Molecular Epidemiology of Antibiotic Resistant *Salmonella* spp. from Farm to Fork in an Intensive Pig Production System in Kwazulu Natal South Africa

Nozipho Pamela Tshakane

218054108

A dissertation submitted in fulfillment of the requirements for the degree of Master of Medical Sciences (Medical Microbiology) in the school of Health Sciences, University of Kwazulu Natal

Supervisor

Professor Sabiha Yusuf Essack

Co-supervisors

Dr. L.K Abia Akebe

Dr. D.G Amoako

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This is a dissertation by manuscript with an overall introduction and final summary.

This is to certify that the content of this dissertation is the original research work of Mrs. Nozipho Pamela Tshakane, supervised by:

Supervisor: Signed [redacted]……..Name: Prof S.Y. Essack Date:12.Nov.2021….

Co-Supervisor: Signed [redacted] Name: Dr L.K Abia Akebe……Date……..12-11-2021

Co-Supervisor: Signed [redacted] ..Name: Dr D. G Amoako….. Date……..12 Nov. 2021

DECLARATION

I, Mrs. **Nozipho Pamela Tshakane**, declare as follows:

1. 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.
2. That my contribution to the project was as follows:
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Signed: 

N.P. Tshakane

Student Number: 218054108

Date: 30/07/2021

DEDICATION

This work is dedicated to my family, my mother Zanele Mahlinza, my daughter Rorisang Blessing Tshakane who has brought a beautiful new meaning to my life and my husband Thapelo Ashely Tshakane.

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Any omissions and shortcomings that may be identified in this piece of work remain the sole responsibility of the researcher.

NP Tshakane

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS AND ACRONYMS

ABR	Antibiotic resistance
AGISAR	Advisory Group on Integrated Surveillance of Antimicrobial Resistance
AGP	Antimicrobial growth promoters
AMR	Antimicrobial resistance
AMU	Antimicrobial use
ARG	Antibiotic resistance gene
AST	Antimicrobial susceptibility testing
BPW	Buffered peptone water
BREC	Biomedical Research Ethics Committee
CDC	Centers for Disease Prevention and Control
CIA	Critically important antimicrobials
CLSI	Clinical and Laboratory Standards Institute
ERIC PCR	Enterobacterial repetitive intergenic consensus polymerase chain reaction
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FAO	Food and Agricultural Organization of the United Nations
LMIC	Low and-middle-income country
MHA	Mueller Hinton agar
MLST	Multi-locus sequence typing
MRDO	Multi-drug resistant organism
NAP	National Action Plan
NDC	Non-digestible carbohydrates
NTS	Non-typhoidal Salmonella
OH	One Health
OIE	World Organisation for Animal Health
PCR	Polymerase chain reaction
PVS	Pig Veterinary Society
RVS	Rappaport-Vassiliadis Soya
SPI	Salmonella pathogenic island
SS	Salmonella Shigella
T3SS	Bacterial type III secretion system
TS	Typhoidal Salmonella
TSB	Tryptone soy broth
UKZN	University of Kwazulu-Natal
VHIA	Veterinary highly important antimicrobial agents
WHO	World Health Organization

LIST OF APPENDICES

ABSTRACT

Antibiotic resistance (ABR) is a worldwide challenge, and, if not resolved, can be a danger to humans, animals and the ecosystem. The inappropriate use and misuse of antibiotics in food animal production creates selection pressure for the development of bacterial resistance. We investigated the molecular epidemiology, antibiotic resistance and virulence of *Salmonella* spp. from farm-to-fork in an intensive pig production system in KwaZulu-Natal. A herd of pigs was followed from birth to slaughter over a period of 4 months. Following ethical approval, a total of 408 samples were collected, which consisted of feces, litter, slurry, hand and nasal swabs from occupationally exposed workers, carcass swabs and rinsate, caecal samples and pork for retail purposes. *Salmonella* was putatively identified using selective media, i.e., Brilliance Salmonella Agar and Salmonella Shigella Agar (SS agar). Identification to species and sub-species level was confirmed by polymerase chain reaction (PCR), where the *invA* gene was used to confirm *Salmonella* spp. and the *iroB* gene for *Salmonella enterica*. Isolates were subjected to antibiotic susceptibility testing using the Kirby-Bauer disk diffusion method against a panel of 14 antibiotics. Isolates were screened for selected virulence genes, *misL, spiC, orfL, sopB, pipD, hilA* and *stn*, conferring intracellular survival (*misL*), type III secretion system (*spiC*), adhesion and autotransporter (*orfL*), type III secreted effector protein (*sopB*), type III secreted effector associated with SPI-1 system (*pipD*), host cell invasion (*hilA*), and enterotoxin production (*stn*) by PCR. Genetic relatedness of the isolates was determined by ERIC-PCR. A total of 399 putative *Salmonella* spp. were detected by selective media, of which 49% (n= 197) were confirmed by the presence of the *invA* gene and 45% (n=179) were identified as *Salmonella enteric*a by the presence of the *iroB* gene. The largest number of Salmonella were isolated from retail meat samples. Antibiotic susceptibility testing showed 10% (n=19) resistance to cefoxitin, 8% (n=16) to amoxicillin and 0.5% (n=1) to gentamicin and chloramphenicol. The isolates carried the *hilA* (*91%*), *stn* (*91%*), *misL* (89%), *pipD* (88%), *spiC* (87%), *orfL* (85%) and *sopB* (72%) virulence genes. The isolates were clonally diverse with 26 ERIC-types and four major ERIC-type groups. The large number of isolates in retail meat samples, their virulence, and, to a lesser extent their antibiotic resistance profiles poses a challenge to the food safety system and requires a comprehensive understanding of molecular epidemiology of the organism so that it's incidence spread can be reduced and better controlled from the primary source within the food chain.

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Salmonella infections are a global public health concern, with approximately 93.8 million cases and 155.000 deaths reported annually (Campos et al., 2019). The most common sources of human infections are food products, especially of animal origin, with pork being among the most relevant. The main symptom of Salmonellosis is diarrhea. Severe cases of invasive *Salmonella* infection such as bacteremia or extra-intestinal infections are uncommon but can occur and affect high-risk groups such as infants, children, the elderly, and immunocompromised patients (Teng et al., 2020). The main source of non-typhoidal Salmonella (NTS) is the gastrointestinal tract of warm-blooded animals, especially food-producing animals, which results in the contamination of food (Campos et al., 2019).

One of the major driving forces behind the inadequate control of this pathogenic bacteria is antibiotic resistance (ABR), which is threatening the interrelated human-animal-environmental health under the One Health framework. In a recent review conducted in the United Kingdom on antimicrobial resistance, it was projected that drug-resistant infections would cause approximately 10 million human deaths by 2050 if the current situation continues (O'Neill, 2016). Resistant bacteria can spread from one geographic area to the next, even worldwide, via the food chain and the environment. One of the sources of ABR in low and middle-income countries (LMICs) is the food production chain (Lekagul et al., 2020).

The rising concern about ABR has drawn attention to the use of antibiotics in livestock. Several antibiotics used in agriculture are categorized as critically important antimicrobials (CIA) in human medicine as per the World Health Organisation (WHO) CIA list. The list consists of 3 categories namely: important, highly important and critically important. The last category is further broken down into high priority CIA which comprises aminoglycosides, aminopenicillins, and carbapenems, and highest priority CIA which includes cephalosporins (third, fourth, and fifth-generation), glycopeptides, macrolides, polymyxin (colistin), and quinolones (World Health Organization, 2019).

Antibiotics have been used in animal farm production since the 1950s to control, treat, prevent disease, and increase productivity. The predicted rise in the demand for livestock production is set to also increase global antimicrobial consumption in livestock (Lekagul et al., 2020). Currently there is a paucity of information on the epidemiology of *Salmonella* spp. from pigs in South Africa. This study

investigated the epidemiology of antibiotic resistant *Salmonella* spp. from farm to fork in an intensive pig production system in Kwazulu Natal, South Africa.

1.2 Literature Review

1.2.1 *Salmonella* spp.

Salmonella is a Gram-negative, facultative, anaerobic, rod-shaped bacilli belonging to the order of Enterobacterales (Jajere, 2019). The pathogen can infect multiple animal hosts as well as humans through a variety of contaminated foods. Contaminated meat is the main foodborne source for Salmonella, which has been detected in retail beef, pork, bison, turkey, chicken, and in other meat types worldwide (Gad et al., 2018). There are two species of *Salmonella*, namely*: Salmonella enterica* and *Salmonella bongori*. *Salmonella enterica* is the most pathogenic species and has >2600 serovars to date (Savas and Çetinkaya, 2020). The pathogen can be classified into two categories: typhoidal serotypes and non-typhoidal Salmonella serotypes (NTS). Typhoidal serovars, namely: *Salmonella enterica* serovars Typhi (*S*. Typhi), *S*. Paratyphi, and *S*. Sendai are human host restricted and cause enteric fever. NTS serovars infect and colonize a broad range of vertebrate animals and cause diarrhea and fever. Moreover, NTS can invade normally sterile sites which can result in bacteremia and meningitis (Wang et al., 2020; Crump et al., 2015).

1.2.2 Brief History

Salmonella was first discovered and isolated from the intestines of pigs infected with swine fever, also known as hog cholera, by Theobald Smith and Daniel Elmer Salmon in 1885. As a result, Joseph Leon Lignières proposed that the pathogen be called Salmonella to honor the American pathologist Dr. Daniel Elmer Salmon (D'Aoust, 1991). The first isolate from a human with typhoid fever was isolated by Gaffky and subsequently studied by Eberth in the 19th century. The bacterium was thought to be an etiological agent of hog cholera (swine fever). In recent years the subject of nomenclature of the genus Salmonella has been debated at length. However, most Salmonella reference centers globally, including the Centers for Disease Prevention and Control (CDC) currently use the nomenclature recommended by the World Health Organisation (WHO), which categorizes the genus of *Salmonella* into two species, namely *S. enterica* and *S. bongori* (Jajere, 2019).

1.2.3 Taxonomy

Salmonella genus is one of the most common foodborne pathogens to be isolated from food animals(Jajere, 2019). Serovars are specific or distinctive groups that are present in a single species of microorganisms such as bacteria and viruses, which share similar distinctive surface structures. The Kauffman and White classification system classifies *Salmonella* further into different serotypes, based

on three major antigenic determinants, namely: somatic (O), capsular (K), and flagella (H). The O antigens located on the outer part of the bacterial cell membrane, are heat-stable, and form the oligosaccharide part of the lipopolysaccharides of the bacterial cells. More than one O antigen can be expressed by a specific *Salmonella* serotype, and they may vary in their chemical constitution. The H antigens are rod-shaped, threadlike structures that form part of the flagella. They are heat-labile and involved in the activation of host immune responses. The surface K antigens are rarely found among most *Salmonella* serotypes. They are heat-sensitive polysaccharides located at the bacterial capsular surface (Guibourdenche et al., 2010). A minority of the serotypes are host-specific and can reside in one or a few animal species: e.g., *Salmonella enterica* serotype Choleraesuis in pigs and *Salmonella enterica* serotype Dublin in cattle. Some serotypes, such as *Salmonella enterica* serotype Enteritidis and *Salmonella enterica* serotype Typhimurium, are important because they can be transmitted from animals to humans globally (Sindiyo, 2019).

1.2.4 Physical and biochemical characteristics

Salmonella is a Gram-negative, rod-shaped bacillus with a size of 0.2-1.5×2-5 μm. The pathogen can be classified as non-fastidious because of its ability to grow and multiply in various environments outside the living host. *Salmonella* spp. are motile by means of a flagella except for *Salmonella* Gallinarum and *Salmonella* Pullorum. Members of this genus are referred to as chemo-organotrophic due to their ability to metabolize nutrients through their respiratory and fermentative pathways (Popoff and Le Minor, 2005). Most of the *Salmonella* serovars, except *S. paratyphi* A and *S. cholearaesuis* produce hydrogen sulphide, and even though they grow in the presence of 0.4-4% of sodium chloride, they do not require sodium chloride for their growth and do not ferment lactose. Almost all serotypes do not produce indole, can ferment a variety of carbohydrates with acid production, hydrolyze urea, and reduce nitrate to nitrite. These properties have been used as a guideline for the development of selective and differential media for culturing, isolation, and presumptive identification of *Salmonella* (Rambach, 1990). These media are MacConkey agar, Salmonella-Shigella Agar (SS), brilliant green agar (BGA), xylose lysine deoxycholate (XLD) agar, lysine iron agar (LIA), Hektoen enteric (HE) agar and triple sugar iron (TSI) agar (Andrews and Hammack, 2001, Anderson and Ziprin, 2001). Few serotypes grow at less optimal conditions of 2-4 °C and higher temperatures of 54°C, but most serotypes grow at a wide range of 5-47 °C with the optimum temperature of 32-35 °C (Pui et al., 2011). Salmonella is heat sensitive and cannot tolerate temperatures higher than 70 °C. Favorable pH ranges from 4 to 9, with optimum values between 6.5 and 7.5. *Salmonella* has been reported to survive in low water activity environments (<0.94), such as dry foods, even though they thrive between 0.99 and 0.94. Salmonella is completely inhibited at a pH of <3.8, a water activity of <0.94 and temperatures of <7 °C (Jajere, 2019).

1.2.5 Epidemiology

The emergence of *Salmonella*, especially multi-drug resistant strains, is a big concern in terms of food safety and public health because the pathogen can be spread to humans through consumption of contaminated pork products, amongst other things (Nair et al., 2018). NTS incidences have increased in these past years resulting in approximately 94 million cases and 155 000 deaths globally, 1.2 million cases, and 450 deaths in the United States; unfortunately, there is extremely limited data for regions such as Southern Asia and the western Pacific (Lammie and Hughes, 2016). Therefore, an in-depth understanding of the epidemiology of *Salmonella* along the food chain is required in order for this problem to be effectively managed (Dang-Xuan et al., 2019).

Studying the epidemiology of *Salmonella* at feed production is vital because feed is the potential source of introducing *Salmonella*. The use of antibiotics in animal feed as growth promoters contributes to the development of antibiotic-resistant *Salmonella* (Castro-Vargas et al., 2020). A large amount of feed is transported and stored regularly in pig production facilities. Even the slightest *Salmonella* contamination at this level has the potential to infect many herds. Therefore, interventions and decontamination steps like heat treatment are needed to avoid the contaminated feed spreading amongst the herds (Rousham et al., 2018). Due to the prevalence of *Salmonella* being too high in pigs in the UK, 19 farms that had maintained a low *Salmonella* seroprevalence were investigated. The 19 "platinum farms" were compared with 38 control farms over 4 years. Faecal and environmental samples were collected, and it was identified that the control farms had a higher percentage (12.1%) of faecal samples positive for *Salmonella* compared to platinum farms (0.4%). The risk factor analyses identified feed deliveries crossing farm perimeter, pelleted feed, regular antibiotic use being associated with the control farms (Smith et al., 2018a). Over and above these measures, the type of feed that is being given to the pigs also plays an important role. A study in Canada aimed to determine the impact of modifying the particle size and processing of the feed on faecal microbiota and *Salmonella* shedding, the pigs were fed a diet of varying processing (pellet or mash) and particle size (500, 750, and 1250 µm) for 21 days. Faecal samples were analyzed for *Salmonella*. It was found that mash processing and large particle size in feed reduces the prevalence of *Salmonella* and yielded beneficial populations of digestive microbiota (Lebel et al., 2017) .

The three main factors during pre-harvest *Salmonella* epidemiology are the introduction, transmission of infection amongst the herds, and antibiotic resistance. In order to effectively control *Salmonella* infection in pigs, one needs to consider minimizing or preventing exposure to *Salmonella*, as well as maximizing resistance. Given the risk factors for *Salmonella*, there are various ways to prevent and control infections, such as pest, bird, and flies control in the holding pens and storage facilities. Moreover, good hygiene practices are crucial in preventing *Salmonella* infections and the spread thereof

in pig production facilities (Smith et al., 2018b). Antibiotics are used at this stage at non-therapeutic levels in livestock production as insurance along with other animal disease risk-management measures. This, then contributes to the emergence and spread of antibiotic resistance (Wall et al., 2016). In a study to analyze bacteriological pollution and detection of antibiotic resistance from the prevailing bacteria resulting from pig farm seepage in Gauteng, South Africa, water samples were collected for six months from various points in the farm for analysis. About 4.30×10^2 to 1.29×10^9 cfu/mL *Salmonella choleraesuis* spp. were isolated, and most were resistant to penicillin G, vancomycin, oxytetracycline, spectinomycin, and lincomycin. It was, therefore, inferred that pig farm seepage could cause drug-resistant bacterial pollution, which can negatively impact the environment by introducing bacteria which harbour antibiotic-resistant genes (Matjuda and Aiyegoro, 2019).

Transportation stress and as well as handling of the pigs can largely increase the number of pigs that exhibit *Salmonella* upon arrival at the slaughterhouse, causing *Salmonella* negative pigs to be infected since positive pigs carry Salmonella in their mouths, gastrointestinal system, and on the skin. Cross-contamination at the abattoir is also a major concern as the bacteria is redistributed during the various slaughter processes. This can be attributed to the fact that there can be meat present from different sources at the slaughterhouse, harboring different pathogens (Castro-Vargas et al., 2020). A study was done to determine the genetic relatedness of isolates from broilers and pigs in abattoirs in Thailand. Fecal samples (604 broilers and 562 pigs) were collected and analyzed for *Salmonella* spp. per ISO 6579:2002. *Salmonella* was detected in 18.05% broiler isolates and 37.54% of pig isolates, and PFGE results showed a high genetic relatedness (85% similarity) between the broiler and pig isolates (Phongaran et al., 2019). Another study was done in Cameroon to investigate pigs and cattle slaughtered in Buea as reservoirs of *Salmonella typhimurium* and the susceptibility of the isolates to antibiotics. A total of 230 samples consisting of ileum, rectum, gall bladder as well as drains swabs were investigated for *Salmonella* using standard microbiological, biochemical and serological methods. Here, 32.6% of the 230 specimens were positive for *S. typhimurium*, with the pigs and abattoir drains having the highest levels of isolation (40%). The antibiotype amoxicillin-doxycycline-ceftazidine was predominant and recorded in 17.3% of the isolates. Multidrug resistance to four antibiotics was evident in 50.7% of the isolates. All the isolates were 100% resistant to tetracycline and ampicillin. Pigs were found to be the reservoirs of *Salmonella typhimurium* in the environment of Buea, Cameroon, meaning foods from these sources had the potential to serve as vehicles for its transmissions to humans if not properly handled (Akoachere et al., 2009).

1.2.6 Virulence factors and pathogenesis

After ingestion and overpowering the resident microbiota, *Salmonella* colonizes the distal part of the small intestine. Ttphoidal Salmonella (TS) have specific virulence factors, which include the typhoid

toxin and virulence capsular polysaccharide (Vi antigen), which are the ones responsible for the development of symptoms and immune invasion. The bacteria then invade the intestinal mucosa through the spleen and liver. It is important to note that these pathogens do not trigger the inflammatory response and infected individuals are most likely to become chronic carriers after recovery (Wang, 2020).

Many virulent factors play a role in the pathogenesis of *Salmonella* infections. The factors include plasmids, flagella, capsule, adhesion systems, and type 3 secretion systems (T3SS) encoded on the *Salmonella* pathogenicity island (SPI)-1 and SPI-2 and other SPIs. *S. enterica*, which is like other enteropathogenic bacteria, can produce different virulent determinants, some of which are part of the adhesion systems, including invasins, fimbriae, adhesins, hemagglutinins, endotoxins, and exotoxins (Foley et al., 2008, Kaur and Jain, 2012). These factors can individually or collectively allow Salmonella to colonize the host by firstly attaching, then invading, surviving and finally bypassing the host's defense mechanism like gastrointestinal proteases, gastric acidity, defensins as well as the aggressins of the intestinal microbiome (Jajere, 2019).

Salmonella pathogenicity islands (SPIs) are gene clusters which are found in certain areas of the chromosomes or plasmids within the bacterial cells, and they carry genes that encode various pathogen factors such as adhesion, invasion, and toxic genes. SPIs are associated with transfer RNA (tRNA) and genetic elements, namely transposons or phage genes, and their composition is different from core genomes (Jajere, 2019).

The *misL, spiC, orfL, pipD, sopB, hilA,* and *stn* are common virulence genes in *Salmonella* of animal origin. Sip proteins (*SipA, SipB, SipC* and *SipD*) are involved in the invasion of cultured epithelial cells. *SipC* is a translocon protein, which is involved in the formation of the SPI-1 T3SS needle complex (Zierler and Galán, 1995, Collazo and Galán, 1997, Scherer et al., 2000, Myeni and Zhou, 2010, Myeni et al., 2013). *SipC* is a *Salmonella* translocon protein which targets F-actin, which is necessary for pathogen internalization as well as promoting *Salmonella* invasion. *SipC* is delivered to the cytosol of the host cell where it can have effector functions, and it also interacts with the intercellular membrane trafficking,and as a result, hinders the correct cellular function by inhibiting the fusion of *Salmonella*-containing phagosomes with lysosomal and endosomal compartments. *SipC* is translocated by the SPI-2 TTSS to the cytosol of macrophages, where it is able to interact with host proteins such as TassC to alter intracellular trafficking (Lou et al., 2019). The *misL* is an SPI-3 encoded protein responsible for membrane insertion and secretion; it is an auto-transported protein only found in pathogenic bacteria. These proteins are made up of N-terminal effector domain and C-terminal conserved domain, which forms a pore in the outer membrane through which the N-terminal domain is translocated. *misL* also has a predicted N-terminal signal sequence necessary for the translocation of the protein across the inner

membrane (Lou et al., 2019). The *orfL* virulence gene is found in SPI-4, it plays a role in adhesion, auto-transportation, and colonisation. The *orfL* gene also has a secretion system which mediates the secretion of toxins and is vital for macrophage survival (Ramatla et al., 2020). The *pipD* is located in SPI-5 and it's a type III secreted effector associated with the SPS-1 system (Zishiri et al., 2016). *pipD* plays a vital role in enteropathogenicity by secreting intestinal mucosal fluids as well as inflammatory responses, which are regulated by SPI-1 and SPI-2 T3SS (Wang et al., 2020). Some virulence genes like *stn* are chromosomally encoded and not situated on SPIs and are also known as *Salmonella* enterotoxin genes. *stn*'s are the dedicated protein secretion system known as type III secretion system (TTSS) which are responsible for the invasion of intestinal epithelial cells and the survival of *Salmonella* in macrophages. This system contributes to pathogenesis by directing secretion and translocation of virulence-associated proteins known as effector proteinsdirectly into the cytosol of the host cell. The *hilA* gene is vital for the regulation of the type III secretion apparatus, as well as activating the expression of invasion genes (Hughes et al., 2008). *hilA* is a transcriptional regulator which is encoded by SPI-1 and directly activates the expression of two SPI-1 genes (*invF* and *sicA*), which encode SPI-1 T3SS apparatus components (Lou et al., 2019). *hilA* gene conferrers pathogenicity by allowing *Salmonella* to invade the epithelial cells (Thung et al., 2018).

1.2.7 Host specificity and adaptation

Host adaptation or specificity can be described as the ability of an organism to cause disease in a particular host regardless of the degree of pathogenicity it exhibits for a different host. Therefore, the host specificity and adaptation of a particular Salmonella is dependent on the serovar's ability to adapt to the host's environment. This ability is dictated by several microbial characteristics responsible for expressions of clinical manifestation in a host (Evangelopoulou et al., 2013). These characteristics include the host's age and immune response, the type of animal species infected, and, most importantly, the infectious dose of the serovar. It has been proven that a particular mechanism responsible for a serovar's virulence in one host can make that serovar less virulent or even avirulent in a different host (Uzzau et al., 2001). For instance, the Choleraesuis serovar is considered a pig-adapted serovar because it only causes severe disease in swine compared to humans. *S. enterica*'s serovars (including Dublin, Paratyphi C, Abortusovis, Pullorim, Gallinarum, Choleraesuis, Enteritidis and Typhimurium), have host specificity that is dependent on gene deletions. The host adaptation process is dependent on two factors: loss of genes and acquisition of new genetic elements encoding specific virulent factors. In a study done to demonstrate how human *Salmonella* clinical isolates were distinct from those of animal origin, *S. enterica* clinical isolates were sourced from humans and animals to determine their virulencecapabilities and the presence of *Salmonella* virulence plasmid encoding the SpvB actin cytotoxin in mice. It was found that all Typhimurium strains from animals were virulent in mice and the strains sourced from humans lacked this ability and vice versa (Jajere, 2019).

1.2.8 Transmission

Salmonella is ubiquitous in the sense that it can be persistent in both water and dry environments for a couple of days up to several months. There are various ways that *Salmonella* can be transmitted. The majority of *S. enterica* serovars are host restricted/specific, as described above. Pig carriers have been an important source of contamination of the environment and at the harvesting stage. What makes these carriers problematic is that they can shed the organism in their feaces without showing any symptoms (Jajere, 2019). A study was done in Kenya and Malawi to investigate *Salmonella* carriage by pigs where a total of 647 faecal and mesenteric lymph node samples were collected during slaughter and analyzed using selective culture methods, antisera testing, and whole-genome sequencing. The prevalence of non-typhoidal *Salmonella* carriage was 12.7% in Busia, 9.1% in Nairobi, and 24.6% in Chikwawa. The discovery of porcine non-typhoidal *Salmonella* carriage in Kenya and Malawi shows the potential zoonotic transmission of diarrhoeal strains to humans in these two countries (Wilson et al., 2020).
Salmonella can be introduced into herds by newly purchased infected pigs. This is because during nursing, piglets are very vulnerable to enteric pathogens such as *Salmonella* spp. Bacterial colonization by these pathogens is due to intestinal dysbiosis, which is usually observed in weaned piglets after a change in diet from milk-based feed to gross feed, as well as the stress associated with co-mingling and new environments. An investigation on the prevalence of *Salmonella* in suckling piglets was undertaken in Spain. Four hundred ninety-five mesenteric lymph nodes and intestinal content from 4-week old slaughtered piglets were analyzed. The overall prevalence in infection and shedding was 36% which indicated that piglets played a vital role in *Salmonella* transmission in the farm (Casanova-Higes et al., 2019).

Pests such as mice, rats, flies, and cockroaches act as reservoirs and play a major role in transmitting *Salmonella* amongst the pigs on the farm. They can carry the pathogen in their intestinal tract asymptomatically without any clinical disease. The mode of transmission is through the food animals ingesting *Salmonella*-infected flies. Rodents can also transmit Salmonella to food animals since they have been associated mostly with the contamination of water, feeds, and stored grains in the farms, and they acquire the bacteria from feaces or sick wild mammals in the farm. A study in Italy that investigated the related risk of *Salmonella* infecting host species such as wild birds, rodents, and arthropods showed that wild animals are exposed to *Salmonella* and can therefore transmit it to food animals on farms (Rubini et al., 2016).

1.2.9 Control of *Salmonella* on farms

Different methods can be used to achieve the control measures on the farm such as antimicrobial medication, vaccination of incoming pigs, preventing infection of the pigs from contamination that may

arise from the environment, nutritional supplements such as probiotics and biosecurity (Tran et al., 2018).

On-farm control measures are the most crucial out of all the stages in the food chain to minimize the risk of feed-borne infections. The feed is produced and transported in large quantities onto the farm, sometimes daily, for use in the pork production industry. Therefore, even a minor Salmonella infection can affect a large herd. Thus, process control and decontamination steps like heat treatment are necessary to prevent the spread of contaminated feed into herds. It is also important to have proper storage for the feed to prevent it from wild birds, pests, and rodents. In a study by (De Lucia et al., 2018), it was found that the feed contamination as a result of fresh wild bird droppings was also introducing *Salmonella* in the farms. Therefore, it is important to ensure that the final feed is protected from contact with reservoir hosts such as birds, rodents, residual contamination in trucks, and contaminated raw materials on the farms and mills. It is also imperative to keep pets such as cats and dogs out of the pig farms and wildlife to prevent *Salmonella* from entering the environment. Dust and aerosols are another form of air transmission that should be avoided (Krijger, 2020).

Maintaining good hygiene standards is very important because weaning needs to take place in a clean environment, and disinfection after each batch is therefore crucial. Sanitary facilities should be readily available for showering and changing clothes and boots before entering the farm. In cases where *Salmonella* is present in the herd, the feed and drinking water need to be treated through acidification by either fermenting the feed or adding organic acids. Lastly, holding pens should be separated by a sufficient height or closed off to prevent the spread of infection (Godyń et al., 2019).

Vaccination seems to be a control measure showing great potential to minimize *Salmonella* in infected farms, but only culling can eliminate infected pigs. It is generally accepted that vaccination can reduce the prevalence of *Salmonella* by preventing it from colonizing the gut and reduce subsequent shedding. Several vaccines have been developed for *Salmonella*, such as inactivated bacterins, which elicit a humoral response, and live vaccines that stimulate cell-mediated immunity. Live vaccines theoretically are the best option because of their ability to stimulate cell-mediated immunity. The only hindrance is licensing, with some of the key issues being clearing the vaccine from the body before the slaughter of the pigs and non-persistence in the environment. Vaccination strategies that involve stimulation of passive immunity from the dam, also known as sow vaccination, and active immunity in the offspring, also known as piglet vaccination, seem to be the most effective control measures (Smith et al., 2018).

Probiotics are non-digestible carbohydrates (NDCs) which include resistant starch and non-starch polysaccharides and oligosaccharides which are resistant to hydrolysis by digestive fluids. Species without a fore-stomach like humans and pigs can resist digestion of NCDs in the upper gastrointestinal tract and reach the ileum and colon, where resident microbes ferment them. Probiotics can be described

as a type of food ingredient that has a favorable direct or indirect impact on beneficial microbiota and intestinal homeostasis and, as a result, can inhibit pathogenic infections by coating the host's epithelial surface, downregulation of adhesion in pathogens, and promotion of beneficial bacteria (Tran et al., 2018).

The importance of animal health and its connection with biosecurity has increased over the years, especially with the emergence of and re-emergence of several diseases that are difficult to control. This is evident in pig farming, as observed with African swine fever or porcine epidemic diarrhoea cases. Biosecurity can be described as the application of measures to reduce the possibility of introducing and spreading pathogens within the farm. In order to develop an effective biosecurity program, one needs to understand how the diseases are transmitted, the risks, which mitigation measures can be more effective, and most importantly, evaluation and improvement of the biosecurity program (Alarcón et al., 2021). In pig farming opportunistic bacteria such as *Salmonella* can complicate infections. The fight against these pathogens will be an ongoing effort, and therefore, biosecurity measures need to be the first-line defense for this battle. A properly implemented biosecurity programme might not eliminate the possibility of disease, but it will reduce the probability. A disease outbreak indicates a breakdown in the programme's implementation. As the industry continues to develop, solid biosecurity programmes are essential for farms to survive and remain profitable in the pig farming business (Kouam et al., 2020). Biosecurity can be divided into two sections, namely internal and external biosecurity. External biosecurity measures include preventing pathogens from entering a herd via purchasing of animal semen, geographic risks (presence of a fence around the farm or bird-proof nets), transport vehicles and visitors. Internal biosecurity measures include management practices, facilities cleaning and disinfection (Allepuz et al., 2018).

1.2.10 Mechanism of action and resistance of selected antibiotics for *Salmonella* infections

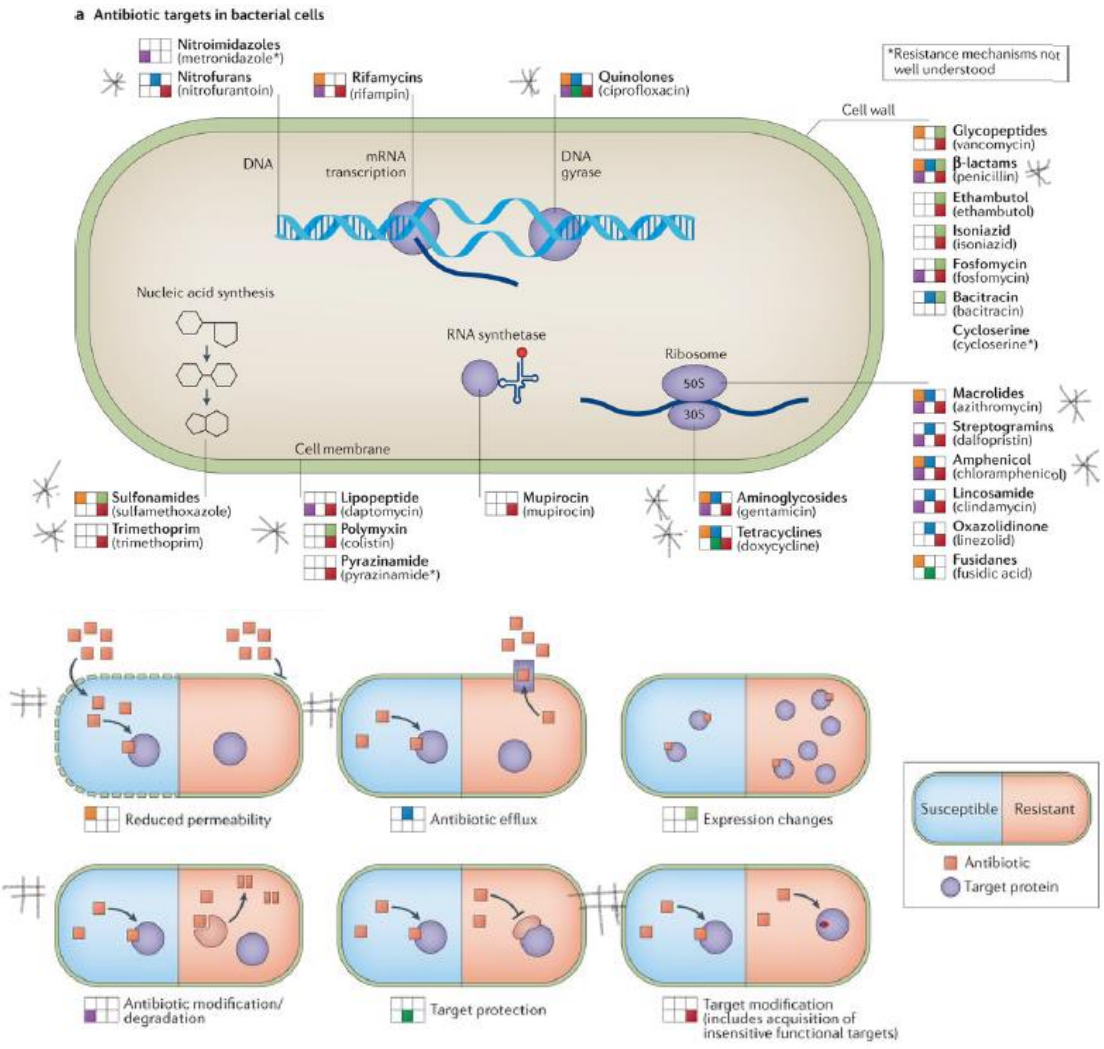

Figure 1: Mechanisms of action and resistance of all clinically available antibiotics. Those denoted with an asterisk (*) are indicated for *Salmonella* spp. while those denoted with a hashtag (#) indicate resistance mechanisms expressed by *Salmonella* spp. (Boolchandani et al., 2019).

1.2.11 *Salmonella* infections in pigs

Salmonella spp. is found in the gastrointestinal tract of most warm-blooded animals and is the leading cause of foodborne outbreaks (Schut et al., 2020). Contaminated meat, including pork, is a common source of infections because swine is a natural host for *Salmonella* spp. (Argüello et al., 2018).

Out of all the serovars that are persistent in pigs, *Salmonella Typhimurium* is of significance due to its role in human salmonellosis and widespread distribution in pig production. It has been reported that the pathogen is detected in high concentrations in faeces within a few hours of ingestion. This was investigated in Spain where 16S rRNA metagenomic sequencing was used to determine the changes in

the gut microbiota of pigs in response to an infection by *Salmonella Typhimurium*. Two-days post-infection, an early infection impacted the microbiome diversity at the mucosa where there was a decrease in representatives of the generally desirable genera such as *Lactobacillus* and *Bifidobacterium.* Severe damage in the epithelium of the ileum mucosa was linked to an increase in Salmonella infection. The observed changes in healthy microbiota and the depletion of beneficial bacteria could contribute to the pathogen's ability to colonize the gut successfully (Argüello et al., 2018).

The prevalence of *Salmonella* shedding is the highest in the nursery stage, and there is a decline during the grower-finisher stage, all the way till slaughter. A trial was conducted in Canada to determine the course of Salmonella shedding as well as antibody response in naturally infected grower-finisher pigs. A total of 45 ten-week-old pigs were taken from a farm with a history of *Salmonella* and housed at a research facility. Faecal samples were collected weekly for 11 weeks, including tissue samples at slaughter. The samples were tested for *Salmonella* using enzyme-linked immunosorbent assay (ELISA). The results showed that 91% of the pigs shed Salmonella over 10 weeks: *S. Typhimurium* was the highest sub-species detected at 28%, *S.* I:Rough-O was the least at 7%. This study indicated that pigs might shed Salmonella into the mid-point of the grower-finisher stage, and there is the possibility of re-infection with different serotypes (Nair et al., 2018).

The indiscriminate use of antibiotics to control salmonellosis in animals has contributed to the emergence and the spread of drug-resistant bacteria in both pathogenic and commensal organisms. In a study conducted to determine the presence, serovar distribution, and antibiotic resistance profiles of *Salmonella* isolated from food animals in South Africa, 1069 rectal and cloacal swabs were collected from pigs (n=322), chickens (n=286), and goats (n=461) from farms situated in Kwazulu-Natal, North West, Northern Cape, and Eastern Cape. The occurrence of *Salmonella* per animal species was highest in pigs (5.90%: n=19) and chicken (3.15%: n=9) while occurrence in goats was the lowest (0.43%: n=2). Most isolates were resistant to at least one antibiotic (n=20: 66.7%) with the resistance being predominant towards trimethoprim (n=11: 36,7%), ampicillin (n=5: 16,7%), oxytetracycline (n=3: 10%) and kanamycin (n=1:3, 3%). There were nine *Salmonella* serovars obtained which included *S.* Techimani, six isolates were assigned to *Salmonella* II and some of the *Salmonella* were untypable (n=6). The results demonstrate the presence of diverse *Salmonella* serovars, which were not previously isolated from food animals in South Africa (*S.* Techimani) (Mathole et al., 2017).

1.2.12 Food animals' production systems

Food animal production systems can be classified into three categories, namely: land-based intensive systems, land-based extensive systems, and organic systems. Land-based intensive systems refer to a large number of animals that are kept in a high-density environment which is often indoors. Animals that are bred in intensive systems tend to have reduced variability in their microbiota and susceptibility

to colonization with certain bacteria (Schokker *et al*., 2014). This, together with the close proximity of the animals in the intensive system can amplify any resistant population of bacteria. Therefore, based on these factors, an intensive system with poor biosecurity and general herd health may be at risk of being colonized by pathogenic strains of bacteria, including drug-resistant bacteria (Zhu et al., 2013). Intensive production is expected to increase in the future due to the ever-growing demand for livestock products. However, intensive systems that have high biosecurity can eliminate many problems such as AMR (FAO, 2016).

Changes in the pig production sector have taken place in many countries in recent decades to increase the production of pig meat per capita and per farm. There has been a shift from small-scale farming, which is extensive, subsistence, and mixed production systems, towards intensive farming, which is at a larger scale, geographically concentrated, commercially focused, and highly specialized. Most small-scale farms have been gradually replaced by large-scale farming after intensive farming first commenced in Thailand as far back as the 1960s. New technology was introduced with intensive farming, such as temperature control between 25 °C and 27 °C since pigs are sensitive to heat stress (Thanapongtharm et al., 2016).

The second category is land-based extensive systems, which can be characterized by low inputs, which in turn yields low outputs, the opposite of intensive systems. Extensive systems consist of free-roaming animals in large quantities. This particular system poses a risk of pathogenic bacteria transmission via exposure to several bacterial species, environmental species such as soil bacteria which may not be present in an intensive system (FAO, 2013b).

Livestock in organic systems that are integrated with an extensive or free-range or outdoor farming model which might be in contact with bacterial population through soil bacteria and effluent may result in organic or extensively produced livestock harboring AMR just like the conventionally produced livestock (FAO, 2016).

1.2.13 Antibiotic use in food animals

There is an increasing demand for animal protein in low-and middle-income countries (LMICs), and as a result, human demand now exceeds the supply. In order to meet this demand, countries like Brazil, Russia, India, China, and South Africa have changed to cost-effective intensive livestock production systems. These production systems require antibiotics to keep animals healthy to ensure productivity. Therefore antibiotic usage in food-animal production systems has risen and it is predicted to rise by 67% between 2010 and 2030 (Van Boeckel et al., 2015). Antibiotics are used in food animal production systems for several reasons, such as for therapy (treatment of sick animals), metaphylaxis (control

treatment for the whole herd in case of a disease outbreak), prophylaxis which is also referred to as prevention treatment and growth promotion (Gemeda et al., 2020).

Exposing bacteria to sub-therapeutic concentrations of antibiotics can have significant impact on ABR evolution (Andersson and Hughes, 2014). Antimicrobial growth promoters (AGPs) are mostly used in intensive food-animal production systems as feed additives and have resulted in gut microbiota alteration of treated livestock, promoting the development and spread of resistance in the animals and the environmental microbiome (You and Sibergeld, 2014). AGPs are used without supervision and veterinary prescription in many countries (Laxminarayan et al., 2013). Besides using AGPs, the animal feed can also be supplanted with other non-antimicrobial compounds such as Sepiolite. This compound has been used as an additive for 31 years now in the EU. It aids with better absorption of nutrients by slowing down the passage of food through the intestinal tract. However, Sapolite can promote the horizontal transfer of resistance plasmids amongst the bacteria (Rodríguez-Beltrán et al., 2013).

Antibiotics can be administered to susceptible but healthy animals to prevent infectious disease from occurring, and this is referred to as prophylaxis. If therapeutic dosing levels are adhered to, then there should be less risk of inducing resistance in exposed bacterial populations, even though such antimicrobial use (AMU) may have a similar effect as growth promoters. There is also a greater risk of survival of exposed bacteria and generation of resistance when antibiotics are administered in groups through water and feed because of variations in consumption by the individual animals and the number of exposed animals (Osei Sekyere, 2014).

Antibiotics can also be administered at therapeutic doses to all animals in a group where some have exhibited infection, known as metaphylaxis. Therefore, metaphylaxis aids in both treatment of the infected animals and prevention for those who are healthy but at risk of infection. The challenge arising with this form of treatment is that broad-spectrum antibiotics are often used in livestock without a prior confirmed diagnosis, such as undertaking antibiotic susceptibility testing. Correct dosing is crucial to ensure that the duration of systematic treatment is only long enough to eliminate the infection in the livestock, as this can result in further selective pressure on the gut microbiota (FAO, 2016a).

WHO CIA serves as a standard for animal producers globally as it guides global retail companies. The list considers global AMU and categorizes compounds as medically important if used in human medicine anywhere in the world. The list was initially published in 2005, the most recent version (sixth version) was released in 2019. There are four categories, namely: critically important, highly important, important and lastly, currently not used in humans. Critically important antimicrobials have two subcategories called highest priority and high priority, which need to meet two criteria. Firstly, it needs to be the only or one of the few available therapies to treat serious bacterial infections in people.

Secondly, they must be used to treat infections where there is evidence of transmission of resistant bacteria or genes in people arising from non-human sources. Highly important antimicrobials should meet either 1 or 2 criteria previously mentioned but not both. Important antimicrobials can be any compounds used in human medicine, meeting neither criteria 1 nor 2 (Scott et al., 2019).

The World Organisation for Animal Health (OIE) also created their list of antimicrobials which are important in veterinary medicine which overlaps with the WHO CIA list. Antimicrobial agents in the OIE List are classified into three categories namely Veterinary Critically Important Antimicrobials Agents (VCIA), Veterinary Highly Important Antimicrobials Agents (VHIA) and Veterinary Important Antimicrobials Agents (VIA).For example, bacitracin, which is a cyclic peptide, is low on the WHO CIA list, but it is rated as 'Veterinary Highly Important Antimicrobial Agents (VHIA). Among the VCIA in the OIE List, some are referred to be critically important for both human and animal health such as fluoroquinolones, third and forth generation of cephalosporins. Colistin was moved to the WHO category of Highest Priority Critically Important Antimicrobials in 2006, therefore it can no longer be used as a growth promoter or preventative treatment applied via feed or water in the absence of clinical signs in the animal that is being treated. It is important to note that both the organizations used different goals and criteria to develop their CIA lists (OIE – World Organization for Animal Health, 2015).

Since AMR poses a big threat to human, animal, and environmental health, it is important that ethical practice guidelines be implemented on how to effectively and responsibly use antimicrobials in the pig industry of South Africa. Members of the Pig Veterinary Society (PVS) of the South African Veterinary Association have committed themselves to putting the guidelines into action to preserve the future effectiveness of the antimicrobials. PVS also has objectives regarding responsible and prudent use of antimicrobials, and implementing practical measures and recommendations which will improve pig health and welfare while preventing and minimizing the selection for the emergence and spreading of antibiotic-resistant bacteria in pigs and humans. Responsible measures include the following:

- Ensuring responsible use of antimicrobial agents in pigs to optimize both their efficacy and safety to comply with the ethical and economic obligation needed to keep the pigs in excellent health.
- Reducing or preventing the transfer of resistant microorganisms within the pigs, the environment, and between pigs and humans.
- Encouraging prudent use of antibiotics in order to ensure that they remain effective.
- Protecting consumer health by ensuring the safety of pork regarding residues of antimicrobial agents.
- Protect the environment by safe disposal of empty or expired products (Pig Veterinary Society of the South African Veterinary Association 2016).

With the increasing demand for pork and pork products in South Africa and globally, it is crucial that any factors that might compromise the quality and safety of the meat products, such as drug-resistant pathogens and antibiotic residues, be investigated. *Salmonella* spp. is on the WHO priority pathogens list for the research and development of new antibiotics. *Salmonella* spp. is one of the leading foodborne pathogens causing outbreaks globally. Understanding the dynamics and epidemiology of *Salmonella* infections in pig production is vital to improve prevention and control measures for Salmonella especially antibiotic-resistant and virulent strains at farm level, as well as reducing the "farm-to-fork" transmission of the pathogen. It was, therefore, necessary to investigate the molecular epidemiology of virulence in *Salmonella* spp. from "farm to fork" in pigs from an intensive farming system.

1.3 Aim

To delineate the molecular epidemiology of *Salmonella* spp. from farm-to-fork in an intensive pig production system in uMgungundlovu District Kwazulu-Natal, South Africa.

1.4 Objectives

- To collect samples collected across the farm- to- fork, i.e., (1) faeces, slurry, and human hand and nasal samples bi-monthly from the farm from birth to sluaghter, (2) transport vehicles, (3) caecal samples and carcass rinsate post-slaughter at the abattoir, and (4) swabs of the retail meat samples.
- To putatively identify *Salmonella* spp. on selective media using Brilliance Salmonella Agar and Salmonella Shigella Agar (SS agar).
- To identify *Salmonella* spp. to species level by PCR using *invA* gene to confirm *Salmonella* spp. and the *iroB* gene to identify *Salmonella enterica*.
- To ascertain the antibiotic susceptibility of isolates against the WHO Advisory Group on Integrated Surveillance of Antimicrobial Resistance (AGISAR) recommended panel of antibiotics by Kirby-Bauer disk diffusion susceptibility tests as per the Clinical and Laboratory Standards Institute (CLSI) guidelines.
- To identify selected virulence genes.
- To undertake strain typing by ERIC-PCR to ascertain clonal relatedness across the farm-to-fork continuum.

1.5 Study Outline

1.5.1 Ethical considerations

Ethical approval was granted by Biomedical Research Ethics Committee (BREC) (Reference No.: BCA4444/16) and the Animal Research Ethics Committee (AREC) (Reference No.: 007/018) of the University of Kwazulu-Natal (UKZN). Section 20A permission to conduct the study was obtained from the Department of Agriculture, Forestry and Fisheries (Reference No. 12/11/1/5). The name of the farm,

abattoir, as well as the employee's names were not disclosed. Human samples in the form of hand and nasal swabs were obtained upon explicit, voluntary informed consent.

1.5.2 Study design and general methodology

The study addresses the molecular epidemiology of *Salmonella* spp. from "farm to fork" in an intensive pig production system in Kwazulu-Natal uMgungundlovu Region, South Africa. Samples along the pig farm-to-fork continuum, (1) samples on the farm (feacal matter, slurry, and human swabs), (2) truck to slaughterhouse, (3) post-slaughter (caecal contents, carcass rinsate, and retail meat swabs) as per WHO-AGISAR sampling method for "farm to fork" were evaluated for *Salmonella* spp. using selective media, i.e., Brilliance Salmonella Agar and Salmonella Shigella Agar (SS agar) (Oxoid, Hampshire, England). Molecular confirmation by PCR targeting the *invA* and *iroB* genes was used to confirm *Salmonella* spp. and *Salmonella enterica*, respectively. Susceptibility profiles were assessed by Kirby-Bauer disk diffusion method using WHO-AGISAR recommended panel of antibiotics for *Salmonella* spp. Virulence genes were detected using conventional PCR, targeting the *misL, sopB, spiC, orlF, pipD, hilA and stn* genes. The genetic relatedness between isolates throughout the continuum was evaluated using ERIC-PCR.

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2 CHAPTER TWO

This dissertation is in a manuscript format as follows:

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Contributions

- Mrs. Nozipho Tshakane, as the principal investigator, worked on the study design with guidance from supervisors, carried out the laboratory work and wrote the manuscript.
- Dr. Daniel Gyamfi Amoako and Dr. Akebe Luther King Abia, as co-supervisors assisted with laboratory protocols and analysis of the results.
- Prof Sabiha Y. Essack, as the principal 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 *Salmonella* spp. from Farm to Fork in an Intensive Pig Production System in KwaZulu- Natal South Africa**

Nozipho Tshakane, Daniel Gyamfi Amoako, Akebe Luther King Abia, Sabiha Y. Essack

[1]**Antimicrobial** Research Unit, College of Health Sciences, University of KwaZulu-Natal, Durban, South Africa.

Corresponding author*

Sabiha Y. Essack,

Antimicrobial Research Unit

College of Health Sciences

University of KwaZulu-Natal

Private Bag X54001

Durban

4000

Email: essacks@ukzn.ac.za

Running title: Antibiotic resistant *Salmonella* spp. from pigs in Kwazulu-Natal, South Africa.

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Abstract

Antibiotic resistance (ABR) is a worldwide challenge, and, if not resolved, can be a danger to humans, animals and the ecosystem. The inappropriate use and misuse of antibiotics in food animal production creates selection pressure for the development of bacterial resistance. We investigated the molecular epidemiology, antibiotic resistance and virulence of *Salmonella* spp. from farm-to-fork in an intensive pig production system in KwaZulu-Natal. A herd of pigs was followed from birth to slaughter over a period of 4 months. Following ethical approval, a total of 408 samples were collected, which consisted of feces, litter, slurry, hand and nasal swabs from occupationally exposed workers, carcass swabs and rinsate, caecal samples and pork for retail purposes. *Salmonella* was putatively identified using selective media, i.e., Brilliance Salmonella Agar and Salmonella Shigella Agar (SS agar). Identification to species and sub-species level was confirmed by polymerase chain reaction (PCR), where the *invA* gene was used to confirm *Salmonella* spp. and the *iroB* gene for *Salmonella enterica*. Isolates were subjected to antibiotic susceptibility testing using the Kirby-Bauer disk diffusion method against a panel of 14 antibiotics. Isolates were screened for selected virulence genes, *misL, spiC, orfL, sopB, pipD, hilA* and *stn*, conferring intracellular survival (*misL*), type III secretion system (*spiC*), adhesion and autotransporter (*orfL*), type III secreted effector protein (*sopB*), type III secreted effector associated with SPI-1 system (*pipD*), host cell invasion (*hilA*), and enterotoxin production (*stn*) by PCR. Genetic relatedness of the isolates was determined by ERIC-PCR. A total of 399 putative *Salmonella* spp. were detected by selective media, of which 49% (n= 197) were confirmed by the presence of the *invA* gene and 45% (n=179) were identified as *Salmonella enteric*a by the presence of the *iroB* gene. The largest number of Salmonella were isolated from retail meat samples. Antibiotic susceptibility testing showed 10% (n=19) resistance to cefoxitin, 8% (n=16) to amoxicillin and 0.5% (n=1) to gentamicin and chloramphenicol. The isolates carried the *hilA* (*91%*), *stn* (*91%*), *misL* (89%), *pipD* (88%), *spiC* (87%), *orfL* (85%) and *sopB* (72%) virulence genes. The isolates were clonally diverse with 26 ERIC-types and four major ERIC-type groups. The large number of isolates in retail meat samples, their virulence, and, to a lesser extent their antibiotic resistance profiles poses a challenge to the food safety system and requires a comprehensive understanding of molecular epidemiology of the organism so that it's incidence spread can be reduced and better controlled from the primary source within the food chain.

1. Introduction

Salmonella infections are a global public health concern, with approximately 93.8 million cases and 155.000 deaths reported annually (Campos et al., 2019). The most common sources of human infections are food products, especially of animal origin, with pork being among the most relevant. The main symptom of Salmonellosis is diarrhea. Severe cases of invasive *Salmonella* infection such as bacteremia or extra-intestinal infections are uncommon but can occur and affect high-risk groups such as infants, children, the elderly, and immunocompromised patients (Teng et al., 2020). The main source of non-typhoidal Salmonella (NTS) is the gastrointestinal tract of warm-blooded animals, especially food-producing animals, which results in the contamination of food (Campos et al., 2019).

One of the major driving forces behind the inadequate control of this pathogenic bacteria is antibiotic resistance (ABR), which is threatening the interrelated human-animal-environmental health under the One Health framework. In a recent review conducted in the United Kingdom on antimicrobial resistance, it was projected that drug-resistant infections would cause approximately 10 million human deaths by 2050 if the current situation continues (O'Neill, 2016). Resistant bacteria can spread from one geographic area to the next, even worldwide, via the food chain and the environment. One of the sources of ABR in low and middle-income countries (LMICs) is the food production chain (Lekagul et al., 2020).

The rising concern about ABR has drawn attention to the use of antibiotics in livestock. Several antibiotics used in agriculture are categorized as critically important antimicrobials (CIA) in human medicine as per the World Health Organisation (WHO) CIA list. The list consists of 3 categories namely: important, highly important and critically important. The last category is further broken down into high priority CIA which comprises aminoglycosides, aminopenicillins, and carbapenems, and highest priority CIA which includes cephalosporins (third, fourth, and fifth-generation), glycopeptides, macrolides, polymyxin (colistin), and quinolones (World Health Organization, 2019).

Antibiotics have been used in animal farm production since the 1950s to control, treat, prevent disease, and increase productivity. The predicted rise in the demand for livestock production is set to also increase global antimicrobial consumption in livestock (Lekagul et al., 2020). Currently there is a paucity of information on the epidemiology of *Salmonella* spp. from pigs in South Africa. This study investigated the epidemiology of antibiotic resistant *Salmonella* spp. from farm to fork in an intensive pig production system in Kwazulu Natal, South Africa.

2. Methodology

2.0. Materials and methods

2.1 Ethical considerations

Ethical approval was granted by Biomedical Research Ethics Committee (BREC) (Reference No.: BCA4444/16) and the Animal Research Ethics Committee (AREC) (Reference No.: 007/018) of the University of Kwazulu-Natal (UKZN). Permission to conduct the study was obtained from the Department of Agriculture, Forestry and Fisheries (Reference No: 12/11/1/5). The name of the farm, abattoir, as well as the employee's names were not disclosed. Human samples in form of hand and nasal swabs were obtained upon explicit, voluntary informed consent.

2.2 Study population and sampling

Samples were collected from a pig farm in uMgungundlovu District, Kwazulu-Natal in South Africa every two weeks from a herd that was followed from birth up until the pigs were ready for slaughter after 4 months. Faecal, slurry, and hand and nasal swabs of occupationally-exposed workers were collected from the farm. Thereafter, samples were collected from the truck transporting the pigs to the abattoir, caecal samples, carcass rinsate samples and retail meat swabs were collected at the abattoir.

Table 1: Farm to fork sampling framework.

Sampling points:	**Week**	**Sources**	**No. of samples per visit**	**No. of isolates recovered**
Farm	1-17	Fresh pig faeces	10	4
		Slurry/ wastewater	6 (1ml each)	15
		Human	6	11
Transport	Week 18	Truck floor	60 (Pooled into sets of 10)	6
Abattoir		Carcass rinsates	48 (Pooled into sets of 12)	0
		Caecal samples	48 (Pooled into sets of 12)	0
Retail meat		Retail meat (Whole Carcass)	20 (Pooled into sets of 5)	39
		Retail meat (Head)	20 (Pooled into sets of 5)	77
		Retail meat (Thigh)	20 (Pooled into sets of 5)	45
Total				197

2.3 Sample collection

Faecal samples were collected using a sterile spatula and transferred to 100 ml wide-mouth plastic containers, immediately after being dropped. Slurry samples were obtained from the outlet of the piggery and transferred into a 100 ml wide-mouth plastic container. Unwashed hands of the farm workers were swabbed on the palms, inbetween fingers, at the back of the hand and the fingernails. Nasal swabs of the same farm workers were also collected using a sterile swab from both nostrils. The truck transporting the pigs from the farm to the abattoir was swabbed before and after loading of the pigs. To collect the caecal samples, sterile knives were used to cut approximately 50g of the intestines into a sterile zip lock bag. The rinsate water used for washing the carcass after slaughter was collected in sterile 100 ml plastic containers. Sterile swabs were used to swab the the head, body, and thigh of the retail meat carcass. After collection the samples were labelled and placed in cooler box with ice to ensure that the samples reached the laboratory at a temperature below 5°C. Samples were processed immediately upon arrival at the laboratory.

2.4 Phenotypic Isolation and identification of *Salmonella*

2.4.1 Enrichment

For the faecal, slurry, and caecal samples, 10g of the sample was weighed out into 90 ml of Buffered Peptone Water (BPW) and incubated at 37°C for 24 hours. Thereafter 1 ml was inoculated into 9 ml of Rappaport-Vassiliadis (RV) soya peptone broth and incubated at 42°C for 24 hours (Gelaw et al., 2018).

2.5 Microbiological analysis

A loopful of inoculum was streaked on Brilliance Salmonella Agar and Salmonella Shigella Agar (SS agar) (Oxoid, Hampshire, England) and incubated at 37°C for 24 hours. Typical colonies, i.e., shiny, purple colonies on Brilliance Salmonella Agar and clear colonies with a black center on Salmonella Shigella Agar, were re-streaked on both media for purification purposes and incubated at 37°C for 24 hours. Single typical colonies were then streaked on Nutrient Agar (Lab M, Lancashire, England) and incubated at 37°C for 24 hours. Afterwards the single colonies were used for DNA extraction. The rest of the single colonies were preserved in cryogenic vials with microbeads containing 1 ml of Tryptone Soy Broth (TSB) (Oxoid, Hampshire, England) with 20% glycerol.

2.6 DNA Extraction

The boiling method was used for the extraction of DNA. Briefly, a loopful of the fresh 24 hour culture was inoculated in an Eppendorf tube containing 300 µL of sterile distilled water. The inoculum was mixed and then placed in a heating block for 15 minutes at 100 °C. Thereafter it was centrifuged at 5000 rmp for 5 minutes. The supernatant containing the DNA was transferred to a sterile tube, and the pellet was discarded (Yamagishi et al., 2016).

2.7 Molecular confirmation

Real time PCR was conducted on the DNA extracted from all the putative *Salmonella* samples. The primers used are listed in **Table S1**. *InvA* gene was used to confirm *Salmonella* spp. The total PCR reaction mixture of 25 µL contained 12.5 µL Luna PCR Master Mix, 1 µL *invA* forward primer, 1 µL *invA* reverse primer, 4 µL of template DNA and 6.5 µL dH_2O. The PCR protocol was as follows: 34 cycles consisting of denaturation for 30 minutes at 95°C, annealing for 30s at 58°C, extension for 1 min at 72 C°. The presence of *iroB* which is unique to *S. enterica*, was used to confirm the sub-species type with the protocol being the same as the one for *invA* gene (Zishiri et al., 2016). *Salmonella enterica* ATCC 10708 strain was used as positive control and nuclease-free water was used for the negative control.

2.8 Antimicrobial susceptibility testing

The *Salmonella* isolates were tested against 14 antibiotics recommended by the World Health Organization Advisory Group on Integrated Surveillance of Antimicrobial Resistance using the Kirby-Bauer disc diffusion method and interpreted according to the Clinical and Laboratory standards Institute guidelines (CLSI, 2019). The European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2017) guidelines were used for antibiotic breakpoints absent from the CLSI, 2019. The panel consisted of, amoxicillin (10 µg), ceftriaxone (30 µg), cefoxitin (30 µg), cefepime (30 µg), imipenem (10 µg), meropenem (10 µg), tetracycline (30 µg), trimethoprim-sulfamethoxazole (25 µg), ,gentamicin (10 µg) chloramphenicol (30 µg), azithromycin (15µg), nitrofurantoin (300 µg) ciprofloxacin (5 µg), perfloxacin (5 µg) and tigecycline (15 µg). Isolates were categorized as susuceptible (S), intermediate susceptibility (I) or resistant (R) as per CLSI, 2019 and EUCAST, 2017 recommendations. *E. coli* ATCC 25922 was used as the control.

2.9 Detection of virulence genes

Seven virulence genes, (*misL, sopB, spiC, orlF, pipD, hilA, stn*) were selected based on their pathogenic potential to humans and food animals (Zishiri *et al.*, 2016, Thung et al., 2018). The total reaction mixture of 25 µL which comprised of 1 µL bacterial DNA 12,5 of Dream Taq mastermix (ThermoFisher Scientific Waltham, MA, USA), 1.5 µL each of forward and reverse primers and 8.5 µL of nuclease-free water (ThermoFisher Scientific Waltham, MA, USA).. For *spiC,* the PCR conditions were as follows: initial denaturation at 94°C for 12min, denaturation at 94 °C for 1 min, annealing at 54 °C for 30 s, extension at 72 °C for 5 min with a total of 34 cycles, for each cycle of the extension, 5 s was added. For *pipD,* initial denaturation at 94 °C for 5 min, 34 cycles for denaturation at 94 °C for 25 s, annealing at 56 °C for 30 s, extension at 72 °C for 50 s, final cycle at 72 °C. For *misL and orfL* the PCR conditions were as follows. initial denaturation at 94 °C for 3 min,35 cycles of denaturation at 94 °C for 1 min, annealing at 58 °C for 1min, extension at 72 °C for 1 min. Final cycle was at 72 °C for 5 min.

The primers used for detecting virulence genes can be found in **Table S2** (Zishiri et al., 2016)**.** PCR was conducted on the T100 Thermal cycler™ (Bio-Rad, Hercules, California, USA). The end products from PCR were loaded on 1% agarose gel containing 5 µL ethidium bromide in 1X Tris-acetate-EDTA (TAE) buffer and run at 120V for 60 minutes. The gels were observed, and the images were captured using the Gel Doc™ XR+ imaging system (Bio-Rad, Hercules, California, USA), and 100-base pair (bp) and 1 kilobase (kb)-DNA ladder (Biolabs, New England, Hertfordshire, UK) were used as reference molecular markers.

2.10 Clonality

Enterobacterial Repetitive Intergenic Consensus -Polymerase Chain Reaction (ERIC-PCR) was used to investigate the genetic relatedness of the isolates. A total of 50 isolates were analyzed, where at least one representative sample was chosen from all the different weeks, sites, sources and based on the unique virulence profiles. DNA was extracted by means of GeneJET Genomic DNA purification kit (ThermoFisher Scientific, Waltham, MA, USA) as per the manufacturer's instructions. The primers ERIC-PCR: 5' ATG TAA GCT CCT GGG GAT TCA C-3' (F) and 5' AAG TAA GTG ACT GGG GTG AGC G-3' (R) (Inqaba Biotechnical Industries (Pty) Ltd., Pretoria, South Africa), were used (Levent et al., 2019).. The total reaction mixture of 25 µL comprised of 1 µL bacterial DNA 12,5 of Dream Taq mastermix (ThermoFisher Scientific Waltham, MA, USA), 1.5 µL each of forward and reverse primers and 8.5 µL of nuclease-free water (ThermoFisher Scientific Waltham, MA, USA). The PCR conditions were one cycle of at 94 °C for 3 min , 30 cycles of 94 °C for 30 sec, 50 °C for 1 min, 65 °C for 8 min and a final step at 65 °C for 16 min and the samples were processed with a positive control *Salmonella enterica* (ATCC 10708) and negative control (nuclease free water) (Levent et al., 2019).

The end products from ERIC PCR were loaded on 1% agarose gel containing 5 µL ethidium bromide in 1X Tris-acetate-EDTA (TAE) buffer and run at 75V for 180 minutes. The gels were observed, and the images were captured using the Gel Doc™ XR+ imaging system (Bio-Rad, Hercules, California, USA). A 100-base pair (bp)-DNA ladder (Biolabs, New England, Hertfordshire, UK) was used as a reference molecular marker. The resulting electrophoretic patterns were analyzed by means of the Bionumerics software 6.6 (Applied Maths NV, Belgium) using the Dice coefficient. Clustering was done by the unweighted pair group with arithmetic averages (UPGMA) using 1% tolerance and 0.5% optimisation. Clusters were identified based on a similarity of 70%.

3. Results

3.1 Prevalence of *Salmonella* .. along the farm to fork continuum

A total of 399 putative *Salmonella* spp. were detected by selective media, 49% (n= 197) were confirmed by the presence of the *invA* gene and 45% (n=179) were identified as *Salmonella enteric*a by the

presence of the *iroB* gene. *Salmonella* spp. isolates were isolated from slurry, faeces and human (hands and nose) on the farm during weeks 1-17, from the truck transporting the pigs to the abattoir in week 18, and from the caeca, carcass rinsate and retail meat at the abattoir in week 18 (Table 1). The largest number of the isolates (161, 81%) was detected in retail meat (the head, thigh and body of the pigs) followed by 7.1% (14) in week 13, a month from slaughter, 4% (8) from faecal samples at week 1, 3% (6) from the truck after loading the pigs for transportation to the slaughter in week 18, 2.5% (5) during week 9 and 1% (2) at week 7 from faecal samples.

3.2 Antibiotic susceptibility testing

The highest resistance was observed in cefoxitin 10% (19), followed by amoxicillin 8% (16) with the least resistance to gentamicin and chloramphenicol 0.5% (1). The isolates were susceptible to the rest of the antibiotics tested. There were no multi-drug resistant isolates detected.

3.3 Detection of virulence genes

The most prevalent virulent genes were *stn* and *hilA*, detected in 91% of the isolates, *misL* was the second highest at 89%, followed by the *pipD* gene (88%), *spiC* (87%) and *orlF* (85 %). The *sopB* gene was the least represented at 72%.

3.4 Enterobacterial Repetitive Intergenic Consensus (ERIC)-Polymerase Chain Reaction (PCR)

Salmonella isolates were grouped into 26 ERIC types with four major ERIC-types. All isolates constituting the largest group X (n=10) were recovered in week 18 from retail meat except for 1 isolate which was recovered at the farm (human swabs) in week 11. The second largest was V (n=5) with two isolates from retail meat (head and thigh), isolated in week 18 and 3 from the farm in week 15 (human and slurry). The third largest groups were S (n=4) during week 18 in retail meat (thigh and body) and F (n=4) with 1 isolate from week 11 in the farm (faecal) and the rest of the isolates from retail meat (head and body).

4. DISCUSSION

Salmonella spp. infection is a worldwide public health issue (Santana et al., 2020). We isolated *Salmonella* spp. at different points along an intensive pig production system. A total of 406 samples were collected along the farm to fork continuum and 198 (47%) were confirmed to be *Salmonella spp.* by the presence of the *invA* gene. This prevalence is quite high compared to the prevalence of only 5.90% (19/322) confirmed *Salmonella* isolates in a previous study aimed at investigating the epidemiology of Salmonella in domestic livestock in South Africa (Mathole et al., 2017). Another example of low prevalence was seen in a study done to investigate the occurrence and antimicrobial susceptibility of *Salmonella* isolates recovered from the pig slaughter process in Romania, where only

4.1% (26/108) of the samples were positive for Salmonella (Morar et al., 2015). The highest number of isolates were recovered from retail meat. This may be attributed to the fact that *Salmonella* carried in the gut of the pigs makes its way to the carcass during slaughter (Jiang et al., 2019).

Antibiotic use in food animal production leads to resistance in bacteria such as *Salmonella*, linking antibiotic resistance in foodborne bacterial enteric pathogens to antibiotic use in food animal production systems (Lekshmi et al., 2017). Ten percent (10%) of isolates were resistant to cefoxitin, a cephamycin that is classified as highly important on the WHO list of critically important antimicrobials for human medicine. Resistance to cefoxitin is mostly due to the production of AmpC β-lactamases that hydrolyze the antibiotic. AmpC β-lactamases, encode resistance to penicillins and cephalosporins. In *Salmonella A*mpC genes are acquired while they are intrinsic in other bacterial species (Edirmanasinghe et al., 2017). The AmpC β-lactamase, bla_{CMY}, has been reported in *Salmonella* serotypes globally as have the extended-spectrum β-lactamases (ESBLs) $bla_{\text{CTX-M}}$ and bla_{OXA} (Bush and Bradford, 2020). Although not evident in this study, plasmids carrying the AmpC β-lactamases also harbor resistance genes to other classes of antimicrobials, thus conferring multidrug resistance (Edirmanasinghe *et al*., 2017). We observed 8% resistance to amoxicillin. Ampicillins and amoxicillin are among the drugs of choice for treating salmonellosis (Tack et al., 2020) and amoxicillin was also used on the farm (personal communication). The antibiotic resistance observed in our isolates was substantially lower than reported in other studies. For example, a study in Brazil that investigated the distribution, diversity, virulence genotypes and antibiotic resistance of *Salmonella* isolated from a pork production chain, reported a high prevalence of resistance against streptomycin (90.5%), tetracycline (88.1%), ampicillin (81.0%), chloramphenicol (71.4%), and ciprofloxacin (50.0%). Susceptibility was only observed to ertapenem, meropenem and kanamycin and 80.9% of the isolates were identified as multidrug resistant and they were resistant to ≥3 antibiotic classes *(*Viana et al., 2019).

Antimicrobial susceptibility could be affected by the methodology used. In a study done in Texas, USA, *Salmonella* isolates were cultured from a sample of commercial broiler caeca with and without selective broth enrichment, followed by AST for a panel of 14 antimicrobials using the Sensititre® platform and a qualitative broth breakpoint assay. The *Salmonella* samples which were enriched in Rappaport-Vassiliadis broth reported lower detectable resistance to amoxicillin/clavulanic acid, ampicillin, azithromycin, cefoxitin, ceftriaxone, nalidixic acid, and meropenem, and increased resistance to streptomycin and tetracycline. These results show that methodology is crucial in the detection and surveillance of antimicrobial resistance phenotypes in *Salmonella* samples (Wang et al., 2021).

We confirmed the prevalence of seven virulence genes: *misL, sopB, spiC, orlF, pipD, hilA, stn* at 89%, 72%, *87% , 85%, 88%, 91%*, and *91%* respectively. These virulence genes are prevalent in *Salmonella* and are involved in invasion of epithelial cells, interaction with the intercellular membrane, chronic

infection, host specificity and adhesion (Zishiri et al., 2016). The greatest attribute that *Salmonella* uses to survive in a host cell is pathogenicity. Pathogenicity enables *Salmonella* to invade and destroy epithelial cells in the host intestines and thereafter to colonize other cell lines. This process is a result of the presence of genetic determinants which are responsible for the virulence in *Salmonella* spp. The seven virulence genes were detected throughout the farm to fork continuum except during transportation.The high prevalence of *stn* gene amongst other genes is in agreement with a study aimed at detecting virulent genes of *Salmonella* serovars from pork in Ahmedabad, Gujarat. All *Salmonella* isolates contained the *stn* gene (Chaudhary et al, 2015). The most common virulence genes detected were *hilA* and *stn* (91%): *hilA* gene encodes an OmpR/ToxR transcriptional regulator which is responsible for activating the expression of invasion genes (Ammar et al., 2016). The *hilA* gene facilitates invasion of the epithelial cells (Thung et al., 2018). High detection of *hilA* gene was also seen in a study done to investigate the occurrence of antimicrobial resistance, biofilms synthesis and virulence genes in Salmonella isolated from pigs on intensive farms (Barilli et al., 2018) .

Other virulence genes detected such as *SipC* is a *Salmonella* translocon protein which targets F-actin, which is necessary for pathogen internalization as well as promoting *Salmonella* invasion. *SipC* is delivered to the cytosol of the host cell where it can have effector functions, and it also interacts with the intercellular membrane trafficking and as a result, hinders the correct cellular function by inhibiting the fusion of *Salmonella*-containing phagosomes with lysosomal and endosomal compartments. (Lou et al., 2019). The *misL* is an SPI-3 encoded protein responsible for membrane insertion and secretion; it is an auto-transported protein only found in pathogenic bacteria. These proteins are made up of N-terminal effector domain and C-terminal conserved domain, which forms a pore in the outer membrane through which the N-terminal domain is translocated. The *misL* gene also has a predicted N-terminal signal sequence necessary for the translocation of the protein across the inner membrane (Lou et al., 2019). The *orfL* virulence gene is found in Salmonella pathogenic island (SPI-4), and it plays a role in adhesion, auto-transportation, and colonisation. The *orfL* gene also has a secretion system which mediates the secretion of toxins and is vital for macrophages survival (Ramatla et al., 2020). The *pipD* gene is located in SPI-5 and it's a type III secreted effector associated with the SPS-1 system (Zishiri *et al*., 2016). This gene plays a vital role in enteropathogenicity by secreting intestinal mucosal fluids as well as inflammatory responses, which are regulated by SPI-1 and SPI-2 bacterial type III secretion system (T3SS) (Wang et al., 2020). Some virulence genes like *stn* are chromosomally encoded and not situated on SPIs and are also known as *Salmonella* enterotoxin genes. *stn*'s are the dedicated protein secretion system known as type III secretion system (TTSS) which are responsible for the invasion of intestinal epithelial cells and the survival of *Salmonella* in macrophages. This system contributes to pathogenesis by directing the secretion and translocation of virulence-associated proteins known as (effector proteins) directly into the cytosol of the host cell. The *hilA* gene is vital for the regulation of the type III secretion apparatus, as well as activating the expression of invasion genes (Hughes et al.,

2008). *hilA* is a transcriptional regulator which is encoded by SPI-1 and directly activates the expression of two SPI-1 genes (*invF* and *sicA*), which encode SPI-1 T3SS apparatus components (Lou et al., 2019). *hilA* gene conferrers pathogenicity by allowing *Salmonella* to invade the epithelial cells (Thung *et al*., 2018). The detection of these virulence genes at the farm level as well as the carcasses of healthy pigs is worrisome because it implies that healthy pigs with no sign of illness can be carriers of pathogenic *Salmonella* (Ferrari et al., 2019).

ERIC-PCR showed that the isolates were very different with 26 ERIC-types (A-Z) and 4 major types in total. Clonal relatedness was seen in C, F,H,S,T,V,W,X which consisted of retail meat, slurry and human isolates. The clonal relatedness in isolates belonging to ERIC-type V, from the slurry, human swabs and retail meat (whole carcass) is of great concern since it is an indication of transmissiom of organisms between the environment, animals and humans within the farm. The isolates were howerever quite diverse in relation to their virulence profiles and this might be due to the fact that only a few number of isolates were selected for the experiment. Such selection is based on the fact that only positive isolates were selected, therefore studies involving a larger number of samples could give a better perspective of the clonality along the food production system. Similar cross contaminaton was also observed in a study which investigated the prevalence, antimicrobial resistance and relatedness of Salmonella isolated from pigs on farms, abattoirs and markets. Results showed *Salmonella* isolates from different parts of the production chain seemed to be highly genetically related, indicating that *Salmonella* could potentially be transmitted from farms to abattoirs and markets (Ma et al., 2017) :Colello et al., 2018). Some isolates which belonged to the same clones harboured the similar virulence genes, even though there was diversity in the resistance profiles. This diversity coupled with the different permutations and combinations of virulence genes in related and unrelated clones indicates the mobility and fluidity of virulence and resistance genes across the farm-to-fork comtinuum although there was no evidence of clonal transmission.

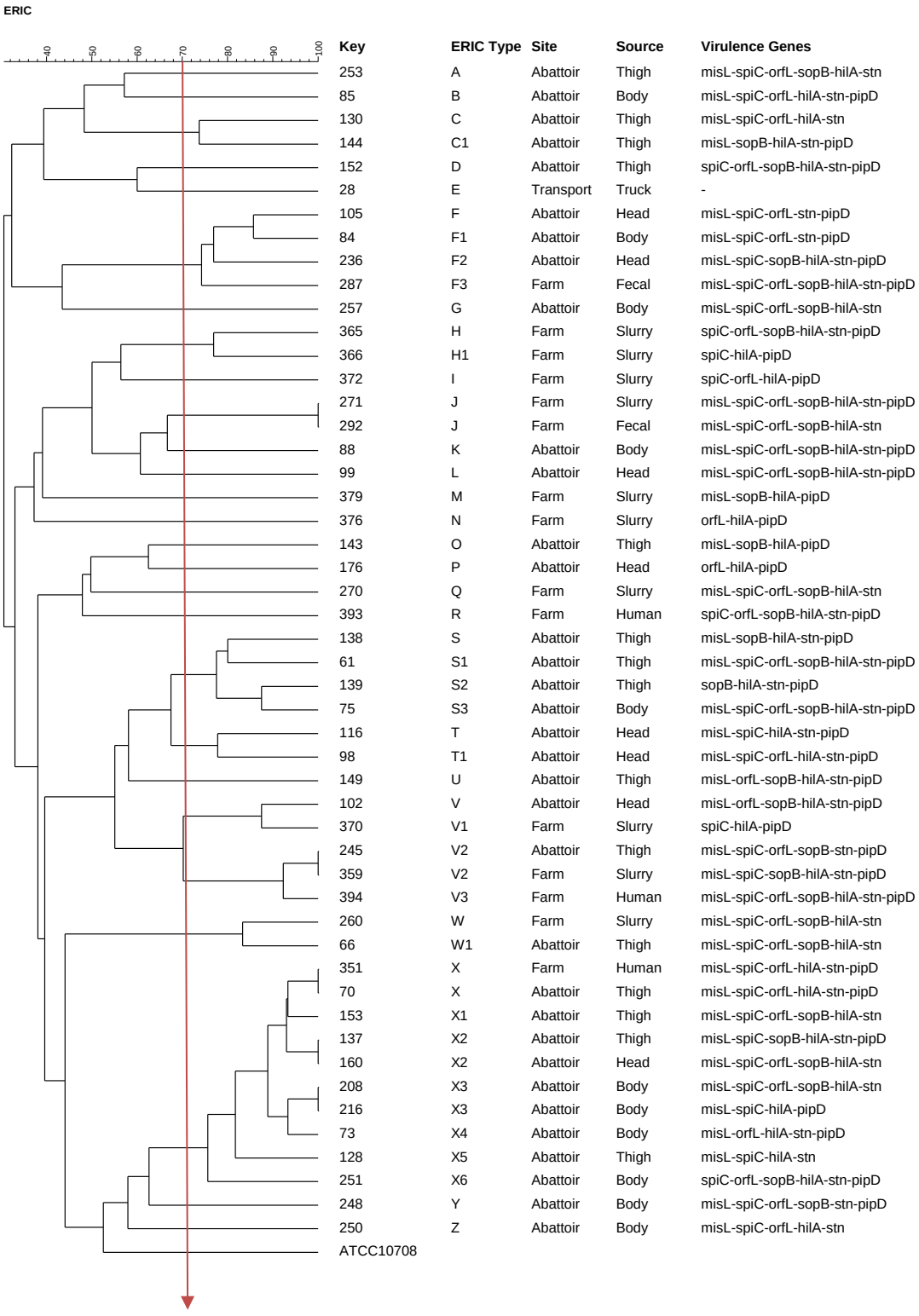
5. CONCLUSION

This study on *Salmonella* in intensively produced pigs carried out at Umgungundlovu District in Kwazulu-Natal, South Africa uses the farm-to-fork approach, Antibiotic resistance was evident in a small number of isolates. There was however, a large number of *Salmonella* isolates that exhibited virulence genes. Of greater concern was the fact that the vast majorit of islates were detected at the abattoir in retail meats, presenting a food safety concern. This requires a comprehensive understanding of molecular epidemiology of the organism so that it’s incidence can be reduced and better controlled from the primary source within the food chain.

Author contributions: Co-conceptualized the study: All. Performed the laboratory work: NPT. Analyzed the data: All. Wrote the paper: NPT. Revision of the manuscript: All.

General Disclaimer: The opinions, results, conclusions and recommendations expressed in this manuscript are those of the author(s) and do not reflect the views of the organizations that provided support for the project. The funders had no role in the study.

Transparency Declaration: Professor Sabiha Essack is the chairperson of the Global Respiratory Infection Partnership and member of the Global Hygiene Council, both sponsored by unconditional educational grants from Reckitt, UK.



Key	ERIC Type	Site	Source	Virulence Genes
253	A	Abattoir	Thigh	misL-spiC-orfL-sopB-hilA-stn
85	B	Abattoir	Body	misL-spiC-orfL-hilA-stn-pipD
130	C	Abattoir	Thigh	misL-spiC-orfL-hilA-stn
144	C1	Abattoir	Thigh	misL-sopB-hilA-stn-pipD
152	D	Abattoir	Thigh	spiC-orfL-sopB-hilA-stn-pipD
28	E	Transport	Truck	-
105	F	Abattoir	Head	misL-spiC-orfL-stn-pipD
84	F1	Abattoir	Body	misL-spiC-orfL-stn-pipD
236	F2	Abattoir	Head	misL-spiC-sopB-hilA-stn-pipD
287	F3	Farm	Fecal	misL-spiC-orfL-sopB-hilA-stn-pipD
257	G	Abattoir	Body	misL-spiC-orfL-sopB-hilA-stn
365	H	Farm	Slurry	spiC-orfL-sopB-hilA-stn-pipD
366	H1	Farm	Slurry	spiC-hilA-pipD
372	I	Farm	Slurry	spiC-orfL-hilA-pipD
271	J	Farm	Slurry	misL-spiC-orfL-sopB-hilA-stn-pipD
292	J	Farm	Fecal	misL-spiC-orfL-sopB-hilA-stn
88	K	Abattoir	Body	misL-spiC-orfL-sopB-hilA-stn-pipD
99	L	Abattoir	Head	misL-spiC-orfL-sopB-hilA-stn-pipD
379	M	Farm	Slurry	misL-sopB-hilA-pipD
376	N	Farm	Slurry	orfL-hilA-pipD
143	O	Abattoir	Thigh	misL-sopB-hilA-pipD
176	P	Abattoir	Head	orfL-hilA-pipD
270	Q	Farm	Slurry	misL-spiC-orfL-sopB-hilA-stn
393	R	Farm	Human	spiC-orfL-sopB-hilA-stn-pipD
138	S	Abattoir	Thigh	misL-sopB-hilA-stn-pipD
61	S1	Abattoir	Thigh	misL-spiC-orfL-sopB-hilA-stn-pipD
139	S2	Abattoir	Thigh	sopB-hilA-stn-pipD
75	S3	Abattoir	Body	misL-spiC-orfL-sopB-hilA-stn-pipD
116	T	Abattoir	Head	misL-spiC-hilA-stn-pipD
98	T1	Abattoir	Head	misL-spiC-orfL-hilA-stn-pipD
149	U	Abattoir	Thigh	misL-orfL-sopB-hilA-stn-pipD
102	V	Abattoir	Head	misL-orfL-sopB-hilA-stn-pipD
370	V1	Farm	Slurry	spiC-hilA-pipD
245	V2	Abattoir	Thigh	misL-spiC-orfL-sopB-stn-pipD
359	V2	Farm	Slurry	misL-spiC-sopB-hilA-stn-pipD
394	V3	Farm	Human	misL-spiC-orfL-sopB-hilA-stn-pipD
260	W	Farm	Slurry	misL-spiC-orfL-sopB-hilA-stn
66	W1	Abattoir	Thigh	misL-spiC-orfL-sopB-hilA-stn
351	X	Farm	Human	misL-spiC-orfL-hilA-stn-pipD
70	X	Abattoir	Thigh	misL-spiC-orfL-hilA-stn-pipD
153	X1	Abattoir	Thigh	misL-spiC-orfL-sopB-hilA-stn
137	X2	Abattoir	Thigh	misL-spiC-sopB-hilA-stn-pipD
160	X2	Abattoir	Head	misL-spiC-orfL-sopB-hilA-stn
208	X3	Abattoir	Body	misL-spiC-orfL-sopB-hilA-stn
216	X3	Abattoir	Body	misL-spiC-hilA-pipD
73	X4	Abattoir	Body	misL-orfL-hilA-stn-pipD
128	X5	Abattoir	Thigh	misL-spiC-hilA-stn
251	X6	Abattoir	Body	spiC-orfL-sopB-hilA-stn-pipD
248	Y	Abattoir	Body	misL-spiC-orfL-sopB-stn-pipD
250	Z	Abattoir	Body	misL-spiC-orfL-hilA-stn
ATCC10708				

Figure 2: Dendrogram of *Salmonella* spp. isolated along the farm to fork continuum.

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Supplementary Material

Table S1: Primers used to confirm *Salmonella* spp. *and Salmonella enterica*

Target gene	***Primer sequence (5'-3')***	***Product size (bp)***	***Reference***
invA (Salmonella spp.*)*	F:TCATCGCACCGTCAAAGGAACC R:GTGAAATTATCGCCACGTTCGGGCAA	*284*	*Li et al. (2012)*
iroB (Salmonella enterica)	*F:TGC GTA TTC TGT TTG TCG GTCC* *R:TAC GTT CCC ACC ATT CTT CCC*	*606*	*(Bäumler et al., 1997)*

TABLE S2: Primers used to identify virulence genes.

Target gene	***Primer sequence (5'-3')***	***Product size (bp)***	***Reference***
misL	F:GTCGGCGAATGCCGCGAATA R:GCGCTGTTAACGCTAATAGT	*400*	Zirishi et al., 2016
spiC	*F:CCTGGATAATGACTATTGAT* *R:AGTTTATGGTGATTGCGTAT*	*309*	Zirishi et al., 2016
orfL	*F:GGAGTATCGATAAAGATGTT* *R:GCGCGTAACGTCAGAATCAA*	*550*	Zirishi et al., 2016
sopB	*F:TCAGAAGRCGTCTAACCACTC* *R: ACCGTCCTCATGCACACTC*	*517*	Thung et al,. 2018
pipD	*F:CGGCGATTCATGACTTTGAT* *R:CGTTATCATTCGGATCGTAA*	*350*	Zirishi et al., 2016
hilA	*F:CGGAAGCTTATTTGCGCCATGCTGAGGTAG* *R:GCATGGATCCCCGCCGGCGAGAT TGTG*	*854*	Thung *et al.,* 2018

stn	*F: TTGTGTCGCTATCACTGGCAACC* *R: ATTCGTAACCCGCTCTCGTCC*	*617*	Thung *et al*,. 2018

3 CHAPTER 3 – CONCLUSION, LIMITATIONS AND RECOMMENDATIONS

This study describes the antibiotic resistance and virulence profiles and genetic relatedness of *Salmonella* spp. isolates from an intensive pig-production farm in the uMgungundlovu district in Kwazulu-Natal, South Africa across the farm-to-fork continuum.

3.1 Conclusions

The following conclusions can be drawn in relation to the objectives of the study.

- *Salmonella* spp. isolates were isolated from the slurry, faeces and human (hands and nose) on the farm during weeks 1-17, from the truck transporting the pigs to the abattoir in week 18, and from the caeca, rinsate from carcass after slaughter and retail meat at the abattoir in week 18.

- The large number of isoaltes in retail meat samples, their virulence, and, to a lesser extent their antibiotic resistance profiles poses a challenge to the food safety system.

- 197 isolates were successfully identified using PCR.

- Antibiotic susceptibility testing showed 10% resistance to cefoxitin, 8% to amoxicillin and 0.5% to gentamicin and chloramphenicol.

- The overall prevalence of the virulence factors detected in the isolates were as follows: *stn* and *hilA*, were detected in 91% of the isolates, *misL* was detected in 89% *pipD* in 88%, *spiC* in 87% and *orlF* in 85 %. The *sopB* gene was the least represented with 72%.

- The clonal relatedness in isolates belonging to ERIC-type V, which were found in the slurry, human swabs and retail meat (whole carcass) is worrying because it is a possible indication of transmissiom of organisms and mobility of genes between the environment, animals and humans within the farm.

3.2 Limitations

- This study was limited to only one pig-production farm and is therefore not representative of the prevalence of *Salmonella* spp. throughout Kwazulu-Natal or South Africa.

3.3 Future recommendations

The following recommendations are made based on the findings of this study:

- More studies need to be done on animal-production systems in order to get a better representation of ABR in *Salmonella* spp. along the farm to fork continuum in South Africa.
- Additional virulence genes should be studied to gain more insight into full virulence potential of isolates e.g. *spvC*, *pagC*, *orgA* and *tolC.*
- Other clonal typing methods such as multilocus sequence typing (MLST) PFGE can be used to obtain a clearer picture of the clonal transmission if any.
- Whole-genome sequencing should be considered in order to obtain an accurate representation of the isolate's genomic profile.
- Serotyping should also be considered in order to have a clear picture of the different sub-species.

Appendix 1: Biomedical 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

22 March 2018

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 2018
Expiration of Ethical Approval:	16 March 2019

I wish to advise you that your application for Recertification received on 12 February 2018 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.

This approval will be ratified by a full Committee at its meeting taking place on 10 April 2018.

Yours sincerely
[redacted]
Mrs A Marimuthu
Senior Administrator: Biomedical Research Ethics

Appendix 2: Animal Research Ethics Committee (BREC) Approval.

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 Reynosfield Estate for this study.
2. Necessary approval must be obtained from other appropriate Ethics Committees 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 Haynes 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

1911 - 2011
100 YEARS OF ACADEMIC EXCELLENCE
Founding Campuses: Edgewood, Howard College, Medical School, Pietermaritzburg, Westville

Appendix 3: Department of Agriculture, Forestry and Fisheries (Approval) Section 20.

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 Henry Gololo • Tel: +27 12 319 7532 • Fax: +27 12 319 7470 • E-mail: HenryG@daff.gov.za
Reference: 12/11/1/6

Prof Sabiha Yusuf Essack
Antimicrobial Research Unit
College of Health Sciences
University of KwaZulu-Natal
Private Bag 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:

Appendix 4: Farm personnel information and consent forms

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.

Participant Consent Form

3.1.1.1.1.1 *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: _____