolates were confirmed to genus and species level using real-time polymerase chain reaction (RT-PCR). RT-PCR was used in this study to provide fast, precise and more accurate results for DNA. The genus and species-specific primers used for amplification (**Table S1**) were purchased from Inqaba Biotechnical Industries (Pty) Ltd. Pretoria South Africa. The *Campylobacter* genus specific *16SrRNA* gene was used to confirm isolates to genus level and species identification focused on two species-specific genes, viz., the hippuricase gene (*hipO)* gene specific for *C. jejuni* and the aspartokinase gene *(asp*) gene specific for *C. coli* (**Table S1**). *C. jejuni* ATCC 33560 and *C. Coli* ATCC 33559 served as positive controls while a reaction mixture without template DNA was used as a negative control.

The reaction was carried out in a total volume of 10µl consisting of 5µl of a 2x Luna® Universal qPCR master mix (Biolabs, New England Ipswich, MA, USA), 0.5µl forward and reverse primer mixture, 3µl sample DNA and 1µl of nuclease free water. The following amplification cycling conditions were used as previously optimised by (Chukwu et al., 2019) with slight modifications: initial activation stage at 95°C for 10min, followed by 40 cycles of denaturation at 95°C for 10sec, specific annealing temperature for each primer (**Table S1**) for 15 sec, an extension at 72°C for 20 sec, and a final extension at 72°C for 5 min. A melt curve was achieved by an increase in melting temperature from 60°C to 95°C (Chukwu et al., 2019; Pillay et al., 2020). Samples were then confirmed as positive through comparison to the standard curve of the positive control. All reactions were performed on a QuantStudio 5 Real-Time PCR System (ThermoFisher Scientific, Waltham, MA USA) and the melt curve analysis was carried out using the QuantStudio Design & Analysis software version 1.4.3 (ThermoFisher Scientific, Waltham, MA USA).

2.8 Antibiotic Susceptibility Testing

Antibiotic susceptibility testing (AST) was performed using the Kirby Bauer Disk Diffusion method on Mueller Hinton Agar that was supplemented with 5% horse blood as recommended by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) and Clinical and Laboratory Standards Institute guidelines (CLSI) (CLSI, 2018; EUCAST, 2020). The seven antibiotics that were tested with their corresponding concentrations were: gentamicin (10µg), streptomycin (10µg), erythromycin (15µg), ampicillin (10µg), ciprofloxacin (5µg), nalidixic acid (30µg) and tetracycline (30µg) as recommended by the WHO-AGISAR guidelines (WHO, 2017a). AST results of ciprofloxacin, erythromycin and tetracycline were interpreted using EUCAST breakpoints while AST results of ampicillin, gentamicin, streptomycin and nalidixic acid were interpreted using CLSI guidelines.

In summary, a sterile swab was used to remove about two to three colonies from a 48-hr culture into 250 ml of sterile distilled water to obtain an inoculum equivalent to 0.5 McFarland standard as recommended by the EUCAST guideline. The suspension was inoculated on Mueller Hinton agar (Biolab, Longmeadow Business Estate South, Modderfontein, South Africa) plates supplemented with 5% defibrinated sheep blood. This was followed by dispensing antibiotics discs onto the agar plates using a multipoint disc inoculator. Plates were then incubated under microaerophilic condition in an anaerobic jar created by CampyGen (Oxoid LTD, Basingstoke UK) at 42°C for 24 hours and re-incubated if there was insufficient growth (Pillay et al., 2020). Results were interpreted by measuring zones of inhibition around each antibiotic disc in millimetres. Zones were recorded and interpreted following EUCAST and CLSI breakpoints. Isolates displaying resistance to one or more antibiotics from three or more distinct antibiotic classes were classified as multi-drug resistant (MDR) isolates. *C. jejuni* ATCC 33560, *C. coli* ATCC33559 were used as control strains (CLSI, 2018; EUCAST, 2020).

2.9 Detection of Antibiotic Resistance and Virulence genes

Isolates were tested for the presence of selected resistance and virulence genes, the former informed by AST results, using Real-time PCR (RT-PCR). The primers used and PCR conditions are as listed in **Table (S2** and **S3)** respectively. Primers were purchased from Inqaba Biotechnical Industries (Pty) Ltd., Pretoria South Africa.

All the isolates that conferred high resistance to ciprofloxacin, tetracycline, erythromycin, and ampicillin antibiotics were screened for the presence of the selected resistance genes viz., *gyrA, TetO, 23SrRNA,* $bla_{OXA\text{-}61}$, and *CmeB* as the most frequently reported antibiotic resistance genes in *Campylobacter* spp. in the literature. Selected PCR products were sequenced to identify known/novel mutations conferring resistance on the ABI 3130XL Genetic Analyser using the Sanger method of DNA sequencing by Inqaba Biotechnical Industries (Pty) Ltd., Pretoria South Africa. The sequences were analysed using the Basic Local Alignment Search Tool® 2.0 software, available from the National Center for Biotechnology Information. Results were compared to other known similar *Campylobacter* gene sequences in GENBANK.

Seven virulence genes involved in adhesion (*cadF, pldA*), thermotolerance (*dnaJ*), invasion (*ciaB*), and toxin production (*cdtA, cdtB and cdtC*) were investigated using primers and PCR conditions described in **(Table S3)**.

The reaction was carried out in a total volume of 10µl made up of 5µl of a 2x Luna® Universal qPCR master mix (New England Biolabs, Ipswich, MA, USA), 0.5µl of each forward and reverse primer, 3µl of template DNA and 1µl of nuclease-free water. The already optimised PCR cycling conditions by

(Chukwu et al., 2019) and (Pillay et al., 2020) were followed. *C. jejuni* ATCC 29428 served as a positive control for the virulence genes tested viz *cadF*, *dnaJ, pldA, ciaB, cdtA, cdtB, cdtC*. *C.jejuni* 33560 was used as a positive control for the resistance genes *tetO*, $bla_{OXA-61,}$ 23S rRNA at position 2074 and 2075 while *C.coli* 33559 was used as a positive control for the *gyrA* and *cmeB* genes. All reactions were performed on a QuantStudio 5 Real-Time PCR System (ThermoFisher Scientific, Waltham, MA USA) and the melt curve analysis was carried out using the QuantStudio Design & Analysis software version 1.4.3 (ThermoFisher Scientific, Waltham, MA USA).

2.10 Clonality- ERIC PCR

The clonality of the isolates was determined by ERIC-PCR using ERIC1R (5'-ATG TAA GCT CCT GGG GAT TCA C-3') and ERIC2 (5'-AAG TAA GTG ACT GGG GTG AGC G-3') primers as described by (Cha et al., 2017), where a sub-sample of isolates was selected based on source and the antibiograms. Briefly, isolates with antibiograms showing similar resistance patterns from different sources along the farm-to-fork continuum were selected in order to determine clonal relatedness between the isolates. The reaction was carried out in a total volume of 20µl consisting of 12.5 µl of DreamTaq Green PCR Master mix (2X) (ThermoFisher Scientific, Vilnius, Lithuania), 0.1µl of each forward and reverse primer, 3µl of template DNA and 4.3µl of nuclease-free water. The PCR amplification conditions were as described by (Cha et al., 2017). The amplified products were electrophoresed on a 1% agarose gel at 75 V for 3hrs in a 1 X Tris-acetate-EDTA (TAE) buffer (Bioconcept Ltd, Allschwil, Switzerland). A 100bp DNA ladder (New England Biolabs, Hitchin, Hertfordshire, UK) was used as the molecular weight marker. The gel images were captured using the Gel Doc™ XR+ imaging system (Bio-Rad Laboratories, Inc. Hercules, California, USA,). The fingerprint patterns were analysed using SYNGENE Bionumerics software version 6.6 (Applied Maths NV, Sint-Martens-Latem, Belgium). A band tolerance of 10% was used for inputting gel images. Cluster generation used Pearson correlation with a 1% optimisation and an unweighted pair group with arithmetic averages (UPGMA) to create a dendrogram. Clusters were determined using a 75% similarity cut-off.

3 Results

3.1 Prevalence of *Campylobacter* spp.

Campylobacter spp. were detected in 378/520 (72.7%) samples across the farm-to-fork continuum with *C. coli* as the most predominant spp. 277 (73.3%), followed by *C. jejuni* 67 (17.7%) and the remainder classified as "other" 34 (8.9%). The *Campylobacter* spp. distribution across the farm-to- fork continuum is illustrated in (**Table 1 and Figure 1)**. Samples analysed from week one collection where the piglets were first introduced into the pigpen did not contain *Campylobacter* spp. and *Campylobacter* was not detected in any human samples.

Table 1. Prevalence of *Campylobacter* spp. across the farm to fork continuum.

				% Positive isolates for		
Site	**Source**	**Total Isolates**	Isolate Proportion	*C. coli* (n=277)	*C.jejuni (n=67)*	Other (n=34)
FARM	Faecal	195/232	51%	181(65.3)	55(82.1)	19(55.9)
	Litter	16/16	4.2%			
	Slurry	44/97	11.6%			
TRANSPORT	Transport crates	24/40	6.3%	17(6.1)	4(5.9)	3(8.8)
ABATTOIR	Carcass Swab	31/39	8.2%	66(23.8)	5(7.5)	11(32.4)
	Carcass Rinsate	25/32	6.6%			
	Caeca	26/44	6.8%			
RETAIL	Retail	17/20	4.4%	13(4.7)	3(4.5)	1(2.9)
	Total	378/520		277	67	34

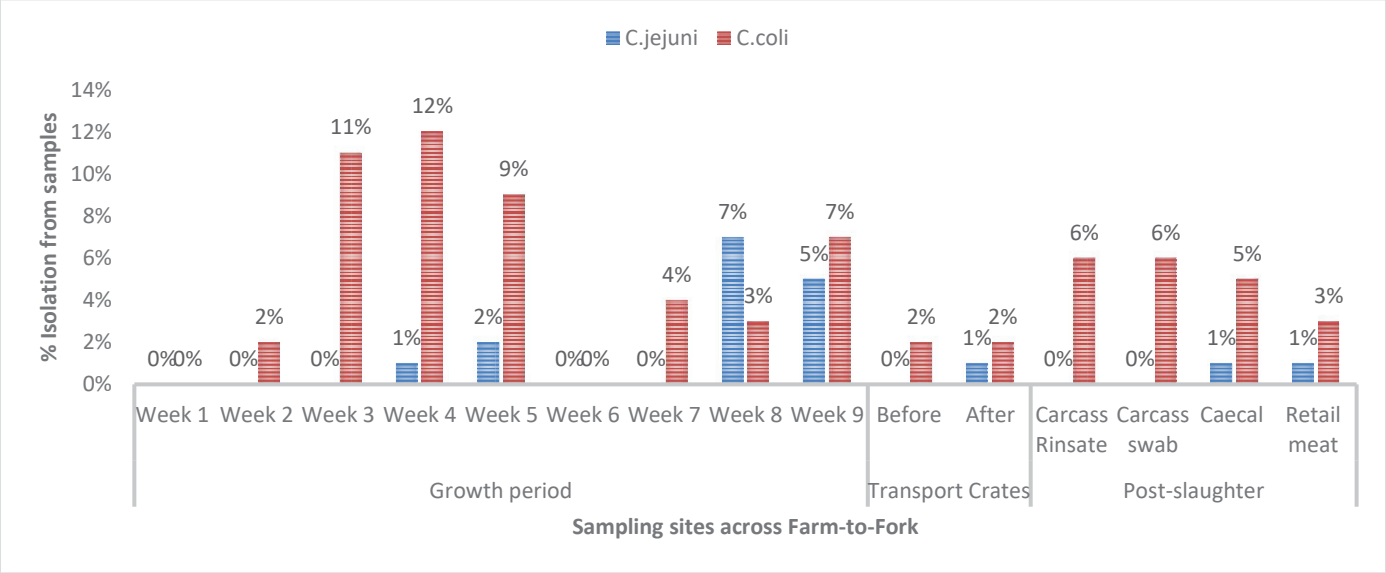


Figure 1: Distribution of *C. coli* and *C. jejuni* spp. along the sampling points of the farm-to-fork continuum.

3.2 Antimicrobial Susceptibility Testing

AST results for *C. coli* and *C. jejuni* revealed high levels of resistance to erythromycin (89% and 99%), tetracycline (82% and 96%), streptomycin (87% and 93%), ampicillin (69% and 85%), followed by relatively lower levels of resistance to ciprofloxacin at (53% and 67%) for *C. coli* and *C. jejuni* respectively. The lowest percentage resistance observed was to gentamicin (12%) in both *C. coli* and *C. jejuni* and nalidixic acid (28% and 27%) for *C. coli* and *C. jejuni* respectively **Table 2.** The "other" species of *Campylobacter* identified in this study also showed the same resistance profiles to the antibiotics tested with high percentage resistance to erythromycin, streptomycin, ampicillin, tetracycline, and ciprofloxacin and low percentage resistance to gentamicin and nalidixic acid **Table 2**.

Table 2. Antibiotic resistance (%) of *Campylobacter* spp. isolated from pigs.

Antimicrobial Class	Antimicrobials	Species and no. (%) of resistant isolates			
		C.coli (n=277)	*C. jejuni* (n=67)	Other (n=34)	Total (n=378)
Aminoglycosides	Gentamicin	34 (12.2)	8 (11.9)	2 (5.8)	44 (11.6)
	Streptomycin	240 (86.6)	62 (92.5)	31 (91.1)	333 (88.0)
Macrolides	Erythromycin	247 (89.1)	66 (98.5)	30 (88.2)	343 (90.7)
Penicillins	Ampicillin	191 (68.9)	57 (85.0)	28 (82.3)	276 (73.0)
Quinolones	Ciprofloxacin	148 (53.4)	45 (67.1)	23 (67.6)	216 (57.1)
	Nalidixic acid	77 (27.7)	18 (26.8)	8 (23.5)	103 (27.2)
Tetracyclines	Tetracycline	227 (81.9)	64 (95.5)	28 (82.3)	319 (84.3)

MDR was noted among 330 out of 378 (87.3%) *Campylobacter* spp. tested (**Table 3)**. Only 48 out of 378 (12.6%) isolates were non-MDR. Separated by species, 236/277 (85.1%) of *C. coli*, 63/67 (94%) of *C. jejuni* and 31/34 (91.1%) of other *Campylobacter* spp. were MDR (**Table 3)**. A total of 39 antibiotic resistance profiles were observed for *C. coli* and *C. jejuni*. *C. coli* displayed 27 antibiotic resistance profiles as compared to *C. jejuni* which showed 12. The most common MDR profiles identified in the current study in both *C. coli* and *C. jejuni* were resistance to five antibiotics (AMP-CIP-ERY-TET-STR) and resistance to four antibiotics (AMP-ERY-TET-STR) as illustrated in **Table 3**.

Table 3. Antibiograms of *Campylobacter* spp. isolated from pigs.

Resistance Patterns	SOURCE	*C. coli* (n=277)	*C. jejuni* (n=67)	Other (n=34)	Total (n=378)
RESISTANCE TO SEVEN ANTIBIOTICS					
AMP+NAL+CIP+ERY+TET+GEN+STR	F, S	10	5	1	16
TOTAL		**10**	**5**	**1**	**16**
RESISTANCE TO SIX ANTIBIOTICS					
AMP+NAL+CIP+ERY+TET+STR	F, L, W, CR, CS, RM	17	7	1	25
NAL+CIP+ERY+TET+GEN+STR	F, S, TC	5	0	0	5
AMP+CIP+ERY+TET+GEN+STR	F, S	11	2	1	14
AMP+NAL+ERY+TET+GEN+STR	F	1	0	0	1
TOTAL		**34**	**9**	**2**	**45**
RESISTANCE TO FIVE ANTIBIOTICS					
AMP+CIP+ERY+TET+STR	F, S, L, TC, CR, CS, CEACA, RM	56	25	13	94
AMP+NAL+ERY+TET+STR	F, CS	4	1	1	6
AMP+NAL+CIP+ERY+STR	CS, CR	4	0	0	4
NAL+CIP+ERY+TET+STR	F, S,	1	2	0	3
NAL+CIP+TET+GEN+STR	S	1	0	0	1
TOTAL		**66**	**28**	**14**	**108**
RESISTANCE TO FOUR ANTIBIOTICS					
ERY+TET+GEN+STR	F	2	0	0	2
AMP+ERY+TET+STR	F, S, TC, CR, CS, CEACA, RM	51	15	6	72
CIP+ERY+TET+STR	F, L, CS, RM	9	0	1	10
AMP+CIP+ERY+STR	CR, CS	9	1	2	12
NAL+ERY+TET+STR	L	1	0	0	1
NAL+CIP+ERY+TET	F, L, CR	3	1	0	4
AMP+NAL+ERY+TET	F	1	1	0	2

NAL+CIP+ERY+STR	CS	0	0	1	1
AMP+NAL+ERY+STR	CR, CS	10	0	1	11
NAL+CIP+TET+STR	F, L, S	11	0	2	13
TOTAL		**97**	**18**	**13**	**128**
RESISTANCE TO THREE ANTIBIOTICS					
AMP+ERY+TET	F, TC	2	0	1	3
NAL+ERY+TET	F	1	0	0	1
ERY+TET+STR	F, L, RM	9	2	0	11
AMP+ERY+STR	F, CR, CS, RM,	12	0	0	12
CIP+ERY+STR	RM	1	1	0	2
AMP+CIP+ERY	CEACA	1	0	0	1
ERY+TET+GEN	F	1	0	0	1
CIP+ERY+TET	CEACA, RM	2	0	0	2
TOTAL		**29**	**3**	**1**	**33**
TOTAL MDR ISOLATES		**236**	**63**	**31**	**330**
PERCENTAGE MDR		**85.1%**	**94%**	**91.1%**	
NONE MDR					**48**

Resistance patterns abbreviations: AMP; ampicillin, ERY; erythromycin, CIP; ciprofloxacin, GEN; gentamicin, STR; streptomycin, TET; tetracycline, NAL; nalidixic acid.

Source abbreviations: F; faeces, S; slurry, L; litter, TC; transport crates, CR; carcass rinsate, CS; carcass swab, RM; retail meat.

3.3 Virulence Genes

Different frequencies of virulence genes were observed by species and across the farm-to-fork continuum **(Table 4, Table S6-S7)**. Higher frequency detection of the genes was noted for the *cdtA* (67.4%), *dnaJ* (61.1%), *ciaB* (48.6%) and *cdtC* (51%) genes in all isolates with small percentages observed for *cdtB* (19.3%), *pldA* (17.4%), *cadF* (5%) **(Table 4)**. Both *C. coli* and *C. jejuni* possessed higher frequency of the *cdtA* (66% and 80%), *dnaJ* (63% and 64%),*ciaB* (51% and 52%) and *cdtC* (53.4% and 46.3%) genes respectively and lower frequencies of *cdtB*, *pldA,* and *cadF* respectively as noted in **(Table 4)**.

Table 4. Virulence genes of *Campylobacter* spp. detected in Pigs.

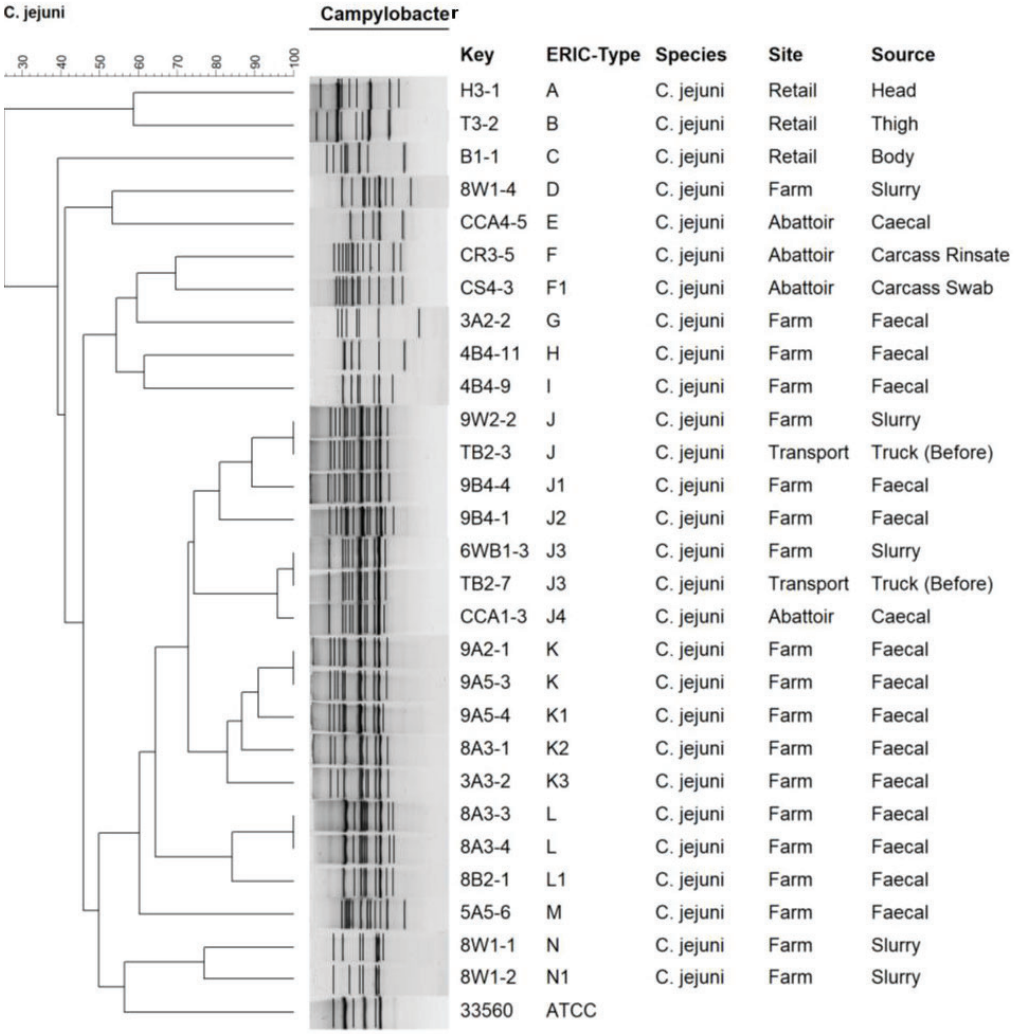
	(%) Total Prevalence of virulence genes			
Virulence gene	*C. coli* (n=277)	*C. jejuni* (n=67)	Other (n=34)	Total (n=378)
cadF	18 (6.5)	1 (1.5)	0 (0)	19 (5)
dnaJ	175(63.1)	43(64.1)	13(38.2)	231(61.1)
ciaB	141(50.9)	35(52.2)	8(23.5)	184(48.6)
pldA	45(16.2)	18(26.9)	3(8.8)	66(17.4)
cdtA	182(65.7)	54(80.6)	19(55.8)	255(67.4)
cdtB	65(23.5)	8(11.9)	0(0)	73(19.3)
cdtC	148(53.4)	31(46.3)	13(38.2)	193(51)

3.4 Detection of antibiotic resistance genes

All the isolates that showed high level phenotypic resistance to the selected antibiotics viz, ciprofloxacin, tetracycline, erythromycin, and ampicillin were screened for the presence of antibiotic resistance genes viz, *gyrA, TetO, 23SrRNA,* $Bla_{OXA-61,}$ and *CmeB*. Tetracycline resistance was mediated by the *tetO* gene in 238/319 (74.6%) of the isolates while resistance to ampicillin could be attributed to the Bla_{OXA-61} gene in just 8/275 (2.9%) isolates. Forty-two isolates 42/378 (11.1%) possessed the *cmeB* gene that encodes a multidrug efflux pump which also contributed to conferring resistance to the antibiotics tested, viz., ampicillin, tetracycline, ciprofloxacin, and erythromycin. All 21 *C. coli* and *C. jejuni* isolates that were tested for the *gyrA* gene exhibited mutation at the Thr-86-Ile region in the quinolone-resistance-determining region (QRDR) of the *gyrA* and all 18 *C. coli* and *C. jejuni* isolates tested for erythromycin resistance exhibited common transitional mutations A2075G and A2074C in the 23S rRNA gene.

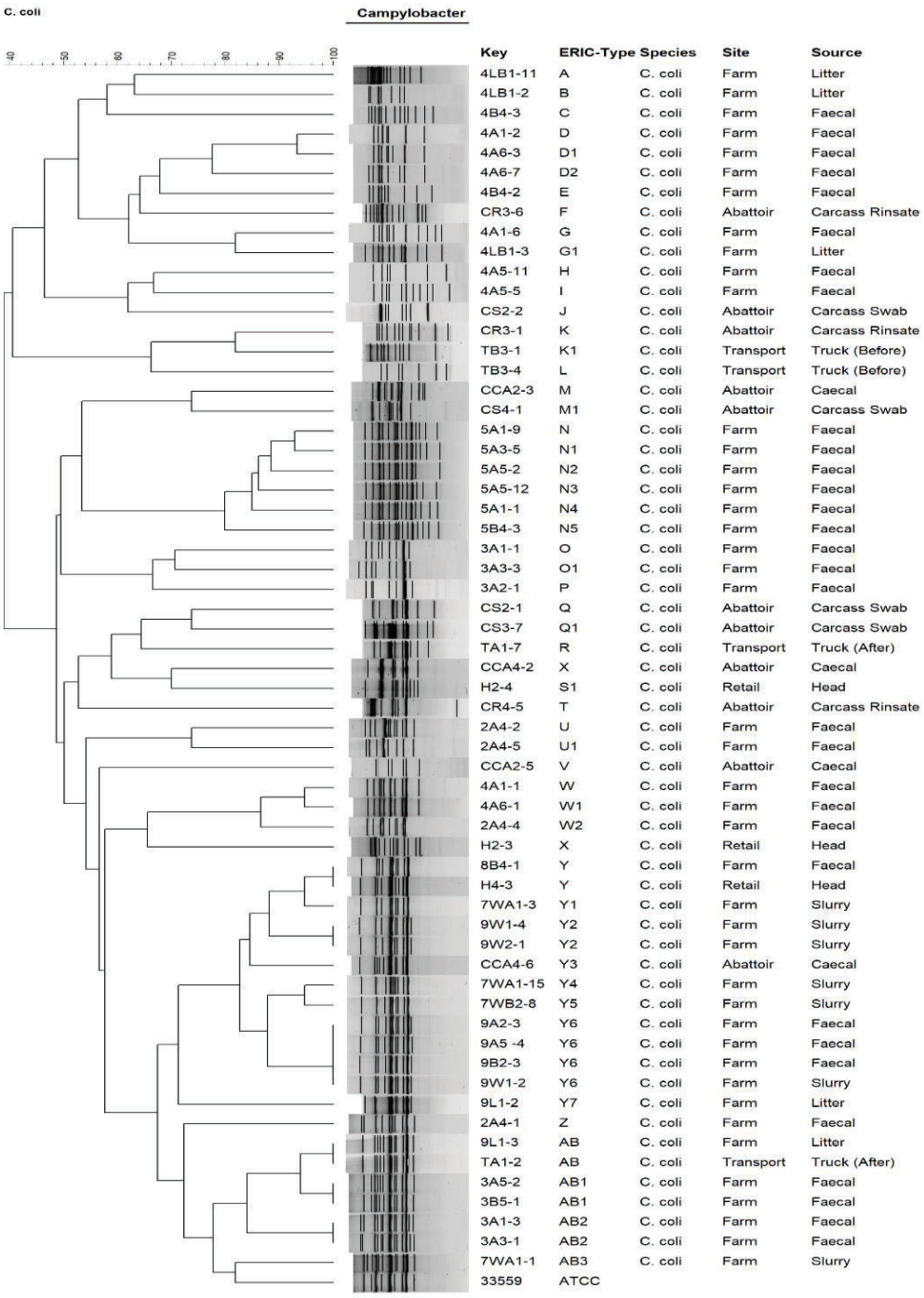
3.5 Clonality

The dendrogram showing ERIC-types of *C. jejuni* and *C. coli* **Figures 2** and **3**, respectively.



Key	ERIC-Type	Species	Site	Source
H3-1	A	C. jejuni	Retail	Head
T3-2	B	C. jejuni	Retail	Thigh
B1-1	C	C. jejuni	Retail	Body
8W1-4	D	C. jejuni	Farm	Slurry
CCA4-5	E	C. jejuni	Abattoir	Caecal
CR3-5	F	C. jejuni	Abattoir	Carcass Rinsate
CS4-3	F1	C. jejuni	Abattoir	Carcass Swab
3A2-2	G	C. jejuni	Farm	Faecal
4B4-11	H	C. jejuni	Farm	Faecal
4B4-9	I	C. jejuni	Farm	Faecal
9W2-2	J	C. jejuni	Farm	Slurry
TB2-3	J	C. jejuni	Transport	Truck (Before)
9B4-4	J1	C. jejuni	Farm	Faecal
9B4-1	J2	C. jejuni	Farm	Faecal
6WB1-3	J3	C. jejuni	Farm	Slurry
TB2-7	J3	C. jejuni	Transport	Truck (Before)
CCA1-3	J4	C. jejuni	Abattoir	Caecal
9A2-1	K	C. jejuni	Farm	Faecal
9A5-3	K	C. jejuni	Farm	Faecal
9A5-4	K1	C. jejuni	Farm	Faecal
8A3-1	K2	C. jejuni	Farm	Faecal
3A3-2	K3	C. jejuni	Farm	Faecal
8A3-3	L	C. jejuni	Farm	Faecal
8A3-4	L	C. jejuni	Farm	Faecal
8B2-1	L1	C. jejuni	Farm	Faecal
5A5-6	M	C. jejuni	Farm	Faecal
8W1-1	N	C. jejuni	Farm	Slurry
8W1-2	N1	C. jejuni	Farm	Slurry
33560	ATCC			

Figure 2: Dendrogram showing ERIC-types of *C. jejuni* isolates based on the similarity index recovered along the farm-to-fork continuum in relation to the site and source. *C. jejuni* ATCC 33560 was used as the control strain. The alphabets A-N shows the main ERIC types and subtypes of each isolate.



Key	ERIC-Type	Species	Site	Source
4LB1-11	A	C. coli	Farm	Litter
4LB1-2	B	C. coli	Farm	Litter
4B4-3	C	C. coli	Farm	Faecal
4A1-2	D	C. coli	Farm	Faecal
4A6-3	D1	C. coli	Farm	Faecal
4A6-7	D2	C. coli	Farm	Faecal
4B4-2	E	C. coli	Farm	Faecal
CR3-6	F	C. coli	Abattoir	Carcass Rinsate
4A1-6	G	C. coli	Farm	Faecal
4LB1-3	G1	C. coli	Farm	Litter
4A5-11	H	C. coli	Farm	Faecal
4A5-5	I	C. coli	Farm	Faecal
CS2-2	J	C. coli	Abattoir	Carcass Swab
CR3-1	K	C. coli	Abattoir	Carcass Rinsate
TB3-1	K1	C. coli	Transport	Truck (Before)
TB3-4	L	C. coli	Transport	Truck (Before)
CCA2-3	M	C. coli	Abattoir	Caecal
CS4-1	M1	C. coli	Abattoir	Carcass Swab
5A1-9	N	C. coli	Farm	Faecal
5A3-5	N1	C. coli	Farm	Faecal
5A5-2	N2	C. coli	Farm	Faecal
5A5-12	N3	C. coli	Farm	Faecal
5A1-1	N4	C. coli	Farm	Faecal
5B4-3	N5	C. coli	Farm	Faecal
3A1-1	O	C. coli	Farm	Faecal
3A3-3	O1	C. coli	Farm	Faecal
3A2-1	P	C. coli	Farm	Faecal
CS2-1	Q	C. coli	Abattoir	Carcass Swab
CS3-7	Q1	C. coli	Abattoir	Carcass Swab
TA1-7	R	C. coli	Transport	Truck (After)
CCA4-2	X	C. coli	Abattoir	Caecal
H2-4	S1	C. coli	Retail	Head
CR4-5	T	C. coli	Abattoir	Carcass Rinsate
2A4-2	U	C. coli	Farm	Faecal
2A4-5	U1	C. coli	Farm	Faecal
CCA2-5	V	C. coli	Abattoir	Caecal
4A1-1	W	C. coli	Farm	Faecal
4A6-1	W1	C. coli	Farm	Faecal
2A4-4	W2	C. coli	Farm	Faecal
H2-3	X	C. coli	Retail	Head
8B4-1	Y	C. coli	Farm	Faecal
H4-3	Y	C. coli	Retail	Head
7WA1-3	Y1	C. coli	Farm	Slurry
9W1-4	Y2	C. coli	Farm	Slurry
9W2-1	Y2	C. coli	Farm	Slurry
CCA4-6	Y3	C. coli	Abattoir	Caecal
7WA1-15	Y4	C. coli	Farm	Slurry
7WB2-8	Y5	C. coli	Farm	Slurry
9A2-3	Y6	C. coli	Farm	Faecal
9A5 -4	Y6	C. coli	Farm	Faecal
9B2-3	Y6	C. coli	Farm	Faecal
9W1-2	Y6	C. coli	Farm	Slurry
9L1-2	Y7	C. coli	Farm	Litter
2A4-1	Z	C. coli	Farm	Faecal
9L1-3	AB	C. coli	Farm	Litter
TA1-2	AB	C. coli	Transport	Truck (After)
3A5-2	AB1	C. coli	Farm	Faecal
3B5-1	AB1	C. coli	Farm	Faecal
3A1-3	AB2	C. coli	Farm	Faecal
3A3-1	AB2	C. coli	Farm	Faecal
7WA1-1	AB3	C. coli	Farm	Slurry
33559	ATCC			

Figure 3: Dendrogram showing ERIC-types of *C. coli* isolates based on the similarity index recovered along the farm-to-fork continuum in relation to the site and source. *C. coli* ATCC 33559 was used as the control strain. The alphabets A-Z shows the main ERIC types and subtypes of each isolate.

4 Discussion

This study describes the prevalence, antibiotic resistance and virulence profiles and clonality of *Campylobacter* spp. recovered across the farm-to-fork continuum in an intensive pig production system in the uMgungundlovu district of KwaZulu-Natal, South Africa. The emergence of antibiotic resistance is considered a cross sectoral problem in the food chain as antibiotic resistant bacteria and genes can spread through the food chain to cause infections in humans (FAO, 2016; Founou, Founou, and Essack, 2016). We showed a *Campylobacter* spp. prevalence of 72.7% (378/520) across the farm-to fork continuum with more *C. coli* (73.3%) detected than *C. jejuni* (17.7%). Different levels of resistance and MDR were observed to the different antibiotic classes across the farm to food continuum. Several antibiograms evidenced MDR profiles in different permutations and combinations, attesting to the complexity and diversity of resistance observed across the continuum. Different frequencies of resistance and virulence genes were also observed in the isolates. ERIC-PCR revealed diversity within the isolates, with some evidence of transmission across the farm-to-fork continuum in clone J and clone J3 in *C. jejuni* and clones Y, N and AB in *C. coli* where isolates originating from different sources belonged to similar clones.

4.1 Prevalence of *Campylobacter* spp.

Gwimi et al. (2015) conducted a study on the prevalence of *Campylobacter* spp. in faecal samples of pigs and humans in Zuru Kebi, Nigeria. The study revealed high percentage (92.66%) of *Campylobacter* spp. in pigs with *C. coli* as the most encountered species at 78.71% followed by *C. jejuni* at 14.03%, *C. upsaliensis* at 5.40% and *C. hyointestinalis* as the least encountered at 1.80%. (Gwimi et al., 2015). The prevalence and antibiotic resistance of *C. coli* and *C. jejuni* in Greek swine farms showed that 49% of the farms were colonised by *Campylobacter* spp. The predominant species was *C. coli* at 77.4% of the isolates followed by *C. jejuni* at 22.2% (Papadopoulos et al., 2020). A study by Igwaran and Okoh (2020) evaluated the prevalence, virulence genes, antimicrobial susceptibility patterns, and resistance gene determinants in *Campylobacter* spp. isolated from retailed meat carcasses in the Eastern Cape Province of South Africa. *Campylobacter* spp. was detected in 28.4% of carcass isolates. *C. coli* had the highest prevalence rate (22.08%), followed by *C. jejuni* (16.66%) and *C. fetus* (3.73%) in all the meat samples. The pork meat samples were colonised with 25.2% of *Campylobacter* spp. (Igwaran and Okoh, 2020).

4.2 Antibiotic resistance profiles and resistance determinants

Antibiotic resistance profiles of *C. coli* and *C. jejuni* across the farm to food continuum were observed. Notwithstanding the higher numbers of farm isolates, resistance was highest at farm level where antibiotic exposure was greatest. The intensive use of antibiotics in intensive food production systems contributes significantly to antibiotic resistance among bacteria in food animals (Van et al., 2020). The South African Veterinary Association (SAVA) policy document on "guidelines for the use of antimicrobials in the South African pig industry" outlines the critical and highly important drugs for swine-veterinary and human-swine-veterinary use in South Africa (SAVA, 2016), Streptomycin, gentamicin, erythromycin, ampicillin, ciprofloxacin and tetracycline are listed among the substances used for pigs that are also critically and highly important for human medicine. There was a correlation between the SAVA list of antibiotics and the resistance observed.

Of note were the higher levels of resistance of *C. coli* and *C. jejuni* isolates to ciprofloxacin (53.4% and 67.1%) compared to nalidixic acid (27.7% and 26.8%) respectively and the lowest percentage resistance to gentamicin (12%) in both *C. coli* and *C. jejuni*. Similar findings were reported in a study conducted in Ghana on antibiotic resistance of *Campylobacter* spp. recovered from faeces and carcasses of healthy pigs where high levels of resistance to erythromycin (100% and 99.5%), tetracycline (89.8% and 96.5%), and ampicillin (84% and 69.8%) were observed for *C. coli* and *C. jejuni* isolates respectively. Here too, was ciprofloxacin resistance (37.7% and 40.2%) higher compared to nalidixic acid resistance (2.9% and 23%) among the *C. coli* and *C. jejuni* isolates respectively. Resistance to the fluoroquinolones is conferred by the point mutations in the *gyrA* gene which encodes DNA gyrase (Luangtongkum et al., 2009) The Thr86-Ile substitution in *Campylobacter gyrA* confers cross-resistance to both ciprofloxacin and nalidixic acid, however, mutations of the *gyrA* region in *C. jejuni* which include Thr86Ala were reported to be responsible for a high-level resistance to nalidixic acid and low-level resistance to ciprofloxacin (Jesse et al., 2006). Differences in *gyrA* point mutations could possibly explain the observed differences for ciprofloxacin and nalidixic acid resistance and more studies need to be conducted to investigate the sequences of *gyrA* and other resistance genes implicated in varying levels of resistance to ciprofloxacin and nalidixic acid in *Campylobacter* spp.

High levels of resistance to erythromycin, tetracycline, streptomycin, ampicillin, and ciprofloxacin were evident in the faecal isolates of animals for both *C. coli* and *C. jejuni* from the farm level compared to other sources, pointing not only to higher exposure levels on the farm, but more importantly, the minimal, resistance transmission across the farm-to-form continuum.

The levels of MDR found in this study with 87.3%, 85.1% and 94% of the total, *C. coli* and *C. jejuni* isolates respectively were similar to those from a study on the antibiograms of *C. jejuni* and *C. coli* isolated from animals, foods of animal origin and humans in Gannavaram, Andhra Pradesh in India that demonstrated MDR of 80%, 89.4% and 100% of the total, *C. coli* and *C. jejuni* isolates (Srinivas et al., 2019).

Different antibiograms were evident amongst *C. jejuni* and *C. coli* isolates irrespective of the source of isolate. A total of 39 antibiograms were observed for *C. coli* and *C. jejuni*. *C. coli* was resistant to more antibiotics with more diverse resistance profiles displaying 27 antibiograms compared to *C. jejuni* that showed 12 antibiograms. The most common MDR profiles in both *C. coli* and *C. jejuni* were resistance to five antibiotics (AMP-CIP-ERY-TET-STR) and resistance to four antibiotics (AMP-ERY-TET-STR). There was evidence that the pig carcasses and final pork products were somewhat contaminated with antibiotic resistant *Campylobacter* isolates, necessitating good food preparation hygiene and proper cooking at high enough temperatures such that meat is not a source of food-borne infection.

Isolates were screened for the presence of the genes causing resistance to ciprofloxacin, erythromycin, tetracycline, and ampicillin. As previously identified in *Campylobacter* studies, the common mutations responsible for fluoroquinolone and macrolide resistance, i.e., Thr-86-Ile region in the QRDR and point mutations at positions 2074 and 2075 in the *23SrRNA* gene were also detected in all our isolates (Sproston, Wimalarathna, and Sheppard, 2018; Di Donato et al., 2020). The presence of the Bla_{OXA-61} gene for ampicillin resistance was detected in only 2.9% of our isolates intimating that other resistance mechanisms to this antibiotic could be involved, such as the cation-selective MOMP in *C. jejuni* and *C. coli* which tends to exclude most β-lactams (Iovine, 2013).

The most common mechanism of resistance to tetracycline reported in *Campylobacter* spp., i.e., the protection of the ribosomal binding site by ribosomal protection protein encoded by *tetO* gene was also detected in 74.6% of the isolates tested, similar to a study on *Campylobacter* spp. from poultry in KwaZulu-Natal, South Africa where all isolates resistant to tetracycline carried the *tetO* gene (Pillay et al., 2020). The *tetO* gene appears to be the most common tetracycline resistance determinant in *Campylobacter* spp. from intensive food animal production in KwaZulu-Natal although efflux genes, *tetA* and *tetB* have also been reported (Sheykhsaran et al., 2019; Igwaran and Okoh, 2020).

Although not all the genes conferring resistance to the tested antibiotics were investigated in this study, the resistance genes detected in the isolates confirmed the phenotypic AST results to a certain extent.

Further studies involving a more comprehensive panel of resistance genes should be carried out to better understand the resistance mechanisms in resistant *Campylobacter* isolates in the intensive pig production industry.

4.3 Virulence Determinants

Different frequencies of virulence genes of *Campylobacter* isolates were observed across the farm-to-fork continuum. The farm level had the highest frequency of the genes detected and this may be due to the fact that the largest number of isolates were from the farm, however, a small percentage of the virulence genes was noted in the retail meat products indicating pathogenic potential for humans. These included, the thermotolerant heat shock gene, *dnaJ* and the genes responsible for expression of the toxins (*cdtA, cdtB, cdtC)*.

The pathogenicity of *Campylobacter* spp. is mediated by several virulence factors. One of these is the ability to adapt and survive under different temperature changes encountered in the food chain i.e. exposure to temperatures of chicken in the intestine at 42ºC, human intestine at 37ºC, and refrigerated foods at 4ºC (Aroori et al., 2013). The response to the temperature stress in *Campylobacter* spp. is carried out by a number of heat shock proteins including dnaJ protein encoded by the *dnaJ* gene (Wafaa et al., 2019). The high frequency of this gene observed in the isolates and its presence in the retail meat products indicates pathogenic potential for humans. The *ciaB* gene (*Campylobacter* invasive antigen B) was also detected in different frequencies throughout the continuum. This is one of the most important genes responsible for *Campylobacter* invasion which aids in the translocation of *Campylobacter* into the host cell thus posing risk to human health through consumption of contaminated meat products.

The *cdtA, cdtB,* and *cdtC* are the three genes that need to be present in a cluster in order to be functionally active for the expression of the cytotoxins that damage the host nuclear DNA leading to cell death. *CdtA* and *CdtC* are mainly responsible for host-cell recognition and the *CdtB* gene is the one that needs to be successfully internalised and delivered into the nucleus of the host cell due to its DNaseI-like activity which breaks the double strand DNA leading to cell death (Biswas et al., 2011; Silva et al., 2011). Although higher frequencies of the three genes in *Campylobacter* isolates were observed for *cdtA* and *cdtC*, *cdtB* was noted in smaller percentage. Nevertheless, the detection of these three genes across the continuum raises the food safety concerns.

Similar findings were reported in a study analysing prevalence of virulence genes in *Campylobacter* spp. isolated from livestock production systems in South Africa, where all the investigated

Campylobacter isolates in pigs harbored the genes responsible for, adhesion and invasion (*cadF*, *ciaB, pldA*), thermotolerance (*dnaJ*), and expressed cytotoxins (*cdtA*, *cdtB*, and *cdtC*). In contrast to our study, their results showed *cdtA* as the least prevalent gene in *Campylobacter* spp. in pigs and *cdtB, cdtC* with high prevalence (Ngobese et al., 2020). Not all the virulence genes involved in *Campylobacter* pathogenesis were evaluated in this study and so other genes may contribute to its pathogenesis.

4.4 Clonality

ERIC-PCR revealed 14 designated ERIC-types (A-N) for *C. jejuni* and 27 designated ERIC-types (A-AB) for *C. coli*. Similar results were reported in a study in South Korea on the distribution and molecular characterization of *Campylobacter* spp. in two poultry processing plants at different processing stages where the *Campylobacter* clones exhibited high variation and no significant relationships to the species or the processing steps (Lee et al., 2017).

A single ERIC type cluster, J-J4, was identified in *C. jejuni* consisting of seven isolates originating from faeces, slurry, truck crates and caeca. Three ERIC type clusters Y-Y7, consisting of 13 isolates originating from faeces, slurry, litter, caeca and retail meat, AB-AB3 consisting of 7 isolates originating from faeces, litter, slurry and truck crates samples, and N-N5 consisting of 6 isolates originating from faeces, litter, slurry, caeca and retail meat samples were elucidated in *C. coli*. There is thus potentially transmission along the food chain where similar clones are found across the continuum and specifically in retailed meat.

5 Conclusion

We found a high prevalence of multidrug resistant *Campylobacter* spp. with some evidence, albeit minimal, of transmission across the farm-to-fork continuum presenting a potential risk to human health. The antibiotic resistance profiles coupled with the pathogenicity profiles exacerbate the risk of food-borne infection, which is further exacerbated by the reduction in antibiotic treatment options. This calls for enhanced antibiotic stewardship, comprehensive biosecurity, and good animal husbandry in intensive pig production together with the implementation of routine surveillance, ideally at a genomic level to understand the trends and molecular epidemiology of antibiotic-resistant *Campylobacter spp*. across the farm-to-fork continuum.

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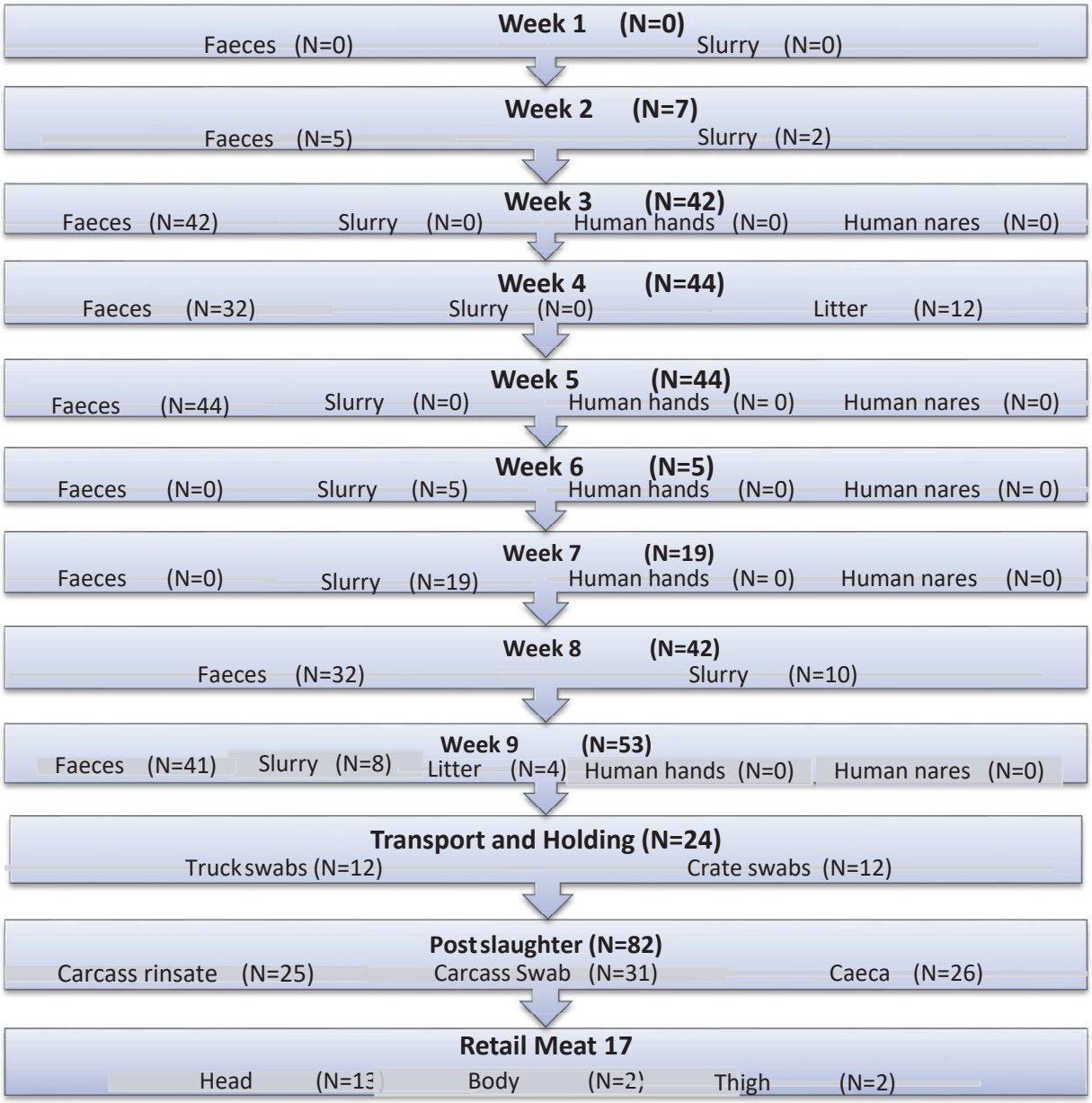

Figure S1: Sampling framework. "N" denotes the number of confirmed *Campylobacter* spp. at each sampling point and source.

Table S1: Primers used for confirmation of *Campylobacter* to genus and species level.

Target gene	Primer Sequence (5' – 3')	Product Size (bp)	Annealing Temperature (°C)	References
Asp	F-GGTATGATTTCTACAAAGCGAG R-ATAAAAGACTATCGTCGCGTG	500	60	(Linton et al., 1997)
hipO	F-GAAGAGGGTTTGGGTGGTG R-AGCTAGCTTCGCATAATAACTTG	735	66	(Linton et al., 1997)
16S rRNA	F-GGATGACACTTTTCGGAGC R-CATTGTAGCACGTGTGTC	816	58	(Linton, Owen, and Stanley, 1996)

Table S2: Primers used for the detection of antibiotic resistance genes.

Target gene	Primer Sequence (5' – 3')	Product Size (bp)	Annealing Temperature(°C)	References
23S rRNA at position 2074	F5'TTAGCTAATGTTGCCCGTACCG R5'AGTAAAGGTCCACGGGGTCTCG	485	59	(Shobo et al., 2016)
23S rRNA at position 2075	F5'TTAGCTAATGTTGCCCGTACCG R5'TAGTAAAGGTCCACGGGGTCGC	486	59	(Shobo et al., 2016)
ccgyrA *ccgyrA*	F-GAAGAATTTTATATGCTATG R-TCAGTATAAC GCATCGCAGC	235	53	(Reddy and Zishiri, 2017)
cjgyrA *cjgyrA*	F-ACGCAAGAGAGATGGTT R-TCAGTATAACGCATCGCAGC	270	45	(Reddy and Zishiri, 2017)
cmeB	F5'GACGTAATGAAGGAGAGCCA R5'CTGATCCACTCCAGCTATG	1166	50	(Shobo et al., 2016)
tetO	F-GGCGTTTTGTTTATGTGCG R-ATGGACAACCCGACAGAAGC	559	49	(Reddy and Zishiri, 2017)
blaOXA-61	F-AGAGTATAATACAAGCG R-TAGTGAGTTGTCAAGCC	372	54	(Reddy and Zishiri, 2017)

Table S3: Primers used for the detection of virulence genes.

Target gene	Primer Sequence (5' – 3')	Product Size (bp)	Annealing Temperature (°C)	References
cadF	F-TTGAAGGTAATTTAGATATG R-CTAATACCTAAAGTTGAAAC	400	43	(Reddy and Zishiri, 2018)
ciaB	F-TGCGAGATTTTTCGAGAATG R-TGCCCGCCTTAGAACTTACA	527	54	(Reddy and Zishiri, 2018)
dnaJ	F-ATTGATTTTGCTGCGGGTAG R-ATCCGCAAAAGCTTCAAAAA	177	50	(Reddy and Zishiri, 2018)
pldA	F-AAGAGTGAGGCGAAATTCCA R-GCAAGATGGCAGGATTATCA	385	46	(Reddy and Zishiri, 2018)
cdtA	F-CCTTGTGATGCAAGCAATC R-ACACTCCATTTGCTTTCTG	370	49	(Reddy and Zishiri, 2018)
cdtB	F-GTTAAAATCCCCTGCTATCAACCA R-GTTGGCACTTGGAATTTGCAAGGC	495	51	(Reddy and Zishiri, 2018)
cdtC	F-CGATGAGTTAAAACAAAAAGATA R-TTGGCATTATAGAAAATACAGTT	182	48	(Reddy and Zishiri, 2018)

Table S4: Antibiotic resistance (%) of *C. coli* across the farm-to-fork continuum.

Antibiotic Panel	Faeces	Litter	Slurry	Transport Crates	Carcass Swab	Carcass Rinsate	Ceacal	Retail Meat
Ampicillin	34%	2%	4%	5%	8%	8%	7%	1%
Nalidixic acid	13%	1%	6%	0%	3%	4%	0%	0%
Ciprofloxacin	26%	3%	8%	2%	3%	4%	5%	3%
Erythromycin	46%	5%	6%	6%	8%	8%	7%	4%
Tetracycline	45%	6%	10%	6%	1%	3%	7%	3%
Gentamicin	10%	0%	2%	0%	0%	0%	0%	0%
Streptomycin	40%	4%	9%	6%	8%	8%	7%	4%

Table S5: Antibiotic resistance (%) of *C. jejuni* across the farm-to-fork continuum.

Antibiotic Panel	Faeces	Litter	Slurry	Transport Crates	Carcass Swab	Carcass Rinsate	Ceacal	Retail Meat
Ampicillin	57%	0%	13%	6%	0%	2%	3%	3%
Nalidixic acid	16%	0%	8%	0%	2%	2%	0%	0%
Ciprofloxacin	43%	0%	13%	0%	2%	3%	2%	5%
Erythromycin	67%	0%	13%	6%	2%	3%	3%	5%
Tetracycline	67%	0%	13%	6%	2%	2%	3%	3%
Gentamicin	8%	0%	3%	0%	2%	0%	0%	0%
Streptomycin	63%	0%	13%	6%	2%	2%	3%	5%

Table S6: Prevalence (%) of virulence genes detected in *C. coli* (n = 277) across the farm-to-fork continuum

Virulence genes	Faeces	Litter	Slurry	Transport Crates	Carcass Swab	Carcass Rinsate	Ceacal	Retail Meat
cadF	2%	0%	3%	0%	1%	1%	0%	0%
dnaJ	26%	6%	8%	3%	7%	8%	1%	4%
ciaB	23%	2%	6%	3%	7%	4%	4%	2%
pldA	11%	3%	1%	0%	0%	0%	0%	1%
cdtA	32%	6%	7%	3%	7%	6%	3%	1%
cdtB	17%	1%	0%	1%	1%	1%	1%	1%
cdtC	21%	2%	5%	5%	8%	5%	5%	2%

Table S7: Prevalence (%) of virulence genes detected in *C. jejuni* (n = 67) across the farm-to-fork continuum.

Virulence genes	Faeces	Litter	Slurry	Transport Crates	Carcass Swab	Carcass Rinsate	Ceacal	Retail Meat
cadF	0%	0%	0%	0%	1%	0%	0%	0%
dnaJ	51%	0%	7%	1%	1%	0%	0%	3%
ciaB	37%	0%	6%	3%	1%	1%	1%	0%
pldA	22%	0%	4%	0%	0%	0%	0%	0%
cdtA	58%	0%	13%	3%	1%	1%	3%	0%
cdtB	7%	0%	1%	0%	1%	0%	0%	1%
cdtC	27%	0%	7%	3%	1%	1%	3%	3%

CHAPTER 3

CONCLUSIONS, LIMITATIONS AND RECOMMENDATIONS

3.1 Conclusions

This study describes the prevalence, antibiotic resistance, and virulence profiles and clonality of *Campylobacter* spp. recovered across the farm-to-fork continuum in an intensive pig production system in the uMgungundlovu district of KwaZulu-Natal, South Africa.

The prevalence of *Campylobacter* spp. was 72.7% (378/520) across the farm-to fork continuum with more *C. coli* (73.3%) detected than *C. jejuni* (17.7%). Different levels of resistance and multi-drug resistance (MDR) were observed. Several antibiograms were evident with MDR profiles taking different permutations and combinations, attesting to the complexity and diversity of resistance observed across the continuum Different frequencies of resistance and virulence genes were also observed in the isolates. ERIC-PCR revealed diversity within the isolates, with some evidence of transmission across the farm-to-fork continuum in clone J in *C. jejuni* and clones Y, N and AB in *C. coli* where isolates originating from different sources belonged to similar clones. Resistance to antibiotics registered for use in food animals in South Africa was evident. This coupled with the pathogenicity of *Campylobacter* spp. elucidated by the virulence profiles presents a risk of food-borne infection. Antibiotic stewardship and continuous monitoring of antibiotic use and resistance in intensive pig production systems is of great importance as is research on alternatives to antibiotic use in food animals.

To the best of our knowledge, this is the first study in South Africa to investigate the molecular epidemiology of antibiotic-resistant *Campylobacter* spp. in pigs using the farm-to-fork approach. The study highlights the high prevalence of MDR *Campylobacter* isolates harbouring both resistance and virulence genes which can serve as a potential reservoir for the transfer of these genes from pigs to humans through the farm-to-fork continuum. This study thus contributes to the integrated approach needed to address antibiotic resistance in the food chain in intensive food animal production.

3.2 Challenges and Limitations

These results represent the findings on a single farm and cannot be generally extrapolated. Isolation of *Campylobacter* is challenging due to the demanding growth conditions of the organism and the phenotypic similarities between species. Not all the resistance mechanisms nor all the virulence factors

of *Campylobacter* were investigated in the study. Other resistance genes involved in resistance mechanisms of *Campylobacter* as well as other virulence factors might have played a significant role in contributing to resistance and virulence mechanisms of the *Campylobacter* spp. investigated in this study.

3.3 Recommendations

There should be more research conducted on Campylobacter isolates from pig farms in South Africa to increase the understanding and knowledge of this emerging pathogen. Other genes known to confer resistance and contribute to virulence should be studied to gain insights into the full resistance and virulence potential of isolates.

Enhanced antibiotic stewardship, comprehensive biosecurity, and good animal husbandry in intensive pig production together with the implementation of routine surveillance, ideally at a genomic level, should be implemented to understand the trends and molecular epidemiology of antibiotic-resistant *Campylobacter* spp. across the farm-to-fork continuum.

3.4 Appendices

Appendix 1: Biomedical Research Ethics Committee (BREC) approval letter.

Appendix 2: Animal Research Ethics Committee (AREC) approval letter.

Appendix 3: Department of Agriculture, Forestry and Fisheries (DAFF) record.

Appendix 4: Farm personnel consent form.

Appendix 1: Biomedical Research Ethics Committee (BREC) approval letter.

UNIVERSITY OF
KWAZULU-NATAL
INYUVESI
YAKWAZULU-NATALI
RESEARCH OFFICE
Biomedical Research Ethics Administration
Westville Campus, Govan Mbeki Building
Private Bag X 54001
Durban
4000
KwaZulu-Natal, SOUTH AFRICA
Tel: 27 31 2604769 - Fax: 27 31 2604609
Email: BREC@ukzn.ac.za
Website http://research.ukzn.ac.za/Research-Ethics/Biomedical-Research-Ethics.aspx

17 May 2019

Prof SY Essack
Department of Pharmaceutical Sciences
School of Health Sciences
essacks@ukzn.ac.za

Dear Prof Essack

Title: One Health approach to the containment of antibiotic resistance.
Degree: Non-degree
BREC Ref No: BCA444/16

RECERTIFICATION APPLICATION APPROVAL NOTICE

Approved:	17 March 2019
Expiration of Ethical Approval:	16 March 2020

I wish to advise you that your application for Recertification received on 29 April 2019 for the above protocol has been **noted and approved** by a sub-committee of the Biomedical Research Ethics Committee (BREC) for another approval period. The start and end dates of this period are indicated above.

If any modifications or adverse events occur in the project before your next scheduled review, you must submit them to BREC for review. Except in emergency situations, no change to the protocol may be implemented until you have received written BREC approval for the change.

The committee will be notified of the above approval at its next meeting to be held on 11 June 2019.

Yours sincerely

[redacted]

Prof V Rambiritch
Chair: Biomedical Research Ethics Committee

Appendix 2: Animal Research Ethics Committee (AREC) approval letter.

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

09 February 2018

Professor Sabiha Yusuf Essack (3951)
School of Life Sciences
Westville Campus

Dear Professor Essack,

Protocol reference number: AREC/007/018
Project title: Antibiotic Resistance & One Health

Full Approval – Field Research Application

With regards to your application received on 06 February 2018. The documents submitted have been accepted by the Animal Research Ethics Committee and **FULL APPROVAL** for the protocol has been granted with the following conditions:

CONDITIONS

1. Samples will only be collected from Baynesfield Estate for this study.
2. Necessary approval must be obtained from other appropriate Ethics **C**ommittees for the human component part.

Please note: Any Veterinary and Para-Veterinary procedures must be conducted by a SAVC registered VET or SAVC authorized person.

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 09 February 2019.

Attached to the Approval letter is a template of the Progress Report that is required at the end of the study, or when applying for Renewal (whichever comes first). An Adverse Event Reporting form has also been attached in the event of any unanticipated event involving the animals' health / wellbeing.

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

Yours faithfully

[redacted]

.....

Prof S Islam, PhD
Chair: Animal Research Ethics Committee

/ms

Cc Academic Leader Research: Dr P Naidoo
Cc NSPCA: Ms Anita Engelbrecht
Cc Registrar: Mr Simon Mokoena
Cc Joseph Baynes Estate (Pty) Ltd

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

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Appendix 3: Department of Agriculture, Forestry and Fisheries (DAFF) record.

agriculture,
forestry & fisheries

Department:
Agriculture, Forestry and Fisheries
REPUBLIC OF SOUTH AFRICA

Directorate Animal Health, Department of Agriculture, Forestry and Fisheries
Private Bag X138, Pretoria 0001

Enquiries: Mr Herry Gololo • Tel: +27 12 319 7532 • Fax: +27 12 319 7470 • E-mail: HerryG@daff.gov.za
Reference: 12/11/1/5

Prof Sabiha Yusuf Essack
Antimicrobial Research Unit
College of Health Sciences
University of KwaZulu-Natal
Private Bac x54001
Durban
4000
essacks@ukzn.ac.za

Dear Prof Essack,

RE: Permission to do research in terms of Section 20 of the ANIMAL DISEASES ACT, 1984 (ACT NO. 35 of 1984)

Your request for permission under Section 20 of the Animal Disease Act, 1984 (Act No. 35 of 1984) to perform a research project/study, refers.

I am pleased to inform you that permission is hereby granted to perform the following research/study, with the following conditions :

Conditions:

1. This permission does not relieve the researcher of any responsibility which may be placed on him by any other act of the Republic of South Africa;
2. Only bacterial cultures emanating from this study may be stored in microbanks in the biofreezer in the access-controlled Antimicrobial Research Unit at UKZN. All other potentially infectious material utilised or collected during the study is to be destroyed at the completion of the study. Records must be kept for five years for audit purposes.
3. A dispensation application must be made to the Director Animal Health in the event that any of the samples/bacterial cultures are to be used for any further studies or distributed outside of the Antimicrobial Resistance Unit of UKZN;

– 1 –

4. The study is approved as per the application form dated 28 August 2018 and the correspondence thereafter. Written permission from the Director: Animal Health must be obtained prior to any deviation from the conditions approved for this study under this Section 20 permit. Please apply in writing to HerryG@daff.gov.za;
5. Pig faecal and litter samples may only be collected from Baynesfield Farm for which a state veterinary letter has been provided;
6. Samples from pig holding pens may only be collected from Cato Ridge abattoir for which a state veterinary letter has been provided;
7. Caecal, carcass rinsates and carcass swabs may only be collected from Cato Ridge abattoir for which a state veterinary letter has been provided;
8. Removal of samples from the abattoir is subject to compliance with the provisions of the Meat Safety Act, 2000 (Act 40 of 2000), as well as written permission from the abattoir owner;
9. The study may only be performed in the Antimicrobial Resistance Unit laboratories at UKZN;
10. Should any aspect of the study change, please contact the Directorate Animal Health to enquire regarding the need for Section 20 permission;
11. If required, an application for an extension must be made by the responsible researcher at least one month prior to the expiry of this Section 20 approval.

Title of research/study: Antibiotic Resistance and One Health
Researcher (s): Prof Sabiha Yusuf Essack
Institution: Antimicrobial Research Unit, College of Health Sciences, University of KwaZulu-Natal
Your Ref./ Project Number: AREC/007/018, BCA444/16
Our ref Number: 12/11/1/5
Expiry date: 2019-04

Kind regards,

[redacted]

DR. MPHO MAJA
DIRECTOR OF ANIMAL HEALTH
Date: 2018 -09- 17

CLASSIFICATION: CONFIDENTIAL

SUBJECT: SECTION 20 APPROVAL FOR: ANTIBIOTIC RESISTANCE AND ONE HEALTH - LJvR

Appendix 4: Farm personnel consent form.

Appendix 1
Participant Information Leaflet – Farm/Abattoir Personnel

You are being asked to volunteer to participate in a research study entitled "One Health Approach to the Containment of Antibiotic Resistance" conducted under the auspices of the South African Research Chair in Antibiotic Resistance and One Health, funded by the Department of Science and Technology via the National Research Foundation (NRF) over a 5-year period 2016-2020.

Your participation is completely voluntary. Please read the following information about the project. If there is anything in this Consent Document that you do not understand, be sure to ask study personnel to explain that portion of the study. If you voluntarily agree to participate, please sign in the appropriate box below.

This study is being conducted at University of KwaZulu-Natal. The overall purpose of this study is to investigate antibiotic resistance in bacteria isolated from pigs and poultry production, including the personnel that work in the production system from farms to abattoirs to butchers and supermarkets. This is called the "farm-to-form" protocol.

Male and female employees in the profession-related to pigs 18 years and older are eligible to participate. We are asking you to take part in this study because you are member of this category of person. The study involves taking a sample/swab of your nares and hands to undertake bacteriological investigations.

Participation in this study is completely voluntary. If you decide not to participate there will not be any negative consequences. Please be aware that if you decide to participate, you may stop participating at any time and you may decide not to provide any samples.

The investigators believe that the risks or discomforts to you and your animals are minimal.
You will not receive any payment for your participation in this study. Your participation will provide information to improve practices in the pig and poultry food production systems.

Please do not hesitate to contact the following person should you have any queries or concerns related to your voluntary participation:

Professor Sabiha Essack
B. Pharm., M. Pharm., PhD
South African Research Chair in Antibiotic Resistance & One Health
Professor: Pharmaceutical Sciences
Director: Antimicrobial Research Unit
College of Health Sciences
University of KwaZulu-Natal
Private Bag X54001
Durban
4000
South Africa
Telephone: +27(0)31 2607785
Telefax: +27(0)31 2607792
Email: essacks@ukzn.ac.za

We thank you for your invaluable time and your assistance.

Appendix 2
Participant Consent Form

I the undersigned…….. certify that I have been invited to participate in a research study entitled "One Health Approach to the Containment of Antibiotic Resistance" conducted under the auspices of the South African Research Chair in Antibiotic Resistance and One Health, funded by the Department of Science and Technology via the National Research Foundation (NRF) over a 5-year period 2016-2020 conducted by Professor Sabiha Essack 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 I am free to contact the researcher the address below:

Professor Sabiha Essack
B. Pharm., M. Pharm., PhD
South African Research Chair in Antibiotic Resistance & One Health
Professor: Pharmaceutical Sciences
Director: Antimicrobial Research Unit
College of Health Sciences
University of KwaZulu-Natal
Private Bag X54001
Durban
4000
South Africa
Telephone: +27(0)31 2607785
Telefax: +27(0)31 2607792
Email: essacks@ukzn.ac.za

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 address below:

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

_____
Signature

Signed this _____ day of _____ 2016 at _____

Witness 1: _____

Witness 2: _____