Molecular Detection and Genetic Characterization of Antimicrobial Resistance Genes in Foodborne Pathogens Isolated from Slaughtered Broiler Chickens in Durban

Submitted by

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PREFACE

The research contained in this thesis was completed by Nelisiwe Mkize under the supervision of Dr Oliver Zishiri and co-supervision of Dr Meenu Ghai from 2014 to 2015 whilst based in the Discipline of Genetics, School of Life Sciences of the College of Agriculture, Engineering and Science, University of KwaZulu-Natal, Westville campus, South Africa. The research was financially supported by College of Agriculture, Engineering and Science.

The contents of this work have not been submitted in any form to another university and, except where the work of others is acknowledged in the text, the results reported are due to investigations by the candidate.

I certify that the above information is correct

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Dr Oliver Zishiri (Supervisor)

Date:

_____

Dr Meenu Ghai (Co-supervisor)

Date

DECLARATION FOR PLAGIARISM

I, Nelisiwe Mkize, declare that:

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DECLARATION FOR PUBLICATIONS

DETAILS OF CONTRIBUTION TO PUBLICATIONS that form part and/or include research presented in this thesis (include publications in preparation, submitted, *in press* and published and give details of the contributions of each author to the experimental work and writing of each publication)

Publication 1- **Prevalence of virulence and antimicrobial resistance genes in Salmonella spp. isolated from commercial chickens and human clinical isolates from South Africa and Brazil**

I Nelisiwe Mkize did 100% of experimental work and writing up, however the other authors edited the paper by adding the information that was not included by me and they also made sure that the article was on a publication standard.

This article was submitted and accepted for publication by Ondersterpoort Journal of Veterinary Research on 11th December 2015

Publication 2- **Genetic characterization of antimicrobial resistance and virulence genes in *Staphylococcus aureus* isolated from commercial broiler chickens in Durban**

I Nelisiwe Mkize did 100% of experimental work and writing up, however the other authors edited the paper by adding the information that was not included by me and they also made sure that the article was on a publication standard.

This article is still under review

Signed

ABSTRACT

Salmonella and *Staphylococcus aureus* are the most troublesome micro-organisms in poultry industry, because their presence poses health hazards and risks to consumers and to the economy of the country. Since they entail virulence characteristic which plays a role in causing illness to hosts and they also have potential to confer resistance toward different antimicrobial agents. Thus can be transmitted from food producing animals to humans through various factors the major one being food chain.

Since antimicrobial agents were introduced in food producing animals for therapeutic and growth promotion purposes, escalating issues of antimicrobial resistance have been reported globally. However speculations about this issue of antimicrobial resistance have been attributed to the extensive use of antimicrobials in animal husbandry as a reason behind the increasing antimicrobial resistance burden. Previous studies have documented that there are genetic determinants involved in order for a bacteria to be pathogenic or resistant toward certain antimicrobial agents. However in South Africa limited work has been done in detecting foodborne pathogens in chicken meat and subsequent screening for genetic determinants that confer virulence and resistance. Therefore, the current study was aimed to investigate the prevalence rates of *Salmonella* spp. and *Staphylococcus aureus* in broiler chicken meat obtained at a farm level and also at a retail level. Furthermore, it was also aimed to investigate the presence of genes encoding for pathogenicity and antimicrobial resistance in detected isolates of *Salmonella* spp. and *Staphylococcus aureus*.

To achieve the aim of the study, chicken samples were collected from slaughterhouses around the Durban metropole whom for confidentiality reasons will remain anonymous. Collected samples were subjected to detection for *Salmonella* spp. as well as *Staphylococcus aureus*. Moreover, for *Staphylococcus aureus* detection, 30 additional samples were sourced from 10 different retail outlets around Durban and were added to 114 samples which is part of portion used previously for *Salmonella* spp. detection. Microbiological techniques were utilized to detect *Salmonella* spp. and *Staphylococcus aureus* and the pathogens were further confirmed using molecular technique (PCR) amplifying *invA* and *nuc* genes respectively. Antimicrobial resistance profiles of confirmed isolates were determined using the phenotypic agar disc diffusion method. Genes encoding for virulence and resistance were screened using PCR.

Among all samples examined, 102 of the 200 were confirmed positive for *Salmonella* spp. and 104 of 194 for the *Staphylococcus aureus*. For *Salmonella* spp. a large proportion (62.5%- 100%) of the isolates was resistant to ampicillin, amoxicillin, bacitracin, erythromycin, kanamycin, trimethoprim-sulfamethoxazole, tetracycline and trimethoprim. Low rates of resistance (0%- 31%) were observed on chloramphenicol and streptomycin. For *Staphylococcus aureus* high levels (58%-100%) of resistance were observe on tetracycline, trimethoprim, cefoxitin, ampicillin, erythromycin and kanamycin, where by low levels were ranging from 0%- 40%. Multidrug resistance was observed on isolates of both *Salmonella* spp. and *Staphylococcus aureus,* almost all isolates detected were resistant to more two antimicrobial agents. Screening of virulence and resistance determinants showed that most of the samples used were harbouring genes encoding for pathogenicity and antimicrobial resistance.

In conclusion, pathogens detected on the chicken meat used in current study were dangerous for consumers, since these pathogens encapsulated genes conferring virulence and resistance, implying that the organism are pathogenic and can be difficult to cure. Therefore it is very crucial for stakeholders involved in production of chicken meat to exercise prudent use of antibiotics and also to make sure that they sell pathogen free products to consumers. Moreover, this calls for department of Health to educate people about the use of antibiotics, hygiene when preparing food and dangers of eating half cooked meat. This can be one of the strategies to combat the escalating antimicrobial resistance burden.

Keywords: Broiler chickens; *Salmonella* spp.; *Staphylococcus aureus*; virulence genes; resistance genes; PCR.

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CONTENT

LIST OF ABBREVIATIONS

AMP	Ampicillin	E	Erythromycin
AMO	Amoxicillin	VA	vancomycin
CDC	Centre for Disease Control and Prevention	W	Trimethoprim
S. aureus	Staphylococcus aureus	FOX	Cefoxitin
C	Chloramphenicol	S	Streptomycin
CLSI	Clinical and Laboratory Standards Institute	k	Kanamycin
°C	Degrees Celsius	spp.	Species
DNA	Deoxyribonucleic acid	CN	Gentamycin
sea	Staphylococcal enterotoxins type A	s	Seconds
ARC	Agricultural Research Council	µg	Microgram
et al	and other people	invA	Invasion A
FAO	Food and agricultural organization	TET	Tetracycline
(T3SS)	III secretion system	mec	Methicillin
PCR	Polymerase chain reaction	coa	Coagulase
STX	Trimethoprim-Sulfamthoxazole	µL	Microliter
SAPA	South African Poultry Association	min	Minutes
TBE	Tris-Borate-EDTA	B	Bacitracin
SABC	South African Broiler Chicken isolates	e.g	for example
NHLS	National Health laboratory Services		
SPI	Salmonella Pathogenicity Island		
SAHC	South Africa Human Clinical isolates		
WHO	World Health Organisation		
XLD	Xylose lysine deoxycholate agar		
BBC	Brazilian Broiler Chicken isolates		
spa	Staphylococcal protein A		
SCCmec	Staphylococcal cassette chromosome mec		

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CHAPTER 1

GENERAL INTRODUCTION

1.1.Introduction

Poultry meat is more popular in the consumer market because of advantages such as easy digestibility and affordability, that why it is accepted by majority of the people worldwide (Yashoda et al., 2001). However, poultry meat and it products are considered as major vehicles for the transmission of foodborne pathogens to humans due to cross contamination events at a farm level and also at retail level (Capita et al., 2007). Most contamination of chicken meat by different food-borne pathogens occurs at farm level from carrier animals excreting the organisms and at the abattoirs during slaughter (Molla et al., 2006). When chickens are slaughtered there are high possibilities of contamination, it can be through contaminated abattoir equipment or through meat contact with the intestinal organs of the carcasses where most bacteria reside (FOA, 2015). However, all abattoirs practise biosecurity and hygienic measures to keep the meat as free from contamination as possible. Needless troublesome foodborne pathogens such as *Listeria* spp., *Campylobacter* spp., *Salmonella* spp., *Staphylococcus* spp. and others still get an opportunity to remain in the meat even though cleansing measures have been undertaken. These foodborne pathogens are associated with various infections in both animals and humans and they have been reported to be zoonotic and possible pathogenic. Zoonotic bacteria are naturally transmitted between animal and humans (Smith et al., 1999; Ribot et al., 2002). Transmission occurs via several pathways but food chain is considered as the major pathway.

Foodborne pathogens such as *Salmonella* species and *Staphylococcus aureus* are responsible for a variety of acute and chronic diseases in both poultry and humans (Mead and Dodd, 1990; Humphrey, 2000; Smyth and McNamee, 2001). The diseases they cause are estimated to be the leading cause of death worldwide, consequently increasing mortality rate (Angulo et al., 2004). Most developing countries have limited or no information on mortality statistics associated with these foodborne pathogens, thus include South Africa, however developed countries like United States of America (USA) and England have information on mortality statistics available to the public. Mead et al. (1999) reported that in USA foodborne

diseases have been estimated to cause about 76 million illnesses, 325,000 hospitalizations and 5,000 deaths. Furthermore, Adak et al. (2002) reported that during year 2000, foodborne pathogens were approximated to be responsible for 1,338,772 cases, 20,759 hospital admissions, and 480 deaths in England and Wales. The worldwide mortality of people who died because of diarrhoea associated with foodborne pathogens was approximately 2.1 million in year 2000 alone (WHO, 2002).

Most of the times the cause of foodborne diseases is attributed to virulence factors encompassed in genome of pathogens (Galan et al. 1992; Groisman et al., 1999) and this factors are responsible for fighting with the immune system of the host. The phenomenon is called pathogenicity and it is measured by the status of virulence of an organism (Casadevall and Pirofski, 2001).Virulence factors assist bacteria to invade the host's cells, cause diseases, which fight with host's immune system. Different virulence factors are characterized in different categories, depending on the functions. Some factors are responsible for adherence, invasion, capsules and exotoxins (Groisman et al., 1999; Casadevall and Pirofski, 2001). Documented information presents speculates which state that virulence factors have a potential to convert harmless bacteria (such as commensal bacteria e.g. *E.coli*) in to dangerous pathogens. Previously there were bacteria that were considered non-pathogenic micro-organisms, but currently they are known to cause infectious diseases (e.g E.coli carrying shiga toxins and intimin proteins) (Wexler, 2007).This is a result of the fact that virulence factors can be transferred from one bacterium to another through horizontal gene transfer and from food producing animals to human beings through various mechanisms, such as direct contact, food chance, environmental interaction and so forth. Previous studies from different developed countries have investigated at molecular level the presence of virulence factors in pathogens affecting food producing animals such as chickens, cattle, goats and others (Peterson, 1996).

Antimicrobial agents are used in veterinary and human medicine for various purposes, but most importantly for therapeutic purposes (Wendlandt et al., 2015). However, since antimicrobials were discovered resistance has been reported globally. Bacterial resistance towards antimicrobial agents hinders treatment efficiency of bacterial infections in both animals and humans (Marshall and Levy, 2011). The prevalence of antimicrobial resistance amongst foodborne pathogens has increased during recent decade (Smith et al., 2015). All this is attributed to extensive, irrational and unwarranted use of antimicrobial agent in veterinary and human medicine. For example in veterinary medicine under poultry industry,

the producers use antimicrobial agents to combat infections. In the case where food producing animals are raised in a confined environment, producers are forced to treat all animals if some of them are sick. Since a massive number animals are overcrowded in one area it is very easy for the infection to spread. The worst part is most of the times it is difficult for producers to distinguish between the sick animals and those that are not sick, especially in poultry industry. So treating the healthy animals with antimicrobial agents at all times creates selection pressure for resistance to these antibiotics. More antibiotics are incorporated in animal feed for growth enhancement; so many producers are inclined to using feed with antibiotics because they produce more productivity in a short period of time. It is for these reasons amongst many other reasons that most researchers have implicated veterinary medicine as the main factor behind the escalating antimicrobial resistance worldwide.

Scientific studies have reported that there are some genetic mechanisms involved in antimicrobial resistance (Hawkey, 1998; Tenover, 2006; Marshall and Levy, 2011). A number of genes have been elucidated to encode for antimicrobial resistance in different microorganisms, for example *tetA, mecA* and *Pse-1* genes which confer resistance to tetracycline, methicillin and ampicillin respectively. (Chopra and Roberts, 2001; Sauvage et al., 2002; Louis and Rice, 2012). In Agricultural sector, anecdotal reports assume that antimicrobial resistant genes arise as a consequence of intensive use of antimicrobials in animal feeds and as a result some environmental factors. The occurrence of resistance genes in food-borne pathogens or in opportunistic bacteria poses a serious threat to humans and animals, since infections caused by these micro-organisms cannot be treated with one common antibiotics (Normark and Normark, 2002; Phillips et al., 2004). Treatment can require a combination of two or more antibiotics or other antibiotics which are more efficient. Dissemination of genes encoding antimicrobial resistance among bacteria is a serious medical problem globally.

1.2.Problem statement

South Africa is one of the countries which are still extensively using antimicrobial agents in poultry production mainly for therapy and growth enhancement. The unwarranted use of antimicrobial agents in poultry production has been speculated to create selection pressure for antimicrobial resistance; such mechanism can be transmitted from chickens to humans

mainly through food chain. Documented information records that poultry production is the largest industry among other agricultural industries in South Africa (SAPA, 2012). So this means that antimicrobial resistance associated with foodborne pathogen in this field should be monitored critically. However there is limited information on the prevalence rate of the presence of antimicrobial resistance foodborne pathogens associated with broiler chicken meat of South Africa. Therefore, such limitation provides scope for studies which will investigate the prevalence of antimicrobial resistance foodborne pathogens associated with broiler chicken meat of South Africa. So it is for this reasons that the study undertaken was implemented.

1.3.Aim and Objectives

The aim of study was to investigate the prevalence rates of *Salmonella spp.* and *Staphylococcus aureus* contamination in broiler chicken meat and to further assess genetic determinants encoding for virulence and antimicrobial resistance. The aim was achieved by paying attention on the following objectives:

- To estimate the prevalence of *Salmonella* spp. and *Staphylococcus aureus* on broiler chicken samples collected at a farm and retail level.
- To evaluate antimicrobial resistance profiles of South African isolates from chickens samples, human, clinical samples and from Brazilian isolates
- To further screen bacterial isolates for genetic determinants encoding for virulence and antimicrobial resistance.

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CHAPTER 2

A REVIEW OF VIRULENCE AND ANTIMICROBIAL RESISTANCE OF FOODBORNE PATHOGENS ASSOCIATED WITH LIVESTOCK

2.1. Introduction

In South Africa, chicken meat remains a viable protein source relative to other meat protein sources and it has also remained reasonably priced in a time of weak economic performance (SAPA, 2012). Worldwide poultry meat is the most consumed animal protein. However, this exerts pressure on poultry producers and other stakeholders to produce more protein in a short period of time for the purpose of catering for increasing consumer needs. Since the 1950, stakeholders involved in poultry industry have been using antimicrobial agents to enhance their goal of increasing productivity in a short period of time. The veterinary use of antimicrobial agents in animal husbandry is to cure, prevent and control diseases (Momtaz et al., 2012; Van et al., 2007). Moreover, they are also used to improve growth and feed efficiency. The main route that incorporates antimicrobial agents as growth promoters is via the feed route. Antimicrobials are added in animal feed as additives, so that animals can obtain them as daily supplements (Mooljuntee et al., 2010).

The use of antimicrobial agents as feed additive in animal husbandry opened doors for the increase productivity in commercial animal farms. In broiler chicken production, antimicrobial agents facilitated growth promotion, which resulted in more muscles thus more meat was produced in a short period of time. In most developed and developing countries where the routine of using antimicrobial agents as feed additives was adopted, an increase in meat production was been achieved. Moreover, antimicrobials that were proven to be first line cures of certain infections caused by foodborne pathogens but as time goes by, antimicrobial resistance cases were reported. Consequently, this has compromised the treatment of animals and humans, since it has yielded an undesirable antimicrobial resistance, which is a global threat. The undesirable antimicrobial resistance has mostly been speculated as consequence of irrational use antibiotics producing animals (Adesiji et al., 2014). Mooljuntee et al. (2010) reported that if food producing animals have been exposed to antimicrobials over a period of time they attain strains of bacteria which are resistant to

antimicrobials. Thus means that those bacterial strains become capable of withstanding attacks from antimicrobial agents. Which on the other hand becomes a threat to animal and human lives, since it inactivates therapy. The phenomenon of antimicrobial resistance has been escalating worldwide thereby, creating public health concerns (Yang et al., 2002; Molla et al., 2006; Padungtod et al., 2006; Bhowmicket al., 2009; Fashae et al., 2010). The danger associated with antimicrobial resistance to animals and humans influenced the European Union in 1998 to prohibit the use of antimicrobials such as tylosin, bacitracin, virginiamycin and spiramycin as animal feed additives (Casewell, et al., 2003; Turnigde, 2004) These antibiotics were banned because their structural analogs were reported to be similar to the antimicrobials used in human medicine. However, South African studies that have been conducted on the use of antimicrobials in animal husbandry have presented evidence that South Africa is still extensively misusing antimicrobials (such as tylosin, and bacitracin) that have been banned in European countries (Eager, 2008; Henton et al., 2011).

Zoonotic foodborne pathogens such as *Listeria* spp., *Campylobacter* spp., *Salmonella* spp. and *Staphylococcus* spp. are associated with various infections in both animals and humans. Food producing animals such as bovine, porcine and avian species have been reported to be reservoirs of zoonotic pathogens (Zhao et al., 2001). Worldwide , studies have been reported demonstrating the presence of *Salmonella* spp., *Staphylococcus* aureus, *Listeria* spp., *Campylobacter* spp., and others in chicken meat and other products (Zhao et al., 2001; Bohaychuk et al., 2005; Manguiat and Fang, 2013; Adeyanju and Ishola, 2014). The escalation of antimicrobial resistance in foodborne pathogens originating from food producing animals such as chicken has been reported mostly in developing countries (Van et al., 2007). In a developing country like South Africa, there is a paucity of studies that have focused on the detection and characterization of antimicrobial resistance bacteria in food producing animals. This demonstrates that South African researchers still have a lot of work to do in this area. Taking into cognizance the few studies that have been conducted in South Africa so far, most have been focused on bovine and swine but not on poultry. The epidemiological information on the outbreaks of bacteria and antimicrobial resistant infections on both animals and humans is limited in South Africa. The paucity of information does not mean that South Africa is not facing crisis of outbreaks of bacterial strains which are resistant toward different antimicrobial agents. The challenge is that most cases are never reported.

Scientific studies have reported that there is an element of genetics involved in the virulence and antimicrobial resistance of microorganisms. A number of genes conferring virulence and resistance have been mapped in different microorganisms. Virulence and antimicrobial resistance genes play a crucial role in maintaining the life span of microorganisms. Virulence genes play a role in well-being of the pathogens, and they are responsible for causing host infections. Resistance genes act as weapons to fight attacks from antimicrobial agents. Each class of antimicrobial agents has its own unique resistance genes encoding for resistance. An example is the Bla_{cmy-2} gene, which confers broad-spectrum resistance to beta-lactam antimicrobials, including ceftriaxone and ceftiofur (Heider et al., 2009). Horizontal gene transfer has been pin pointed as the main mechanism responsible for distribution of virulence and resistance determinants from one microorganism to another (Cruz and Davies, 2002).

South Africa has a huge burden of infectious diseases such Human Immunodeficiency Virus (HIV), Tuberculosis (TB) and several others. Some of these diseases are resistant to treatment for example resistant TB in KwaZulu-Natal. This results in South Africa being a country with a high prevalence of people with compromised immune systems. The lives of immuno-compromised individuals are further threatened when they are infected by foodborne pathogens. It is an unfortunate situation when an immunocompromised individual is infected by a pathogen which is resistant towards a first line antimicrobial agent because the therapy is delayed or rendered ineffective. Thus, the immune system becomes weaker as the patient waits in limbo for alternative drugs and this aggravates high morbidity and mortality.

Since chicken meat is a major vehicle of foodborne pathogens, it is speculated to entail genes encoding virulence and antimicrobial resistance. It is prudent for scientists to have scientific understanding on this complex phenomenon. Against this background, the purpose of this review is to explore worldwide documented information on virulence and antimicrobial resistance genes associated with *Salmonella* spp. and *Staphylococcus aureus*.

2.2. The use of antimicrobials in the poultry industry

Introduction of antibiotics into commercial feed for pigs, cattle and chickens emanated after the emergence of antimicrobials in 1940s (Aarestrup et al., 2008). Antimicrobial agents are currently used in food animals to treat, prevent and control diseases and also to enhance feed efficiency (Swartz, 2002). The use of antimicrobial agents in food animal production provides has resulted in some benefits, including improved animal health, higher productivity and, in some cases, reduction in foodborne pathogens (Mathew et al., 2007). However, the use of antimicrobial agents for non-therapeutic use in food animals influenced researchers and stakeholders involves in animal and human medicine to raise concerns about the possible outcomes which can possibly result in undesirable consequences (Gorbach, 2001; Levy, 2002; Cabello, 2006). With regards to growth promotion most animal feeds are supplemented with varying concentrations of antimicrobial agents and livestock are fed with such feed on a daily basis. Aarestrup et al. (2008) reported that the quantity of antimicrobial agents used in food producing animals is higher than what are used in humans worldwide. In the animal production industry, antimicrobial agents are generally applied after or before the onset of a disease condition and used according to label instructions or according to veterinary physician instructions. In food-producing animals such as chickens, antimicrobials are given as group treatment because individual animal treatments are often impractical. Normally antimicrobials are added in water so that the whole flock will get treatment regardless of disease status. This is advantageous since injecting each and every chicken in farm house will be very difficult and time consuming. However, this practise has its setbacks; one of them is that not all chickens will be infected when they are treated. So antimicrobial application will not create selection pressure on targeted bacteria, but also on other bacteria (Wendlandt et al., 2015). Thus, creating evolution of antimicrobial resistance in unaffected chickens.

Different pathogens cause a range of diseases in poultry. Poultry staphylococcal infections include omphalitis, septicaemia, bumble-foot, arthritis and several others. All these infections are prevalent in poultry husbandry and can be treated with various antimicrobial agents such as ampicillin, erythromycin, penicillin, streptomycin, vancomycin and several others. Poultry salmonella infections vary according to severity. Such infections include depression, paratyphoid and pullorum. Most of Salmonella based infections in poultry involve diarrhoea and antibiotics such as tetracycline, gentamycin, ampicillin, amoxicillin, kanamycin and others are routinely used for treatment. The presence of bacterial infections in poultry industry is a driving force for farmers to use antimicrobial agents quite often. Some

antimicrobial agents (tetracycline, penicillin, etc.) which are used on food producing animals have been reported to be either related or identical to the antimicrobials that are used in human medicine. Tollefson and Karp (2004) reported that antimicrobials such as caphalosporins, penicillin, tetracyclines, and flouroquinolones are used in food animals and also for treating foodborne infections in humans. Thus cause problems because foodborne pathogens are becoming more exposed to antibiotics since they encounter with them on daily basis through animal feed, which then leads to the development of antimicrobial resistance. Development of antimicrobial resistance has been increasing since the increased inclusion of antimicrobials in animal feed. This had influenced some countries to prohibit the use of antibiotics as feed additives in their animal husbandry. In 1986 the use of antimicrobial agents as growth promoters was prohibited in Sweden (Weirup, 2001). This momentous event was followed by the prohibition of antimicrobials such as avoparcin, tylosin, spiramycin, bacitracin and virginiamycin from being used in animal feeds in several European countries (WHO, 2001). Phillips et al. (2004) reported that these antimicrobials were banned because of their structural relatedness to antimicrobials that are commonly used in human medicine. South African studies that have been conducted on the use of antimicrobials in food producing animals have revealed that the country is still using antimicrobials that have been banned in other countries. Tylosin, bacitran and virginiamycin were banned in some European countries, but Eagar et al, (2008) has report that these drugs are still authorized for use in livestock such as poultry and cattle for therapy and growth promotion purposes in South Africa. A survey that was conducted by Henton et al. (2011) on antimicrobial usage in South Africa with specific reference to food producing animals demonstrated during the period of 3 years (2002-2004) the majority of consumed antimicrobials were from classes of macrolides, tetracyclines, sulphonamide group and penicillins respectively (Table 2.1).

Table 2.1: Some antimicrobial agents registered for veterinary use in South Africa

Antimicrobial class	Antimicrobial	Food animal
Aminoglycosides	Gentamycin sulphate*	Poultry, swine , turkey
Macrolides[1st]	Tylosin*[√]	Cattle, poultry, swine
Nitroturans	Nitrovin	Poultry
Oligosaccharides	Avilamycin	Poultry
Penicillins[4th]	Penicillin*	Poultry , swine
Polipeptides	Bacitracin*[√]	Poultry, swine
Streptogramins	Virginiamycin[√]	Cattle, swine, poultry
Sulphonamide[3rd]	Sulfamethazine*	Cattle, swine
Tetracycline[2nd]	Chlortetracycline*	Cattle, swine, poultry
Quinolone	Nalidixic acid	Poultry, swines

[1st, 2nd, 3rd and 4th] is order of major consumption in South Africa from 2002-2004(Henton et al., 2011)

[√] still in use in South Africa but banned in European countries

*Used to treat human infections

Several researchers have emphasized that it very crucial for each state to know the amount of antimicrobials used in animal production per annum. According to Wegener (2012) a major factor for continuation of antimicrobial resistance pathogens in animal reservoirs is the amount and patterns of antimicrobial agents used on food producing animals. Knowledge on these factors can play a very pivotal role in enlightening stake holders involved in livestock production that overuse of antibiotics results in drug resistance problems which consequently negatively affect both animals and humans. In South Africa data on the antimicrobial amounts utilized in livestock production is limited, and this constitutes to the limited information on the amount of antibiotics used in food animals per annum (Henton et al., 2011). In addition counterfeiting of original drugs is one of the causes of limited data on antibiotic consumption in food producing animals. As a result, the quantity

of counterfeit drugs which enter South Africa illegally is not recorded on the pharmaceutical data base. Therefore, the data generated by the Pharmaceutical Industry Association of South Africa (PIASA) has to be reported with caution.

2.3. Evidence linking the connection between the use of antimicrobial agents in food producing animals and antimicrobial resistance among pathogens isolated from humans.

A number of reported cases and epidemiological studies have provided evidence linking the connection between the uses of antimicrobial agents in food animals with the emergence of antimicrobial resistance bacteria in humans. Marshall and Levy (2011) and Angula et al (2004) reported on a historical study which occurred way back in 1975 where the effect of introducing low dose oxytetracycline as a growth promoter in a chicken farm was evaluated. During the study samples were collected from the chickens and farm dwellers. The control samples were collected on families in a neighbouring area. The investigations showed that the chickens were colonized by tetracycline resistant *E.coli*. Moreover, tetracycline resistant *E.coli* was also found in farm dweller families and the level was greater than that of results obtained from the control samples. Fey et al (2002) reported on a case which happened years ago where a 12 year old veterinarian's child was ill. When the child was treated they found out that the child was infected by ceftriaxone resistant *Salmonella*. Follow up was conducted and it was revealed that the father had been treating several herds which were affected by *Salmonella*. Investigations were then taken further by collecting samples from the ill and healthy cows. By using pulse-field gel electrophoresis they obtained results which showed genetic similarities between the ceftriaxone resistant *Salmonella* strain isolated from the child and the ceftriaxone resistant *Salmonella* strain isolated from the sick cows. No information was provided concerning the extensive use of ceftriaxone on infected cows, however it is generally known that ceftriaxone is often used in cattle production. Outcomes of the investigations provided evidence that there was transmission of ceftriaxone resistant *Salmonella* strain from the cattle to a child. On another occasion an American epidemiological study, was conducted after a number of sick patients were found to be infected by fluoroquinolone-resistant *Campylobacter* (Kassenborg et al., 2004). Investigations revealed that the patients were more likely to have consumed poultry meat (chicken or turkey). The investigators drew the conclusion based on the fact that poultry imports are prohibited in America, so poultry meat was the pivotal source of domestically

acquired fluoroquinolone resistant *Campylobacter* infections (Kassenborg et al., 2004). Furthermore, in another study by Winokur et al (2001) which was based on *E.coli* clinical samples collected from cattle, swine and humans, the findings demonstrated that all isolates were resistant to extended spectrum cephalosporin. Moreover, the CMY-2 gene screened from all the isolates was found to be genetically identical, suggesting the occurrence of transmission. Lastly, a study conducted from 1998-1999 by CDC found similarities between quinupristin resistant *Enterococcus faecium* which was isolated from chicken meat purchased in grocery stores from four different states and human stool which was submitted to clinical laboratories by non-hospitalized volunteers. The study was conducted prior to approval of quinupristin use in humans so the observed resistance similarities suggested that dissemination of quinupristin resistant *Enterococcus faecium* did occur.

2.4. Zoonootic bacteria in poultry industry

Microbiological studies have revealed various pathogens that are found in food producing animals. Most of the pathogens have capabilities to be transferred from animals to humans, where they normally cause infections. Countries such as United State of America, Australia and Netherlands have surveillance systems for zoonotic pathogens (in food producing animals and lot of information has been documented. However, in South Africa there is a paucity of information on the prevalence of zoonotic pathogens in the poultry industry. So far a few reported studies that have been conducted on prevalence of foodborne pathogens in South Africa have been mostly done in Gauteng province (Van Nierop et al., 2005). However, there are few studies that have been conducted in KwaZulu-Natal and other provinces (Eager et al., 2008). Against this background, the current review will be focused more on two foodborne pathogens, namely *Salmonella* spp. and *Staphylococcus aureus.*

2.4.1. Salmonella

Salmonella belongs to the family Enterobacteriaceae (Guthrie, 1991).The genus Salmonella contains two species; *S. enterica* and *S. bongori*. There are six subspecies that are differentiated within *S. enterica* based on their biochemical and genomic characteristics (Guthrie, 1991). Different serovars of Salmonella have been identified globally and they are still being identified adding to 2500 serovars that are currently known (Foley et al., 2008).

Epidemiological studies have associated *Salmonella* species with certain diseases in both animals and human, collectively known as Salmonellosis (WHO, 2005).

Salmonellosis diseases; have been reported in both developed and developing countries as a threat to animals and human health. Many diseases associated with Salmonella infections have been reported, however gastroenteritis is dominantly reported across the globe. Retrospective studies have recorded epidemiological incidences for Salmonella infections in humans (Foley et al., 2008). In European countries and in United States Salmonella incidences are known (WHO, 2005) because their surveillance systems for the pathogen are well developed unlike in some African countries where there is lack of Salmonella surveillance systems (Akinyemi et al., 2012). In South Africa there is a surveillance system for foodborne pathogens and *Salmonella* is included as a pathogen of interest. However the limited information on the prevalence of salmonellosis at a provincial and national level shows that the system is not well developed and there is still so much work to be done.

Salmonella spp. can colonize and disseminate in a community of animals and human in several ways. Ingestion of contaminated food, direct contact and poor hygienic environments are some ways in which the pathogen can be disseminated. In humans, the main source of Salmonella infections has been speculated by many researchers as the food of animal origin (Swartz, 2002; Hasan & Aylin, 2009; Lestari et al., 2009). Moreover the reason behind this is explained by the ability of *Salmonella* spp. to survive in various food producing animals such as cattle, pigs, goats and chickens. The production of broiler chickens and their health is maintained using antimicrobial agents. Therefore, this creates a conducive platform for *Salmonella* spp. to accumulate resistance and to proliferate.

2.4.1.1. Salmonella pathogenicity

There are non-pathogenic and pathogenic Salmonella strains that affect animals and humans in different ways. In avian species the well-known pathogenic strains are *Salmonella Gallinaram* and *Salmonella Pollurum*. These two strains have a potential to cause severe infections which can result in mortality of the birds (Revelledo and Ferreira, 2012), affecting the production. Salmonella strains that are based on humans such *Salmonella Typhimurium*, *Salmonella Newport*, *Salmonella enteritis* and others are considered as non-host specific pathogens for avian species. Birds infected by non-host specific salmonella strains do not comprise any visible symptoms; instead they became carriers of the pathogen. Figure 2.1

illustrates that non-host specific Salmonella strains are associated with food poisoning. Figure 2.1 further illustrates that the non- host specific *Salmonella* spp. has a potential to colonize the alimentary tract and ceca of a chicken without manifestation of symptoms. As speculated by various researchers (Revelledo and Ferreira 2012; Foley et al., 2008) Figure 2.1 also shows that how carcass contamination occurs during the slaughtering process. After slaughter meat producers have various meat cleansing processes, however, all those process are not accurate enough to eradicate pathogens completely. When the meat is contaminated chances of consumers contracting the pathogen through food are increased.

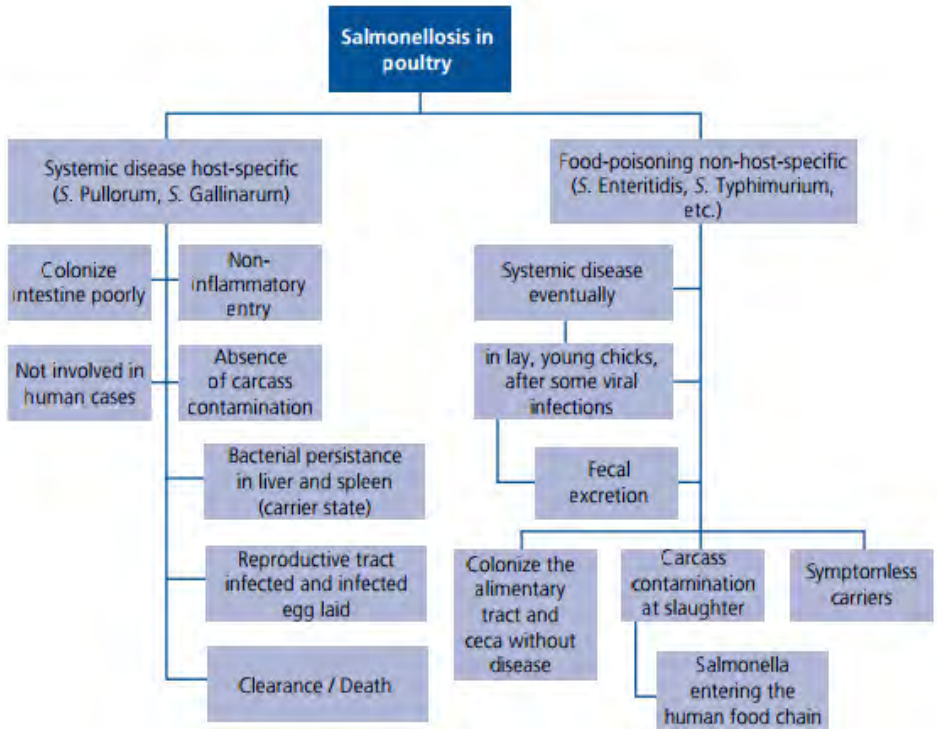


Figure 2.1: Illustration of how host specific and non-host specific Salmonella strains operate in poultry (Revelledo and Ferreira 2012**)**

Pathogenicity of *Salmonella* spp. plays a crucial role for the pathogen to colonize, survive and grow inside the host. There are various systems involved during colonization and survival inside the host, however most of these systems are engineered by genomic islands namely Salmonella Pathogenicity Islands (SPIs) (Marcus et al., 2000).There are more than five SPIs associated with *Salmonella* spp. that have been discovered so far (Winner et al., 2008). These SPIs are located in the chromosomal structure of *Salmonella* spp. (Figure 2.2) and they entail genetic determinants which confer virulence. To date, they are about 60 virulence genes known to be associated with *Salmonella* spp. and all these virulence genes have different roles and different locations where most of them are located in SPI 1, 2, 3, 4, 5

and others (Marcus et al., 2000); Mueller et al., 2008). Amongst the known *Salmonella* Pathogenicity Islands, the SPI-1 is considered as the largest, thus it accommodates a large number of virulence genes compared to others. SPI-1 is associated with type III secretion system (T3SS) and this system is known to assist *Salmonella* to suppress the host immune system by delivering a cocktail of effector proteins (Mueller et al., 2008). Which interfere with host cells and cause destruction in the host cell signalling pathways (Marcus et al., 2000) T3SS has been defined as multiprotein organelles (Foley et al., 2008; Winner et al., 2008) which span the bacterial cell envelop and convey effector proteins into host cells through a needle-like structure formed in a target host membrane (Cosart & Samsonite, 2004; Bhavas et al., 2007; Mueller et al., 2008). Based on Figueroa & Holden, (2012) SPI-1 virulence genes associated with T3SS are normally expressed as a consequence of response sensed by bacteria in the intestine of an infected host. The sensing triggers activation of T3SS upon contact with host's epithelial cells, promoting conveying of effectors across the host cell plasma membrane (Galan, 2001). SPI-2 and SPI-3 are also associated with T3SS; however these islands harbour virulence genes playing different roles. For SPI-2 associated genes it has been reported that some of the genes show close evolutionary similarities for some salmonella servers (Eswarappa et al., 2008). On the other hand, Barrow et al. (1994) hypothesized that the explanation behind can be coevolution of gene sequences for the survival in avian host, because survived reticuloendothelial system has been shown importance for host specificity in chickens.

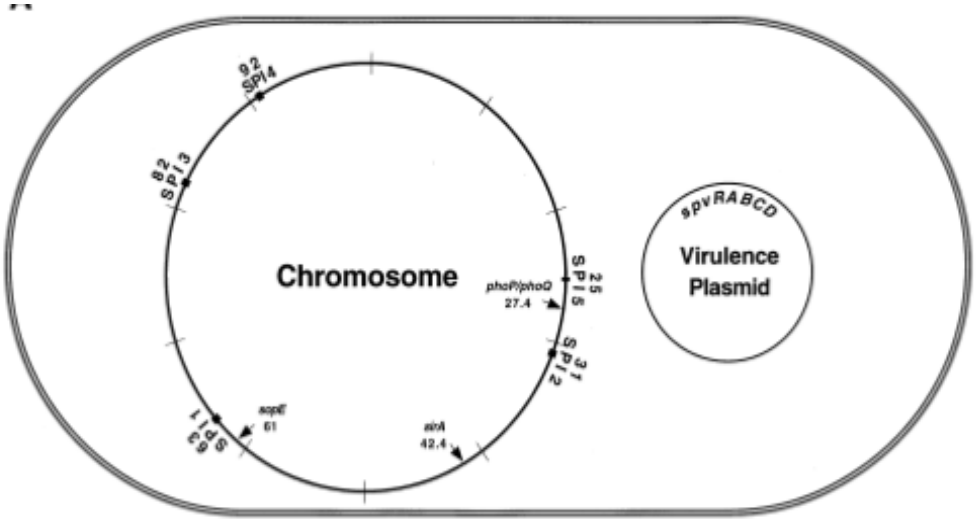


Figure 2.2: Diagram presentation for distribution of Salmonella Pathogenicity Islands (SPIs) and virulence genes in a *Salmonella* spp namely *S. typhimurium* (Marcus et al., 2000

The Invasion A (*InvA)* gene is one of the genes located in SPI-1, it a virulence gene that is considered important (Malorny et al., 2003). It plays a crucial role in life of *Salmonella* spp. since it encodes for a protein which is required to invade host epithelial cells. This gene is conserved across all *Salmonella* spp. and it is considered as preferable marker for rapid detection of *Salmonella* spp. in clinical samples and in food products (Malorny et al., 2003).Another virulence gene that is considered important for salmonella is *cdtB*, this gene is responsible for production of toxin which is used as a weapon for defense (Skyberg et al., 2006). *IroN* gene is responsible for iron uptake, which is required for growth of salmonella (Kaneshige et al., 2009). SopB gene is responsible for manifestation of infection specifically diarrhoea, in humans. Thus is accomplished by the activation of secretory pathway by the *sopB* gene altering the ion balance within the host cells, consequently secreting a fluid which causes diseases (Norris et al., 1998; Wallis & Galyov, 2000). There are different genetic determinants playing a crucial role in serving the purpose of virulence. Some functions of virulence determinants are to monitor the development of new strains and to colonize the host. Therefore it is crucial to study the nature of virulence in microorganism such as *Salmonella.* Moreover availability of information on the danger of *Salmonella* spp. virulence to humans and animals can promote understanding which can make the surveillance system of this organism better (Marcus at al., 2000).

Table 2.2: Some virulence genes associated with Salmonella strains

Virulence gene	Location	Gene function	Broad action	Reference
invA	SPI-1	T3SS apparatus	Invasion of macrophages	Oliveira et al., 2002
sitC	SPI-2	T3SS	Iron uptake	Smith et al., 2015
sifA	SPI-2	T3SS	Intercellular survival and replication in SCV	Ibarra & Steele-Mortimer, 2009
SpiC	SPI-2	T3SS	Survival macrophage	Uchiya& Nikai, 2008
misL	SPI-3	Associated with intramacrophage survival	Survival macrophage	Dorsey et al., 2005
mgtC	SPI-3	T3SS	Vital for bacterial growth at low Mg^{+2} concentrations inside the host cell	Alix & Blanc-Potard, 2008
orfL	SPI-4	Adhesin/ autotransporter	Colonization	Hensel, 2004
pipD	SPI-5	Type III secretion effector associated with SPI-1 system	Enteritis	Hensel, 2004
sopE	SPI-5	Effector protein	Required for full virulence in a murine model	Streckel et al., 2004
spvC	Virulence plasmid	Effector protein	Suppression host immune system	Neumann et al., 2014

2.4.1.2. Salmonella outbreak rates in retail broiler chicken meat

In 2013 there was an outbreak of *Salmonella* Heidelberg in Foster Farm, California (CDC, 2014). The investigations indicated that seven strains of *Salmonella* were involved in the outbreak and the company was forced to recall all its chicken produced at its three plants (CDC, 2014). According to CDC (2014) 42% of people who were sick because of Salmonella infections after eating chicken meat from Foster Farms were hospitalized. Other incidents occurred in India between April 2008 and October 2009, where 500 disease outbreaks in 265 broiler farms were reported. (Nazir et al., 2012). Samples were taken for investigation and it was confirmed that 8.4 % of the outbreak was a results different salmonella species.

In the United States poultry has been estimated as a contributor of 17 % Salmonella food poisoning outbreaks. The prevalence rates of *Salmonella* contamination in developed and developing countries are not the same, since most developed countries have surveillance and control systems aimed to decrease *Salmonella* contamination rate. In South Africa published information on salmonella infections on poultry is limited. This does not mean there are no cases of Salmonella outbreak in poultry industry of South Africa, they do occur and most of them are not reported. The main problem is some poultry farmers do not report salmonella infections outbreak because they want to protect their product reputation. Currently, information available is the one formulated at Bacteriology Laboratories situated at Onderstepoort Veterinary Institute (ARC-OVI) in Pretoria. According to Kidanemariam et al., (2010) all South African *Salmonella* isolates of animal origin are serotyped at the Agriculture Research Council (ARC). This makes the institute to have some records for pathogenic *Salmonella* strains of animal origin, but the problem is that these strains are only detected on sick animals. However, literature makes it clear that sometimes an animal or a human can be infected by a certain pathogen but not show any symptoms. Therefore, this highlights the need for *Salmonella* surveillance even on healthy animals.

Contamination of chicken meat by *Salmonella* spp. is due to various factors and cases are reported every year, where different countries show different prevalence's. In a previous South African study conducted by Van Nierop et al.(2005) where 99 Fresh and frozen whole chicken carcasses were collected from butcheries, supermarkets and street vendors around Gauteng province, the prevalence of *Salmonella* spp. contamination obtained after investigation was 19.2 %. These findings are in accordance to findings obtained by Cortez et al. (2006), however, they are relatively low compared to findigs obtained on a similar studies

(Antunes *et al*., 2003; Capita *et al*., 2007; Hao Van *et al*., 2007; Chuanchuen and Padungtod, 2009) which reported *Salmonella* spp. prevalence of 57%, 49%, 60% and 53.3% respectively. The presence of this pathogen in poultry production is a burden because it affects productivity and creates a bad reputation for producers. Furthermore, it has a bad impact on the economy of the country.

2.4.1.3. Reported Salmonella cases in humans

Salmonella cases in humans have been reported worldwide, however information on the organisms' prevalence is still more available in developed countries compared to developing countries. Up to now, some developing countries do not even have surveillance systems for this organism yet literature has stipulated *Salmonella* spp. as problematic zoonotic pathogens globally. So there is a need for Salmonella based surveillance systems in all countries regardless of the standard of living. In 2012 Center for Disease Control and prevention released estimates for the United States' foodborne illnesses, hospitalizations, and deaths from 2000 to 2008. The estimates shown that 1,000,000 people reported sick, 19,000 got hospitalized and 380 died because of *Salmonella* spp., nontyphoidal (CDC, 2009). Whereas, for *S. enterica* serotype typhoidal 1,800 people reported sick, 200 were hospitalized and no death was report (CDC, 2009).

In South Africa documented information on *Salmonella* spp. outbreaks on humans is limited, but there are few cases that have been previously confirmed in all provinces across the country (Tollefson and Karp, 2004). National Institute for Communicable Diseases is a division of National Health Laboratory Services (NHLS), which is responsible for surveillance systems of communicable diseases in humans. Salmonella is very dangerous in immuno-compromised patients. When an HIV positive patient is infected by *Salmonella* the patient's health is threatened because it has been reported that it enters into bloodstream of the patient and is transported into different part of the body. Thus, increasing chances for a patient to be sick as result of *Salmonella* based infections. National Institute for Communicable Diseases provides information obtained from surveillance systems which include about 31 hospitals in all provinces across South Africa. In surveillance systems, *Salmonella* spp. are included with regard to them being organisms with the ability to cause opportunistic diseases associated with HIV (GERMS-SA, 2013). Table 2.3 adopted from GERMS-SA Annual Report 2013, presents reported cases of Salmonella in humans of South Africa in 2013. In 2013 Gauteng was the leading province in terms of Salmonella cases reported, it was followed by Western Cape, then KwaZulu-Natal. There are various factors

involved behind the number of cases reported in each province, geographical location, state of hygiene and sanitation practice being some of them. This highlights a need for the enhancement of surveillance systems and for people to be educated about foodborne pathogens such as *Salmonella.*

Table 2.3: Number of invasive and non-invasive non-typhoidal Salmonella cases reported to GERM-SA, by province, South Africa, 2013, n=2,995 (Including audit reports, missing isolates, mixed and contaminated cultures) (adopted from GERM-SA, 2013

Province	**Non-invasive, non-typhoidal *Salmonella* isolates**	**Invasive, non-typhoidal *Salmonella* isolates**
Eastern cape	198	44
Free State	72	19
Gauteng	992	315
KwaZulu-Natal	305	121
Limpopo	18	7
Mpumalanga	128	42
Northern Cape	15	5
North west	58	6
Western Cape	512	138
South Africa	2 298	697

The main problem affecting the South African Health Department is that most bacterial infections are never reported since some infections caused by *Salmonella* spp. last for few days so they are neglected. Although results from GERM-SA have been made available to anyone who wants to access but there are not a true representative of what is happening in South Africa in terms of *Salmonella* spp. infections. The reason is GERMS is a medical aid scheme for government employees. Therefore, the figures come from records from sick employees covered by the scheme and it excludes high percentage of South Africa citizens.

2.4.1.4. Antibiotics resistance of salmonella in chickens and human

The first ceftriaxone-resistant salmonella was reported in the United States, literature records that the case occurred in a child of a veterinarian who was treating several cattle herds for severe diarrheal diseases (Tollefson and Karp, 2004). This was confirmed by

comparing the salmonella isolates from the child with the isolates from the ill cattle. Findings have shown that some salmonella isolates from the ill cattle treated by the veterinarian were closely related to the salmonella isolated from the child (Fey et al., 2000). Antimicrobial agents such as trimethoprim, ampicillin, gentamicin, tetracycline and others are used as first line treatments for infections caused by *Salmonella* species in both human and animal medicine (Carattoli et al., 2002). However, development of resistant *Salmonella* species towards antimicrobials have been reported worldwide and it is increasing in an alarming rate, threatening health of animals and humans (Breuil et al., 2000;Carattoli et al., 2002; Van et al., 2007; Wannaprasat et al., 2011; Meng et al., 2011). Su *et al*. (2004) declared that antimicrobial resistance depends on a drug of choice used and also on a bacterial strain involved during the infection. Infections caused by *Salmonella* spp. can be difficult to treat, simple because increasing antimicrobial resistance in *Salmonella* has a potential to delay therapy by limiting therapeutic options available for clinical cases. According to Frye and Jackson (2013) *Salmonella* isolates resistant to various antimicrobials have been isolated worldwide from both human and animal clinical samples, fresh food produce and from health food producing animals ever since the use of antimicrobials was established. Moreover *Salmonella* that is resistance to more than one antibiotic have been reported as well.

A Brazilian study by Oliveira et al. (2005) whereby *Salmonella* spp. isolates from 22 broiler chickens were tested against twelve antimicrobial agents reported resistance prevalence rates of 91% to streptomycin, 86.4% to nitrofurantoin, 91% to tetracycline and 90, 9% to sulphonamide. A similar study reported that obtained salmonella isolates were resistance to tetracycline (84%), Streptomycin (12%), nalidixic acid (60%) and nitrofurantoin (32%) (Ribeiro et al., 2007). Another similar study reported resistance to streptomycin (73.7%), nitrofurantoin (52.3%), tetracycline (31.6%), and nalidixic acid (21%) (Duarte'et al., 2009). Human isolates based study conducted in the same country by Oliveira et al. (2006) on antimicrobial resistance of Salmonella from food involved in reported salmonellosis cases reported an antimicrobial resistance prevalence of 21, 5% towards nalidixic acid, 12, 7% to gentamycin and 11, 4% to streptomycin. All these studies have reported antimicrobial resistance prevalence rates that are not in agreement, this shows that antimicrobial resistance testing depends on many factors since all these studies were conducted in Brazil yet they report different prevalence rates. Moreover, these findings also show that salmonella isolated from Brazil are resistant to a variety of antimicrobials.

Information available suggests that salmonella infections caused by resistant strains can be more severe than infections caused by sensitive strains (Helmet al., 2002). This is supported by the information presented by CDC study on 24 Salmonella outbreaks which demonstrated that resistant salmonella resulted in High hospitalization rate compared to outbreaks by susceptible salmonella (WHO, 2003).Information associate with virulence and antimicrobial resistance *Salmonella* of chicken origin is limited in South Africa, Therefore this limitation provides a scope for studies that will focus on investigation the presence, virulence and antimicrobial resistance of Salmonella in chicken meat of South Africa and also on imports.

2.4.2. Staphylococcus aureus in poultry industry

Staphylococcus species are Gram positive bacteria that are normally found in animals and humans (Lowy, 1998). There are about 40 known staphylococcus species (Bannerman, 2003), some are of animal origin and some are of human origin. Some examples of staphylococcus species are *Staphylococcus epidermidis*, *Staphylococcus haemolyticus*, *Staphylococcus capitis, Staphylococcus warner, Staphylococcus xylosus* and *Staphylococcus aureus*. These bacteria are mostly found on mucosal surfaces and on the skin of both animals and humans. Staphylococci species are commensal, most of them have no harm on humans and animals, but there have been reported to play a role in the spread of antimicrobial resistance between bacteria (Summers, 2002).

Staphylococcus aureus is the most widespread staphylococcus; it has been reported to be the most harmful species than others because it produces toxins which are responsible for staphylococcal food poisoning. Staphylococcal food poisoning occurs when someone has ingested food that is contaminated with *Staphylococcus aureus* which is harbouring toxins producing components. The staphylococcal food poisoning causes various number diseases in humans, such as bones, joints, respiratory, skin and soft tissue diseases (Lowy, 1998). The prevalence of *Staphylococcus aureus* infections worldwide has pointed this organism as one of the leading causes of food-borne diseases (Jablonsky and Bohach, 1997). Documented information shows that this organism is responsible for high morbidity and mortality in children worldwide; however more evidence has been reported intensively in developing countries (Berkely et al., 2005; Enwere et al., 2006; Sigauque et al., 2009).

The main sources of *Staphylococcus aureus* are food producing animals such as bovine, poultry, swine and others. The bacteria can be passed on from food producing animals to

humans through food and direct contact with contaminated carcasses. Mead et al (1995) reported that Staphylococcus aureus expresses different key properties that promote it to survive, colonize and disseminate in commercial poultry process plants. These key properties are called virulence factors and collectively are responsible for pathogenicity of this organism. Examples of virulence factors(virulence genes) are staphylococcal protein A (*spa*), coagulase protein (*coa*), Staphylococcal enterotoxins A to E (*sea, seb, sec, sed and see*), collagen adhesin gene (cna), toxic shock syndrome toxin 1 (tst), exfoliative toxins (eta, atb), leucocidins (lukE-lukD, lukM), and so forth (Montanaro et al., 1999; Mehrotra et al., 2000; Akineden et al., 2008; Pereira et al., 2009). Staphylococcal virulence factors play the main role in causing staphylococcal infections in animals and humans. Various studies have detected the presence of virulence factors in the *Staphylococcus aureus* isolated from different types of food products from livestock (Mehrotra et al., 2000; Kitai et al., 2005; Normanno et al., 2005; Pereira et al., 2009; Pu et al., 2011; Abdalrahman et al., 2015). The presence of virulence factors on *Staphylococcus aureus* isolates from food origin, implicate the possible for virulence factors to be disseminated throughout the communities. The spread of such factors can also results in genetic alteration of virulence factors since evolution is at works and this can cause more harm on host carrying Staphylococcus aureus with dangerous virulence factors.

A number of antibiotics are used to cure infections caused by *Staphylococcus aureus* in both animals and humans. However, since the animal in feed use of antibiotics was permitted *Staphylococcus aureus* developed resistance against an array of antimicrobials agents. The most crucial resistance involving *Staphylococcus aureus* is methicillin resistance. Methicillin resistance *Staphylococcus aureus* (MRSA) has been reported both animals and humans worldwide and it is a serious growing problem for many countries (Van Loo et al., 2007). MRSA emerge mainly when *Staphylococcus aureus* has acquired a genetic determinant known as *mecA* which encodes a modified penicillin binding protein (PBP2a) that has a low affinity for β-lactams(Lim and Strynadk, 2002; Fuda et al., 2004). Production of penicillin binding protein (PBP2a) with low affinity results in *Staphylococcus aureus* strains being resistant to the entire group of β-lactams and other groups of antimicrobial agents (Lim and Strynadk, 2002). Contamination of retail meats by MRSA have been reported for bovine, poultry and swine products and this has recently drawn some attention (Lee, 2003; Gundoga et al., 2005; Kitai et al., 2005; De Boer et al., 2009; Pu et al., 2011; Kelman et al 2011). In South Africa there is limited information on *Staphylococcus aureus* presence and resistance

to commonly used antimicrobial agents. This highlights the need for studies that will investigate the presence, virulence and antimicrobial resistance of *Staphylococcus aureus* strains isolated from poultry products of South Africa.

2.4.2.1. *Staphylococcus aureus* virulence

Staphylococcus aureus virulence is very complex and depends on an array of virulence genes. Virulence genes involved in this microorganism are clustered under two categories namely cell-surface-associated (adherence) and secreted (exotoxins) factors (Diep and Otto, 2008). Cell surface virulence factors play their role by allowing *Staphylococcus aureus* to adhere to surfaces/tissues consequently avoiding or invading the immune system of the host (Foster and Höök, 1998; Foster, 2005). Secreted factors are responsible for production of toxins, which interferes host cell lines consequently causing harmful effects (Dinges et al., 2000; Lin and Peterson, 2010). Cell surface virulence factors includes microbial surface components recognizing adhesive matrix molecules (MSCRAMMs), capsular polysaccharides and Staphyloxanthin. Capsular polysaccharides are responsible for enhancement of bacterial colonization and persistence on mucosal surfaces. Secreted factors include superantigens, cytolytic toxins, various exoenzymes and miscellaneous proteins (Dinges et al., 2000; Harraghy et al., 2003; de Haas et al., 2004; Lee et al., 2004)

The MSCRAMMs entail staphylococcal protein A *(spa*), Fibronectin-binding proteins (*FnbpA* and *FnbpB*), Collagen-binding protein and Clumping factor proteins (*ClfA* and *ClfB*)(Vaudaux et al., 2002; Lee et al., 2004). All these MSCRAMMs have different functions. Staphylococcal protein A *(spa)* encode for a gene (*spa* gene) which binds to IgG thus interfering with opsonisation and phagocytosis. According to Koreen et al (2004) *spa* gene entail a polymorphic region X which is known to poses various number of repeated 24 nucleotide bases. Thus has allowed researchers to use this gene to investigate diversity of *Staphylococcus aureus* isolates by genotyping the gene using molecular techniques. Fibronectin-binding proteins (*FnbpA* and *FnbpB*) functions are to attach to fibronectin and plasma clot; whereby collagen-binding protein is for adherence to collagenous tissues and cartilage and lastly clumping factor proteins (ClfA and ClfB) are for Mediation of clumping and adherence to fibrinogen (Lee et al., 2004; Lin., et al., 2010).

Superantigens are virulence factors which belong to secreted factors category. Superantigens are divided into two groups' namely staphylococcal enterotoxins (SEA, B, C, D, E, G and Q) and toxic shock syndrome toxin-1 (TSST-1). These superantigens have the

ability stimulate proliferation of T-lymphocytes and they cause toxic shock syndrome and food poisoning. Different studies have reported presence of superantigens in staphylococcus aureus strains of chicken meat origin (Thomas et al., 2007; Oguttu et al., 2014). The coagulase enzyme is also a virulence factor in addition to its important role in *Staphylococcus aureus* diagnosis. This enzyme is encoded by *coa* gene, which is used a molecular detection to determine if the *Staphylococcus aureus* strain is coagulase positive or negative. The *coa* gene entail conserve polymorphic regions that can be utilized to investigate diversity among *Staphylococcus aureus* (Shopsin et al., 2000).

There is a need for the understanding the mechanisms of staphylococcal virulence factors in poultry industry. This will help in preventing and decreasing colonization of broiler chickens by dangerous strains of *Staphylococcus aureus*. Moreover it has also been reported that an improved understanding of *Staphylococcus aureus* pathogenesis carries the promise of identification of new targets for novel therapies for preventing and treating both acute and chronic *Staphylococcus aureus* infections in animals and humans (Barrow and Wallis, 2000).

2.4.2.2. *Staphylococcus aureus* reported outbreaks in South Africa poultry industry

In South Africa there is a lack of pathological reports on spontaneous *Staphylococcus aureus* outbreaks associated with poultry and it products. This does not mean that there are no cases at all, but the challenge is farmers never report such cases. However the presence of staphylococcus aureus in poultry industry, have been noted by researchers who have conducted studies investigating the presence of this organism at a farm level and also at a retail level. A poultry process plant study conducted by Geornaras and von Holy, (2001) which investigated the antimicrobial susceptibilities of isolates of *Staphylococcus aureus*, *Listeria* species and *Salmonella* serotypes associated with poultry processing on presented results which showed that there was presence of *Staphylococcus aureus* in this poultry plant located in Sandra where 25,000 birds are slaughtered every day. Another study conducted in Nkonkobe Municipality, South Africa reported that out of 150 farm animals including chickens used for investigating the presence of *Staphylococcus aureus*; provided results which showed that a total of 120 Staphylococcus isolates were detected there were resistant to different antimicrobials (Adegoke and Okoh, 2013). The presence of Staphylococcus was also observed on the ready to eat chicken meat, which was collected in Tshwane Metropole during the study undertaken by Oguttu et al. (2014). The study investigated the food value

chain of ready to eat chicken meat and associated risk for staphylococcal food poisoning in Tshwane Metropole. Apart from the presence of *Staphylococcu aureus* results also show the 1.3% probability for food poisoning due to consumption of contaminated meat by staphylococcal enterotoxins.

Information presented by research studies which have focused on investigating the presence of *Staphyloccocus aureus* associated with South African broiler chicken meat and it products; shows that this microorganism is present in the countries poultry industry and it has a potential to disseminated to different environmental if not monitored properly

2.4.2.3. *Staphylococcus aureus* resistance to antibiotics

Staphylococcus aureus has a potential to be resistant to any group of antimicrobial agents, and it resistance has been noted under veterinary and human medicine sector. Amongst all types of resistant *Staphylococcus aureus*, methicillin resistant *Staphylococcus aureus* (MRSA) is regarded as crucial, since it had been proven as the cause of acquired infections associated with high rate of bacterial mortality worldwide (Tiemersmaetal., 2004). MRSA strains are now reportedly been isolated in livestock meat (beef, pork and chicken) and different food products such as diary milk and it products. The presence of MRSA in food products posing a threat over a potential spread of MRSA to consumers via the food chain (Voss et al., 2005; De Neeling et al., 2007; Wulf, and Voss, 2008). Contamination of chicken meat by Staphylococcus aureus have been detected in countries like Netherlands, Japan, Brazil, United State of America, Nigeria and so forth (Kitai et al., 2005; Kwon et al., 2006 ;de Boer et al., 2008; Momtaz et al., 2013; Islam et al., 2014; Ugwu et al., 2015). In South Africa there is limited information on MRSA isolated form chicken meat.

Contamination of meat with resistant *Staphylococcus aureus* strains is normally speculated to a consequence of cross contamination from the intestines of the animal to the carcass at a slaughterhouse and this resistance can be transmitted to farm workers and it can further disseminate to different environments through retail meat and direct contact with meat handlers (Weese et al., 2010). Since antimicrobial resistance in foodborne pathogens is reported as an escalating burden threatening human health, therefore there is a need for studies which will investigate mechanisms involved in antimicrobial resistance of foodborne pathogens such *Staphylococcus aureus*. Information from such studies will provide more

understanding which will assist in mitigating antimicrobial resistance of troublesome foodborne pathogens.

2.5. Mechanisms of action and resistance in antibiotics

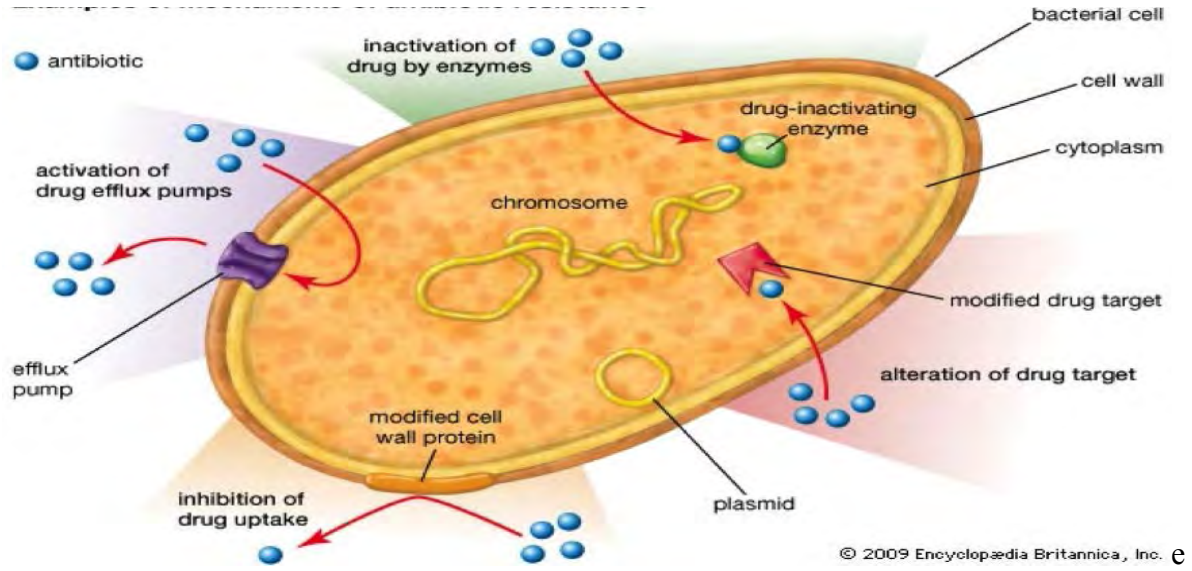
Antibiotic resistance is a phenomenon that constitutes a threat to a successful treatment of bacterial infectious disease. Since the discovery of antimicrobial agents and their introduction to different fields, antimicrobial resistance emerged and has been reported across a variety of microorganisms (McDermott et al., 2003). Threatening lives of humans, animals and plants, since the commonly used antibiotics are no longer effective as they use to be before. Antimicrobial resistance can be classified into three different categories namely intrinsic, mutational and acquired resistance (Woodford and Ellington, 2007).

Intrinsic resistance is the natural ability for an organism to resist antibiotic activity. There are bacteria which lack some components which are required to interact with antimicrobial for effectiveness of the drug to prevail. However, absence of such components limits the effectiveness of the antimicrobial, for example Gram negative bacteria lack component which as responsible for an uptake of vancomycin resulting from inability for drug to enter outer membrane. In this way antimicrobial resistance is constituted (Poirel et al., 2007).

Mutational resistance are due spontaneous chromosomal changes which can involve one or more nucleotide consequently attributing to genetically altered bacterial population. Bacteria are generally known to have short generation times and are able to evolve in what is perceived to be real-time (Woodford and Ellington, 2007). So mutations are important for them to survive under rapidly changing conditions. Acquired resistance refers to acquisition of genetic determinants encoding resistance from another microorganism (Magnet and Blanchard, 2005).

Bacterial resistance depend on the pathogen involved and also on the antibiotic used to suppress or completely eliminate growth of that particular microorganism. Bacterial cells become resistant to antibiotics in different mechanisms (McDermott et al., 2003). Wright, 2005; Magnet and Blanchard, 2005). These mechanisms include Ribosomal protection, Efflux pump, enzymatic oxidation and modification of bacterial cell wall. During ribosomal protection an antibiotic is blocked from binding to the ribosome, whereas during the enzymatic oxidation the acetyl group is added to the molecule, causing the deactivation of the

drug. Moreover, during efflux pump there are resistant genetic determinants involved, these determinants encodes for a membrane that actively pumps out the drug out of the cells. And lastly th



e cell wall is modified in a manner that an antimicrobial is inhibited to enter the cell. All these mechanisms are well illustrated diagrammatically on figure 2.3. Antimicrobial resistance mechanism in different microorganisms by antimicrobial class is as follows:

Figure 2.3: Diagram to illustration mechanisms of bacterial resistance**.**

Adopted from: (https://thetripletcode.wordpress.com/2014/02/23/antimicrobial-resistance-a-ticking-time-bomb-2/)

2.5.1. Aminoglycosides

Aminoglycosides includes antibiotics such as amikacin, gentamicin, kanamycin and tobramycin (Mascaretti, 2003). Such antibiotics possess a broad spectrum with many desirable features for the treatment of life threatening diseases (Gilbert, 1995). The aminoglycosides kill bacteria by inhibiting protein synthesis as they bind to 16S rRNA and also by disrupting the integrity of the cell wall (Wimberly et al., 2000; Schluenzen et al., 2000; Vakulenko,and Mobashery, 2003). However, soon after introduction of

aminoglycosides, these drugs also experienced resistance in different bacteria. Murray (1991) stipulated that the emergence of resistance strains has reduced the potential of aminoglycosides in empiric therapy. In bacteria, generally resistance to aminoglycosides is mostly due inactivation of enzymes. There are three enzymes associated with inactivation. These enzyme are categorised into three groups namely acetyltransferases, phosphotransferases and nucleotidyltransferases (Magnet and Blanchard, 2005; Gebreyes and Altier, 2002). When these enzymes are inactivated resistance emerges, thus limiting the effectiveness of any aminoglycoside drug used. The enzyme inactivation has been associated with high level of aminoglycosides drug resistance compared to other resistance mechanisms such as ribosomal alteration (Poehlsgaard and Douthwaite, 2005) and loss of permeability (Davies and Wright, 1997).

Documented evidence demonstrated that aminoglycoside strains emerge because of acquiring of genes encoding for aminoglycoside resistance (Elango et al., 2014). Various genes conferring resistance to different aminoglycoside antimicrobials have been reviewed in literature (Ramirez et al., 2013; Frye and Jackson, 2013; van Hoek et al., 2011; Ramirez and Tolmasky, 2010). Most aminoglycosides resistance genes encode resistance specific to each antimicrobial within the aminoglycoside group for example anti(3")-Ia confers resistance to streptomycin only. However, in some case it is possible to find a gene which encodes resistance to two or more aminoglycosides for examples aph(3')-II gene confers resistance to kanamycin, gentamicin and tobramycin .

There are many aminoglycosides resistance genes that have been reported and some are still newly discovered (Kao et al., 2000; Tsai et al., 1998); Chow et al., 1997). Examples of aminoglycosides resistance genes are aac(60)-Ie-aph(200)-Ia aph(2")-Ib , aph(2")-Ic , aph(2")-Id , aph(3")-IIIa, aac(6')-Ii , ant(3")-Ia ,ant(4")-Ia, ant(6')-Ia, ant(2')-Ia, ant(2')-Ib, ant(2')-Ic (vesque et al ., 1995 Noppe-Leclercq et al, 1999; Le ´ Chow, 2000). A number of studies have confirmed presence of aminoglycosides antimicrobial resistance genes in retail meat (; Sheikh et al., 2012; Momtaz et al., 2013; Santos et al., 2014). Chen et al (2004) reported on a study where presence of aminoglycosides resistance genes was detected of Salmonella isolates which were isolated on retail meat of various animals(chicken, turkey, beef and pork). On this study six different aminoglycosides resistance genes were detected (aadA1, aadA2, aacC2, Kn, aph(3)-IIa, and aac(3)-Iva) and they were reported to be diverse. The presence aminoglycosides resistance genes in bacterial isolates detected from retail meat is a threat to humans, since dissemination of such genes is possible.

2.5.2. Beta-lactams

Beta lactams are made of three major groups namely penicillins, cephalosporins, and carbapenems. There are many antimicrobials which belong to these groups, examples include penicillin, ampicillin, amoxicillin, methicillin, cefoxitin and so forth. These antimicrobials are mostly used and they have saved countless lives and some of them still continue to be mainstay of therapy for bacterial infections in humans and animals (Bartlett, 2003). Beta lactam antimicrobials exhibit their bactericidal effects by inhibiting enzymes involved in cell wall synthesis. During the treatment, the antimicrobial binds to cell wall synthesis enzymes known as penicillin binding proteins (PBPs), thereby inhibiting the peptidoglycan synthesis (Ghuysen, 1991). The inhibition of PBPs weakens the cell wall resulting in inhibition of cell growth and possible cell death.

Extensive use of β-lactams antimicrobials in human and veterinary medicine has resulted in emergence of resistance which is increasing at a significant rate and has become a common problem worldwide. β-lactams resistance is based on four mechanisms which are inactivation of antimicrobial by β-lactamase, modification of target PBPs, impaired penetration of drug to PBPs and Efflux(as illustrated by Figure 3) (Lakshmi et al., 2014; Louis and Rice, 2012; Jacoby, 2009; Poirel et al., 2007). β lactam mechanism is different in Gram negative bacteria as it is in Gram positive bacteria. In gram-positive bacteria, β-lactam resistance most commonly results from expression of intrinsic low-affinity penicillin-binding proteins. Whereas in gram-negative bacteria, expression of acquired β-lactamases presents a particular challenge owing to some natural spectra that include virtually all β-lactam classes (Louis and Rice, 2012).

The most common mechanism of resistance in β lactams is the secretion of β lactamase and this particularly occurs in Gram negative bacteria. β lactamase enzyme confer resistance by disrupting the rings of β lactam breaking amide bonds, preventing drug effectiveness (Tenover, 2011; Gupta, 2007). β lactamase based resistance mechanisms are very complex in manner that they are differentiated in different classes namely A, B, C and D (Danel et al., 2007; Philippon, et al., 2002; Bush et al., 1995). These classes have been reviewed in different literature (Drawz and Bonomo, 2010; Poole, 2004). There are genes encoding for β lactamase resistance. In most bacterial species these genes are chromosomal encoded, but they can also be found in plasmids in bacteria such as *Salmonella* (Mascaretti, 2003).

Examples of β lactamases resistance genes are *blaCMY, blaTEM, blaSHV, blaPER, blaPSE, blaOXA and blaCTX-M* and most of these genes have been detected in pathogens such Salmonella (Winokur et al., 2001), E. coli (Bortolaia, et al., 2010), Enterobacteriaceae (Canto´n et al., 2008) and so forth. Furthermore, these genes has been detected on bacteria isolated from food producing animals meat(Frye et al., 2008; Hasman et al., 2005; Zhao et al., 2001) and in humans (Whichard et al 2007).

Non β-lactamases resistance of beta lactams antimicrobials have been reported in both Gram genitive bacteria (Richter et al., 2001; Watanabe et al., 2004) and Gram positive bacteria (Kuroda et al., 2003; Alba et al., 2002). Production of altered PBP2b gene associated with non β-lactamases resistance have been reported (Nagai et al., 2002; Chesnel et al., 2003;) contributing to reduced affinity for β-lactams antimicrobials which then results in drug resistance. The example of this type of resistance is methicillin resistance in *Staphylococcus aureus*. Methicillin resistance in *Staphylococcus aureus* is a clinical challenge affecting both veterinary and human medicine worldwide. A certain region in *Staphylococcus aureus* genome called Staphylocococcal cassette chromosome mec (SCCmec) harbors genes which confers resistance to methicillin. The well-known gene responsible for methicillin resistant *Staphylococcus aureu*s (MRSA) is *mec A* gene. However other genes playing a role in MRSA, namely pbpB (Pinho et al., 2001), murF (Sobral et al., 2003) have been discovered. *Mec A* gene encodes for PBP2a, which consequently plays a role in MRSA. The production of PBP2a is not a problem in MRSA, but to other microorganisms as well (Sauvage et al., 2002). This has warranted to novel studies, which are focusing on synthesis new drugs which will target PBPs (Zervosen et al., 2004; Pechenov et al., 2003).

2.5.3. Tetracyclines

A derivative of tetracycline which is used in livestock production is oxytetracycline. Tetracycline has a broad spectrum of activity against variety of bacteria both Gram positive and Gram negative (Speer et al., 1992). It frequently used in poultry because is it relatively cheap, has less side effects and effective against a wide variety of microorganisms (Moellering, 1990; Chopra and Roberts, 2001). The use of tetracycline in veterinary medicine mainly include low dose use for growth promotion, treatment of gastrointestinal, respiratory and skin bacterial infections, infectious diseases of locomotive organs and of genito-urinary tract as well as systemic infections and sepsis (Prescott et al., 2000). During treatment tetracycline inhibits bacterial protein synthesis by binding to the 30S bacterial ribosome and

preventing access of aminoacyl tRNA to the acceptor (A) site on the mRNA-ribosome complex (Chopra and Roberts, 2001). The use this drug have had its own successes previously, however currently it efficacy is compromised since various strains of bacteria have been reported to entail features which makes them resistant.

Documented information records that pathogens use three strategies to exert resistant to tetracycline. The three strategies include limiting the access of tetracycline to the ribosomes, altering the ribosome to prevent effective binding of tetracycline, and producing tetracycline-inactivating enzymes (Goodson, 1994; Speer et al., 1992). All these resistance mechanisms are engineered by different antimicrobial resistance genes. Each mechanism has it genes conferring resistance to tetracycline. For efflux pump, here are the resistance genes involved. *tet(A), tet(B), tet(C), tet(D), tet(E), tet(G), tet(H), tet(I), tet(J), tet(K), tet(L), tet(Y), tet(30), tet(31), tet(34), tet(35)* in Gram-negative bacteria and *by: tet(K), tet(L), tetA(P), tet(V), tet(Z), tet(33), tcr3 or otr(B)* in Gram-positive bacteria (Miranda et al., 2003; Roberts, 2003; Tauch et al., 2002; Tauch et al., 2000). All these genes are responsible for pumping out tetracycline as it is illustrated in (Figure 3). For Ribosomal protections mechanism eight tet genes are no known to be responsible for resistance so far and they are *tet(M), tet(O), tet(S), tet(W), tet(Q), tet(T), tetB(P)*and otr(A). They are generally associated with conjugative transposons, which have preference for the chromosome (Roberts, 1997). Speer et al (1991) reported that for enzymatic modification and inactivation only one gene namely *Tet(X)* has been known to be responsible for this resistance mechanism. Most genes have been isolated in foodborne pathogens of poultry origin and they have potential to be transmitted to other from pathogens to the other via horizontal gene transfer.

2.6. Dissemination of virulence and antimicrobial resistance genes

Genetically dissemination of virulence and antimicrobial genes involves a phenomenon known as horizontal gene transfer (HGT). HGT refers to the transfer of genes between microorganisms mostly asexually (Gyles and Boerlin, 2014). According to McGowan et al. (1998) HGT plays a very important role on evolution of virulence and antimicrobial resistance genes. During HGT virulence and antimicrobials resistance genes are transmitted from one organism to another using different mechanisms known transformation, transduction and conjugation (de Vries and Wackernagel, 2004; Brabban et al., 2005 Kelly et al., 2009).

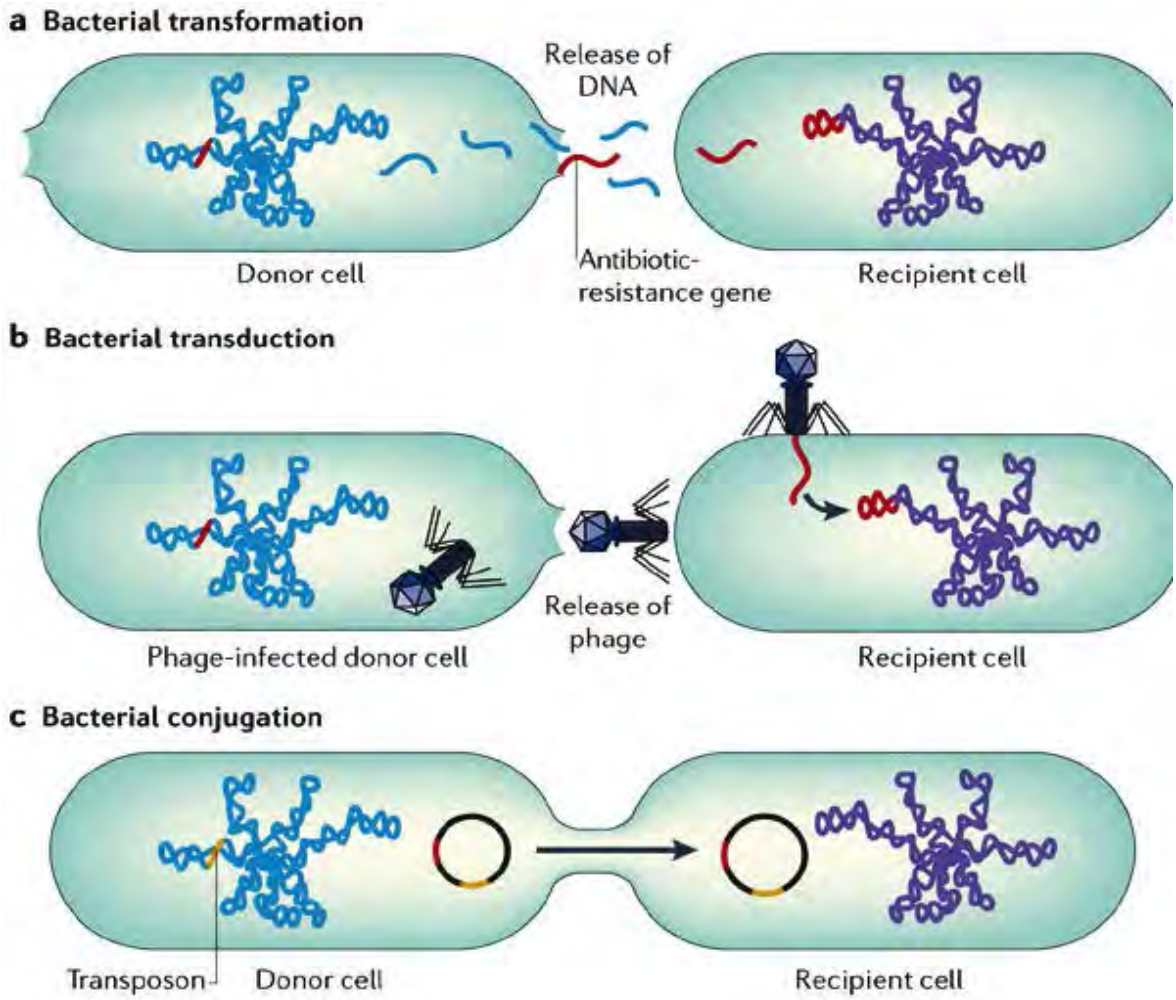


Figure 2.4: Mechanisms illustrating Horizontal gene transfer (Furuya and Lowy, 2006).

For genes to be transmitted through transformation the donor cell need release it genetic material and the recipient cell uptake the naked genetic material from the environment and incorporate it with it genetic material (Figure 2.4a). de Vries and Wackernagel (2004) mentioned that most bacteria are naturally transformable meaning the entail ability to uptake naked DNA from the environment. Horizontal gene transfer through transduction is bacteriophage mediate. The bacteriophage take genetic material from the donor cell goes with it and release it into a recipient cell (Figure 2.4b). Studies have shown evidence that shiga toxin genes (*sxt1* and *sxt2*) (Wick et al., 2005) in E.coli are mostly transferred through and enterotoxins genes in Staphylococcus aureus (*sea, see*) (Novick et al., 2001). Conjugation transfer involves direct contact of the two cells and genetic material is transported via genetic mobile element known as transposon (Figure 2.4c). Conjugation is known to be the most occurring mechanism because majority of gene transfer even in bacteria favours it (Thomas and Nielsen, 2005).

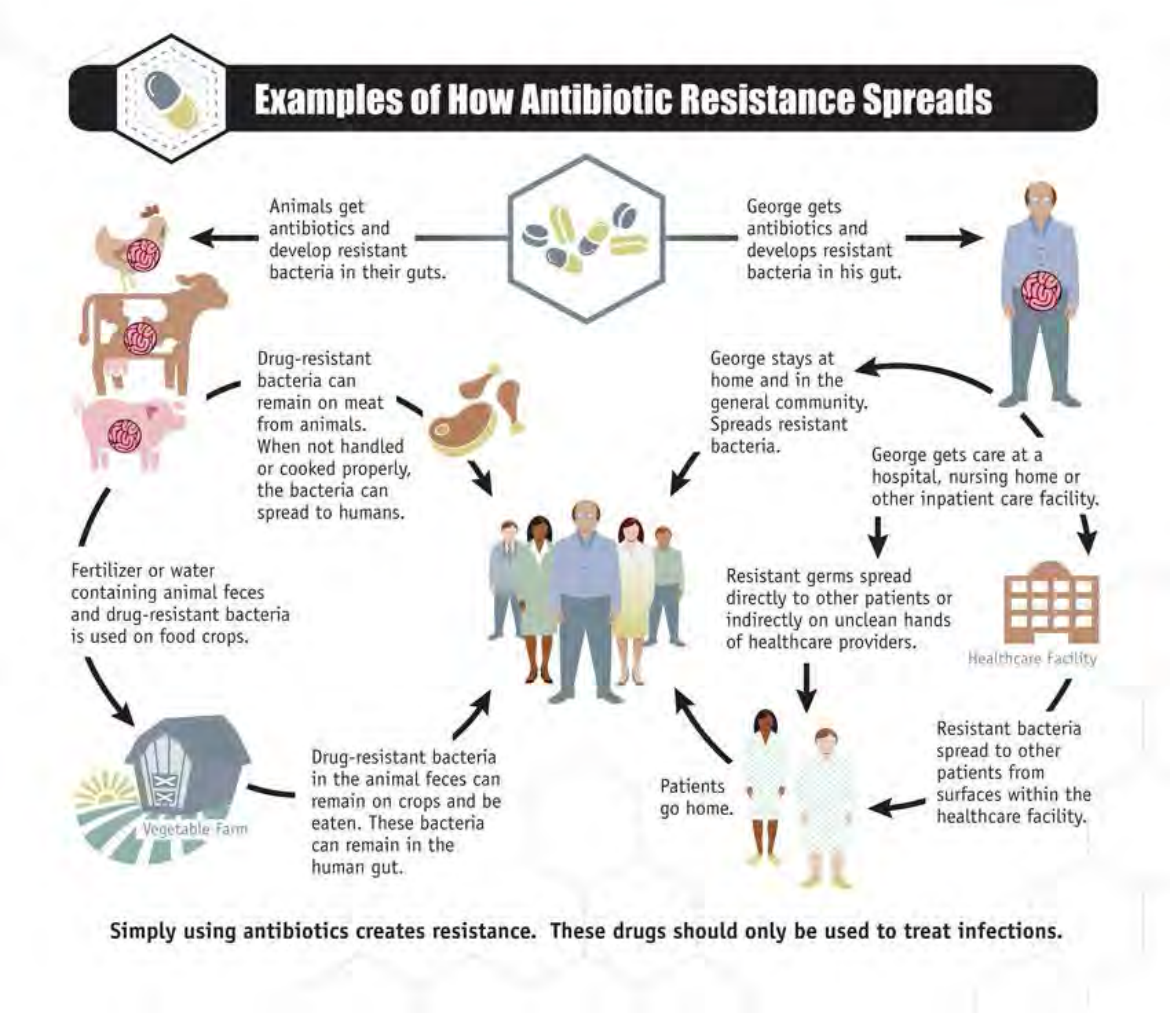


Figure 2.5: Illustration of dissemination of antimicrobial resistance through the community

Bacterial virulence and antimicrobial resistance is also disseminated easily and rapidly throughout the community in form of interconnect chain. Figure 2.5 illustrates how resistance is disseminated; pathogenicity is also disseminated in the same manner. The interconnected relationship showing how resistance virulence is spread shows that it is difficult to control the spread of virulence and resistance in agricultural sector alone, instead all sectors involved need to work together so that measures to decrease spreading can be effective.

2.7. Conclusion

Literature reviewed showed the presence of *Salmonella* spp. and *Staphyloccoccus aureus* in broiler chicken meat. Moreover it showed the presence of genetic determinants conferring virulence and resistance in the two foodborne pathogens mentioned above. It critical that agricultural use of antibiotics be recognized as one of the major contributors to

the development of resistant micro-organisms which results in life-threatening human infections.

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CHAPTER 3

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Prevalence of virulence and antimicrobial resistance genes in Salmonella spp. isolated from commercial chickens and human clinical isolates from South Africa and Brazil

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Abstract

Salmonellosis is a significant public health concern around the world. The injudicious use of antimicrobial agents in poultry production for treatment, growth promotion and prophylaxis has resulted in the emergence of drug resistant strains of *Salmonella*. The current study was conducted to investigate the prevalence of virulence and antimicrobial resistance genes from Salmonella. spp isolated from South African and Brazilian broiler chickens as well as human clinical isolates. Out of a total of 200 chicken samples collected from South Africa 102 (51%) tested positive for *Salmonella* spp. using the InvA gene. Of the overall 146 Salmonella spp. positive samples which were screened for the iroB gene most of them were confirmed to be *Salmonella enterica* spp. giving the following prevalence rates: 85% of human clinical samples, 68.6% of South African chicken isolates and 70.8% of Brazilian chicken samples. All *Salmonella* isolates obtained were subjected to antimicrobial susceptibility testing with 11 antibiotics. *Salmonella* isolates from South African chickens exhibited resistance to almost all antimicrobial agents used, such as Bacitracin (97%), tetracycline (93%), Trimethoprim-Sulfamthoxazole (84%), Trimethoprim (78.4%), Kanamycin (74%), gentamicin (48%), ampicillin (47%), amoxicillin (31%), chloramphenicol (31%), erythromycin (18%) and Streptomycin (12%). All samples were further subjected to PCR in order to screen some common antimicrobial and virulence genes of interest namely sipC, pipD, misL, orfL, pse-1, tet A, tet B, ant (3")-la, sul 1 and sul. All *Salmonella* positive isolates exhibited resistance to at least one antimicrobial agent however; antimicrobial

resistance patterns demonstrated that multiple drug resistance was prevalent. The findings provide evidence that broiler chickens are colonized by pathogenic *Salmonella* spp. harbouring antimicrobial resistance genes. Therefore, it is evident that there is a need for prudent use of antimicrobial agents in poultry production systems in order to mitigate the proliferation of multiple drug resistance across species.

Keys words: *Salmonella* spp.; antimicrobial resistance; chicken; human; susceptibility; virulence gene

3.1. Introduction

The increasing human population around the world places huge demand on food to ensure the survival of mankind. This exerts pressure on a number of food industries such as poultry production systems, where growth promotion agents have to be utilized in an effort to satisfy the increasing food demand. The presence of Salmonella spp. in chicken and related products has been proven to be unsafe for human consumption (CDC, 2013). *Salmonella* is classified as one of the common zoonotic foodborne pathogens causing outbreaks and sporadic cases of gastroenteritis in humans throughout the world (Humphrey, 2000). In the United States of America a total of 19,531 infections, 4,563 hospitalizations, and 68 deaths associated with foodborne diseases were reported in 2012 (CDC, 2013). Epidemiological studies have reported numerous times that foods of animal origin, particularly poultry, are major vehicles associated with illnesses caused by Salmonella (Dallal et al., 2010). Salmonella can grow as surface-associated aggregates on food surfaces and equipment (Chia et al,. 2009), commonly described as biofilms. These cells which develop as biofilms are potential sources of contamination on food products which can result in infection within a human host (Chia et al,. 2009).

The reservoir of Salmonella is the gastro-intestinal (GIT) tract of a wide range of domestic and wild animals, and a variety of food products of both animal and plant origin are potential sources of infection (Thorns, 2000; Thong et al., 2002). Salmonella spp. has approximately 2500 serovars associated with it. These serovars are separated basing on differences in their lipopolysaccharide layer with regards to their somatic (O) and flagellar (H) antigens (Amagliani et al., 2012). With regard to the O antigen, Salmonella spp. is divided into 50 serogroups, and then further divided into greater than 2500 serovars based on

the H antigens present (Amagliani et al., 2012). Majority of serovars of Salmonella spp. belong to S. enterica and the most common serovar associated with zoonotic infection being S. enteriditis, followed by S. typhimurium (Amagliani et al,. 2012). Serovars which are generally found in food products of animal origin include S. enteriditis, S. typhimurium, S. gallinarum, S. weltevreden and S. infantis among others (Foley et al., 2011). Salmonellosis in both humans and animals results from various vehicles of Salmonella serovars such as S. enteritidis, S. infantis, S. kentucky, and S. heidelberg, these vehicles which cause infection appear to be more prevalent in poultry than in any other food animals (Foley et al., 2011).

The ability bacteria to infect host relies on genetic determinants called virulence genes, located in Salmonella Pathogenicity Islands (SPI). According to Groisman et al. (1999) SPIs are portions of DNA that have been acquired from other microorganisms by horizontal gene transfer and they are not present in non-pathogenic strains. At least 60 virulence genes associated with SPIs (Groisman and Ochman, 1997) have been mapped so far and they all serve different functions. Some facilitate colonization for the pathogen to survive under host defense and some are responsible for multiplication inside the host. However, during contamination the host infection outcomes depend on various factors such as age, environment and genetics, influencing the host status (van Asten and Dijk, 2005). A well-known virulence gene in Salmonella spp. is Salmonella invasion gene A (invA) which is responsible for host invasion (Galan et al., 1992). This gene is very vital because it is conserved in all Salmonella spp. Hence, it is used by researchers as a marker to detect this pathogen isolated from different origins. Various studies from both developed and developing countries have been focused on investigating the presence of genes encoding for virulence in Salmonella spp. These countries include United States of America (Zou et al., 2011), Senegal and Gambia (Dione et al. 2001), Brazil (Dias De Oliveira et al., 2003; Borges et al., 2013) and Nigeria (Smith et al., 2015). In South Africa information on the prevalence of virulence genes in Salmonella spp. of animal and human origin is limited. Therefore, these amongst other facts motivated us to embark on the current study.

The use of antimicrobial agents in poultry production, for treatment purposes, growth promotion and prophylaxis raises major concern with regard to antimicrobial resistance and multidrug resistance which are frequently observed among many Salmonella serovars (Duong et al., 2006). Increasing evidence demonstrates that antimicrobial usage in animals promotes the emergence of a wide range of resistant zoonotic pathogens, such as Salmonella, which compromises the effectiveness of antibiotic treatments used in humans when an infection occurs (Gyles, 2008). The variety of antibiotics that are administered in veterinary practice

therapeutically has caused selective pressure resulting in an increase in genetic sequences that confer resistance to microorganisms. Antimicrobial-resistant Salmonella has been recognized as a public health concern for decades in developed and developing countries and the evolving resistance in this pathogen limits the therapeutic options available to physicians for the treatment of human Salmonellosis (Foley et al., 2011). Extensive usage of antibiotics in chicken production systems, for incorrect purposes such as growth promotion results in resistance of bacteria to these antimicrobial agents. Bacteria use both natural and acquired resistance mechanisms to protect themselves from agents which could harm them. Acquired resistance arises from mutations, gene transfer by conjugation or transformation, transposons, integrons, and bacteriophages (Cogliani et al., 2011).

It is therefore, necessary to determine bacterial resistance to antibiotics of all classes, the phenotypes they exhibit and the mutations which are responsible for resistance to these antibiotics using molecular genetic analysis methods. Impact of antimicrobial resistance on human health is of great concern for the treatment of various infections that arise from food animal origin (Glenn et al., 2011); where combinations of broad spectrum antibiotics need to be administered in order to control infection. Therefore, understanding the mechanisms of antibiotic resistance, location of genes on a chromosome or plasmid and their expression will assist in developing, screening and control strategies that are desperately needed in order to reduce the spread of resistant bacteria and their evolution. Against this background, the current study aimed to investigate the prevalence of virulence and antimicrobial resistance genes in chicken samples from South Africa as well as imports from Brazil and human clinical isolates. Genetic characterization of the antimicrobial resistance and virulence genes present within Salmonella spp. is essential in understanding the pathogenicity and prevalence of resistance which exists in this zoonotic foodborne pathogen.

3.2. Materials and methods

3.2.1. Sample Collection

Broiler chicken caecum samples were collected on the day of slaughtering from poultry slaughterhouses within the Durban metropolitan area in KwaZulu-Natal province of South Africa between March and October 2014. Samples were collected in batches of 25 per month. In total 200 samples were randomly collected over the eight months period. All samples were aseptically collected in plastic screw top tubes containing 45 ml of 0.1% w/v peptone-water

and stored on ice until transported back to the University of Kwa-Zulu Natal (Westville Campus) where enrichment of the samples were done on arrival. South Africa imports more than 50% of chicken products from Brazil because domestic production cannot meet the current demand. Whenever chicken products are imported in batches from other countries such as Brazil quality assurance, routine disease surveillance, screening and testing are conducted before the products are conveyed to the food chain. It was therefore, crucial to include some samples which originated from Brazil in our study. Therefore, Salmonella isolates from Brazilian broiler chickens (24) and human clinical samples (20) from patients emanating from the coastal region of KwaZulu-Natal province in South Africa were provided by the National Health Laboratory Service of South Africa in order to embark on this study.

3.2.2. Enrichment

Enrichment was carried on South African (SABC) chicken samples only. Ten ml of rinse peptone-water from the samples were incubated at 37°C for 24hrs. After incubation 0.1ml aliquots from the peptone-water samples were inoculated into tubes containing 10ml of Rappaport Vassiliadis (RV) broth medium and incubated at 42°C for 48hrs (Ahmed & Shimamoto, 2012).

3.2.3 Microbiological analysis

After enrichment, a loopful of the broth culture was streaked on plates of xylose-lysine-deoxycholate (XLD) agar and incubated at 37 °C for 24 hours a. Typical phenotypic characteristics of black colonies were regarded as positive Salmonella spp. Suspected Salmonella colonies were picked and inoculated on TSB broth and incubated while shaking at 37 °C for 24 hours. The resulting culture was used for DNA extraction and some was used for susceptibility tests. The remaining culture was used for 60 % glycerol stocks which was then stored at -80 °C for future purposes.

3.2.4 DNA Extraction

Genomic DNA of all Salmonella isolates was extracted from the culture using ZymoResearch Fungal and Bacterial Genomic DNA MiniPrep^{TM} kit following manufacturer's instructions. A positive Salmonella spp. control was prepared by isolating genomic DNA from a reference strain of known Salmonella broth culture. After DNA

extraction NanoDrop Spectrophotometer was used to check the concentration and quality of the isolated DNA and extracted DNA was then stored at -20°C until use in PCR.

3.2.5. Confirmation Salmonella spp. using Polymerase Chain Reaction (PCR)

PCR was performed on the DNA extracted from all detected and obtained samples. The *invA* gene was used to confirm the presence of Salmonella spp. A 25 µl PCR reaction was used for amplification of the *invA* gene. The primers set used for detection of *invA* gene are presented in Table 1.The PCR reaction was carried out in a total volume of 25 µl containing12.5 µl Green Taq PCR Master, 1 µl invA primer (forward), 1 µl *invA* primer (reverse), 4 µl of template DNA and 6.5 µl dH_2O. Amplification was carried out in thermo-cycler using 34 cycles consisting of denaturation for 30 seconds at 95 ^oC, annealing for 30 seconds at 58 ^oC, extension for 1 minute at 72 ^oC and final extension for 5 minutes at 72 ^oC. PCR products were run on a 1.5 % agarose gel using electrophoresis at 70 Volts for 60 minutes to detect a 284 base pair product size of the *invA* target gene. Furthermore, the iroB gene that is unique for Salmonella enterica species was used to confirm the identity of the species. The primers used are presented in Table 2.1 and everything was done following the same procedure used for *invA* gene amplification except for annealing temperature in this case it was 55 ^oC for 40 seconds.

Table 3.1: Primers used to confirm *Salmonella* spp.

Target gene	Primer sequence (5'→ 3')	Product size (bp)	Reference
invA [a]	F: TCATCGCACCGTCAAAGGAACC R:GTGAAATTATCGCCACGTTCGGGCAA	284	Li *et al*. 2012
iroB [b]	F:TGC GTA TTC TGT TTG TCG GTCC R:TAC GTT CCC ACC ATT CTT CCC	606	Baumler *et al*. 1997

3.2.6. Detection of virulence genes

The primer sets utilized for detection of virulence genes are depicted in Table 2. The PCR reaction was carried out in a total volume of 25 µl and under the following conditions: *sipC* gene (initial denaturation at 94 °C for 12 minutes, 1 minute of denaturation at 94 °C, 30 seconds of annealing at 54 °C and 5 minute of extension at 72 °C for a total of 34 cycles; 5 seconds were added to the extension time each cycle); *misL* and *orfL* genes (3 minutes at 94 °C, 35 cycles of 1 minute at 94 °C, 1 minute at 58 °C and 1 minute at 72 °C and finally 5 min

at 72 °C) and for *pipD* gene (94 °C for 5 minutes, 34 cycles of 25 seconds of denaturation at 94 °C, 30 seconds of annealing at 56 °C and 50 seconds of extension at 72 °C and a final cycle at 5 minutes at 72 °C. Gel electrophoresis of amplified products was then carried out in 1.5% agarose in a 1X TBE buffer containing GelRed. After the gels were ran, PCR products were visualized using ChemiDoc^{TM} imaging system.

Table 3.2: Primers used to detect virulence genes in Salmonella spp. (Hughes et al., 2008)

Target gene	**Primer sequence (5'→ 3')**	**Size (bp)**	**Annealing temperature**	**Mechanism of resistance**	**Broad action**
spiC	F:CCTGGATAATGACTATTGAT R:AGTTTATGGTGATTGCGTAT	309	54	Type III secretion system	Survival in macrophages
misL	F:GTCGGCGAATGCCGCGAATA R: GCGCTGTTAACGCTAATAGT	400	60	Involved in intramacrophage survival	Survival in macrophages
orfL	F:GGAGTATCGATAAAGATGTT R:GCGCGTAACGTCAGAATCAA	550	60	Adhesin/ autotransporter	Colonization
pipD	F: CGGCGATTCATGACTTTGAT R: CGTTATCATTCGGATCGTAA	350	56	Type III secretion effector associated with SPI-1 system	enteritis

3.2.7. Antimicrobial susceptibility testing

Antimicrobial resistance of 146 Salmonella spp. isolates were tested against seven antimicrobial agents using the Kirby-Bauer disc diffusion method on Mueller Hinton Agar following the guidelines of the Clinical and Laboratory standards Institute (CLSI)(CLSI, 2008). The antimicrobials selected were those commonly used in poultry industry and these being Gentamicin (10µg), Amoxicillin (10µg), erythromycin (10µg), chloramphenicol (30µg), tetracycline (10µg), trimethoprim (1.25µg) and ampicillin (10µg) bacitracin (10µg), streptomycin (25µg), Trimethoprim-Sulfamthoxazole (25µg) and Kanamycin (30µg). The

Oxoid antibiotic discs were impregnated with the concentrations of each antibiotic as mentioned above. Firstly, Mueller Hinton Agar was inoculated with 0.1ml of nutrient broth samples, which had been inoculated with a loopful of glycerol stocks of positive samples then incubated at 37°C for 24 hours. With use of a glass hockey stick the culture was spread on the agar for even distribution of the organism which demonstrated presence of Salmonella spp., after PCR, thereafter, discs impregnated with antibiotics were evenly placed on plates and the plates incubated at 37°C for 24hrs. The inhibition zones were measured and scored as sensitive, intermediate susceptibility or resistant according to the CLSI recommendations. Escherichia coli ATCC 25922 was used as a reference strain for antibiotic disc control (Bacci et al. 2012).

3.2.8. Detection of antimicrobial resistance genes

Genomic DNA of Salmonella spp. extracted was used for detection of antimicrobial resistance genes. The primer sets utilized for detection of antimicrobial resistance genes are recorded on Table 3.3. The PCR reaction was carried out in a total volume of 25 µl and using the following conditions*: pse-1 gene* (initial denaturation at 94 °C for 12 minutes, 1 minute of denaturation at 94 °C, 30 seconds of annealing at 57 °C and 5 minutes of extension at 72 °C for a total of 34 cycles; 5 seconds were added to the extension time each cycle); *ant (3")-la* gene (3 minutes at 94 °C, 35 cycles of 1 minutes at 94 °C, 1 minute at 58 °C and 1 minute at 72 °C and finally 5 minutes at 72 °C); *tet A* and *tet B* gene (94 °C for 5 minutes, 34cycles of 25 seconds of denaturation at 94 °C, 30 seconds of annealing at 55 °C and 50 seconds of extension at 72 °C and a final cycle at 5 minutes at 72 °C. *Sul1* and *sul2* detection were carried the same way as tetracycline genes, but with annealing temperatures of 65 °C and 52 °C respectively. Gel electrophoresis of amplified products was then carried out in 1.5% agarose in a 1X TBE buffer containing GelRed. After the gels were ran, PCR products were then visualized using ChemiDocTM imaging system

Table 3.3: Primers used to screen antimicrobial resistance genes in *Salmonella* spp.

Antimicrobial agent	Target gene	Primer sequence (5'-3')	Size (bp)	References	Mechanism of resistance
Ampicillin	*pse-1*	F:CGCTTCCCGTTAACAAGTAC R:CTGGTTCATTTCAGATAGCG	419	Baca *et al*. 2012	
Gentamicin	*ant (3")-la*	F:GTGGATGGCGGCCTGAAGCC R:ATTGCCCAGTCGGCAGCG	526	Bacci *et al*. 2012	Aminoglycoside adenyltransferase
Tetracycline	*tetA*	F:GCTACATCCTGCTTGCCTTC R:CATAGATCGCCGTGAAGAGG	210	Bacci *et al*. 2012	Efflux
	tetB	F:TTGGTTAGGGGCAAGTTTTG R:GTAATGGGCCAATAACACCG	659	Bacci *et al*. 2012	Efflux
Sulfamethoxazole	*Sul1*	F:GCG CGG CGT GGG CTA CCT R:GAT TTC CGC GAC ACC GAG ACC AA	350	Poppe *et al.* 2006	Dihydropteroate synthase inhibitor
	Sul2	F:CGG CAT CGT CAA CAT AACC R:GTG TGC GGA TGA AGT CAG	720	Poppe *et al.* 2006	Dihydropteroate synthase inhibitor

3.3. Results

3.3.1 Culture identification

Out of 200 samples collected from South African broiler chicken (SABC) slaughterhouses, only 102 (51%) were confirmed positive for Salmonella spp. The 102 Salmonella isolates together with the 24 Salmonella isolates from Brazilian chickens (BBC) and 20 human clinical isolates (SAHC) obtained from National Health Laboratory Services (NHLS) made the total of samples used in this study to be 146. Out of 146 samples screened for the iroB gene most of them were confirmed to be Salmonella enterica spp. giving the following prevalence rates 17 (85%) of human clinical samples, 70 (68.6%) of South African chicken isolates and 17 (70.8%) of Brazilian chicken samples. Figure 3.1 and Figure 3.2 illustrate the amplification PCR products for *invA* and *iroB* gene respectively.

3.3.2 Detection of virulence genes in Salmonella. spp.

PCR was used to screen for all 4 virulence genes and all genes screened were depicted in Figure 3, 4, 5, and 6. All the amplicon sizes were consistent with the sizes that were expected. Table 2.4 depicts that *sipC* (47%), *pipD* (35%), misL (2%) and *orfL* (20.6%) genes were harboured in South African broiler chicken isolates. It was also demonstrated that Brazilian broiler Salmonella isolates were found to harbour all genes with following prevalence rates; *sipC* (83%), *pipD* (87.5%), *misL* (29%) and *orfL* (25%). The same table presents results for South African human clinical isolates that were harbouring 85% of *sipC* gene followed by *pipD* (80%), then *misL* (75%) and lastly 20% of *orfL*.

Table 3.4: Prevalence of detected virulence in Salmonella isolates of three different origins

Origin	Number of Isolates (n)	Virulence genes				
		invA	***sipC***	***pipD***	***misL***	***orfL***
SABC	102	102 (100%)	48 (47%)	36 (35%)	2 (2%)	21 (20.6%)
BBC	24	24 (100%)	20 (83%)	21 (87.5%)	7 (29%)	6 (25%)
SAHC	20	20 (100%)	17 (85%)	16 (80%)	15 (75%)	10 (20%)
Total	146	146 (100%)	85 (58%)	73 (50%)	24 (16%)	37 (25%)

SABC- South African Broiler Chicken Isolates

BBC- Brazilian Broiler Chicken Isolates

SAHC – South African Human Clinical Isolates

3.3.3 Antimicrobial susceptibility testing

A total of 146 Salmonella isolates from different origins were tested for resistance towards eleven antimicrobial agents using the disc diffusion method. The incidences of resistance for all isolates tested are presented in Table 2.5. Salmonella spp. isolated from South African Broiler Chicken (SABC) showed resistance toward all eleven antimicrobials and the highest rates of resistance observed were, bacitracin (97%), tetracycline (93%), trimethoprim-sulfamthoxazole (84%), trimethoprim (78.4%), kanamycin (74%), gentamicin (48%), ampicillin (47%), amoxicillin and chloramphenicol (31%), erythromycin (18%) and streptomycin (12%). Isolates from Brazilian broiler chickens also showed resistance towards all antimicrobials tested and 100% isolates showed a complete resistance to ampicillin and bacitracin, amoxicillin and tetracycline (83%), trimethoprim (66.7%), erythromycin (62.5%), trimethoprim-Sulfamthoxazole (50%), kanamycin (16.7%), gentamycin and Streptomycin (12.5%) and chloramphenicol (4.2%). Salmonella isolates from South African human clinical isolates showed resistance to nine antimicrobial agents. Highest resistance rates were observed from the following antimicrobial; bacitracin (100%), erythromycin and amoxicillin (30%). Multi drug resistance was also observed across all isolates tested and Table 3.6 summarizes the resistance patterns of Salmonella isolates in the current study. The resistance

patterns serves evidence for multi-drug resistance and from Table 3.6 it can be reported that Salmonella isolates used have a potential to confer resistance to more than 2 antimicrobial agents.

Table 3.5: Antimicrobial susceptibility tests on Salmonella isolates of different origins

	SABC (n= 102)			BBC (n= 24)			SAHC (n=20)		
	No of isolates (%)								
Antibiotics	R	I	S	R	I	S	R	I	S
AMP	48 (47)	41 (40)	13 (13)	24 (100)	0	0	3 (15)	8 (40)	9 (45)
AML	32 (31)	45 (44)	25 (25)	20 (83)	3 (12.5)	1 (4.2)	6 (30)	5 (25)	9 (45)
B	99 (97)	1(1)	2 (2)	24 (100)	0	0	20 (100)	0	0
C	32 (31)	30 (29)	40 (39)	1 (4.2)	2 (8.3)	21 (88)	0	1 (5)	19 (95)
CN	49 (48)	26 (26)	27 (26.5)	3 (12.5)	3 (12.5)	18 (75)	0	0	20 (100)
E	18 (18)	13 (13)	71 (69.6)	15 (62.5)	6 (25)	3 (12.5)	6 (30)	10 (50)	4 (20)
K	75 (74)	9 (9)	18 (18)	4 (16.7)	0	20 (83)	1 (5)	0	19 (95)
S	12 (12)	8 (8)	82 (80)	3 (12.5)	15 (62.5)	6 (25)	4 (20)	3 (15)	13 (65)
SXT	86 (84)	0	16 (15.7)	12 (50)	4 (16.7)	8 (33)	3 (15)	0	17 (85)
TE	95 (93)	7 (7)	0	20 (83)	0	4 (16.7)	2 (10)	1 (50	17 (85)
W	80 (78.4)	4 (3.9)	19 (18.6)	16 (66.7)	3 (12.5)	5 (20. 8)	4 (20)	2 (10)	14 (70)

AMP- Ampicillin, AML- amoxicillin, B- Bacitracin, C- Chloramphenicol, CN- Gentamycin, E- Erythromycin, K- Kanamycin, S- Streptomycin, STX- Trimethoprim-Sulfamthoxazole, TE-Tetracycline, W- Trimethoprim

SABC- South African Broiler Chicken Isolates

BBC- Brazilian Broiler Chicken IsolatesSAHC – South African Human Clinical Isolates

Table 3.6: Antibiotic resistance patterns of Salmonella Isolates illustrating multiple-drug resistance

Antibiotic resistance patterns	**No. of isolates (%)**		
	SABC	**BBC**	**SAHC**
AMP, TE	32 (31.4)	20 (83)	1 (5)
AML, TE	31 (30.4)	16 (66.7)	2 (10)
B,TE	95 (93)	20 (83)	2 (10)
TE, W	74 (72.5)	14 (58)	1 (5)
S, TE	12 (11.8)	3 (12.5)	0
K, TE	71 (69.6)	3 (12.5)	0
SXT,TE	69 (67.6)	10 (41.7)	0
E,TE	14 (13.7)	12 (50)	2 (10)
C, TE	19 (18.6)	1 (4.2)	0
AMP, AML, TE	30 (25)	16 (80)	1 (5)
AMP, C, TE	10 (9.8)	1 (4.2)	0
AMP, B, TE	32 (31.4)	20 (83)	1 (5)
B, E, W	18 (17.6)	12 (50)	4 (20)
E, SXT, W	15 (14.7)	9 (37.5)	1 (5)
S, SXT W	11 (10.8)	3 (12.5)	0
B ,TE, W	74 (72.4)	14 (58)	1 (5)
AML, AMP, TE, W	23 (22.5)	12 (50)	0
AML, AMP, TE, B	28 (27.5)	16 (80)	1 (5)
AML, AMP, E, TE	12 (11.8)	10 (41.7)	0
B, E, SXT, W	15 (14.7)	8 (33)	0

AMP- Ampicillin, AML- amoxicillin, B- Bacitracin, C- Chloramphenicol, CN- Gentamycin, E- Erythromycin, K- Kanamycin, S- Streptomycin, STX- Trimethoprim-Sulfamthoxazole, TE-Tetracycline, W- Trimethoprim

SABC- South African Broiler Chicken Isolates

BBC- Brazilian Broiler Chicken Isolates

SAHC – South African Human Clinical Isolates

3.3.4 Molecular detection of antimicrobial resistance genes in Salmonella spp.

Antimicrobial resistance genes were detected on all 146 Salmonella isolates, regardless of antimicrobial susceptibility. Observed PCR results indicated that detected Salmonella spp. contained antimicrobial resistance genes, which are known to confer resistance. There were six antimicrobial resistance genes screened in total. The screened genes were pse-1, *ant (3'')-la, tet A, tet B, sul 1* and *sul 2*; the phenotypes of the genes are illustrated in figures 2.7, 2.8, 2.9, 2.10, 2.11 and 2.12 respectively. The prevalence rates of the genes detected are presented in Table 2.7. In the South African broiler chicken isolates the most prevalent antimicrobial resistance gene that was detected was pse-1 gene (56%), known to confer resistance to ampicillin. This gene was followed by *tet A* (44%), *ant (3'')-la* (32%) and *tet B* (28%) known to confer resistance to tetracycline, gentamicin and tetracycline respectively as depicted in Table 2.7. In the Brazilian broiler chicken isolates *tet A* and *sul 1* genes (83%) were most the prevalent genes, followed by sul 2 (79%), ant (3")-la (75%), pse-1 (63%) and tet B (33%). Finally, in the South African human isolates the gene that showed the highest prevalence was *ant (3'')-la* (80%), then followed by *tet A* (70%). The *tet B, sul 1* and *sul 2* genes all exhibited 60% prevalence and lastly pse-1 gene reported (50%) prevalence. These results are partly consistent with the antimicrobial susceptibility testing because most of genes were detected in isolates that showed resistance

Table Table 3.7: Prevalence of antimicrobial resistant genes screened from 146 *Salmonella* isolates

Antibiotics	**Resistances genes**	**No. of isolates (%)**		
		SABC *n= 102*	**BBC *n= 24***	**SAHC *n=20***
Ampicillin	*pse-1*	57 (56)	15 (63)	10 (50)
Gentamicin	*ant (3")-la*	33 (32)	18 (75)	16 (80)
Sulfamethoxazole	*sul1*	44 (43)	20 (83)	12 (60)
	sul2	43 (42)	19 (79)	12 (60)
	Sul1, sul2	18 (17.6)	18 (75)	10 (50)
Tetracycline	*tetA*	45 (44)	20 (83)	14 (70)
	tetB	29 (28)	8 (33)	12 (60)
	tetA, tetB	15 (14.7)	6 (25)	7 (35)

List of figures for Salmonella manuscript

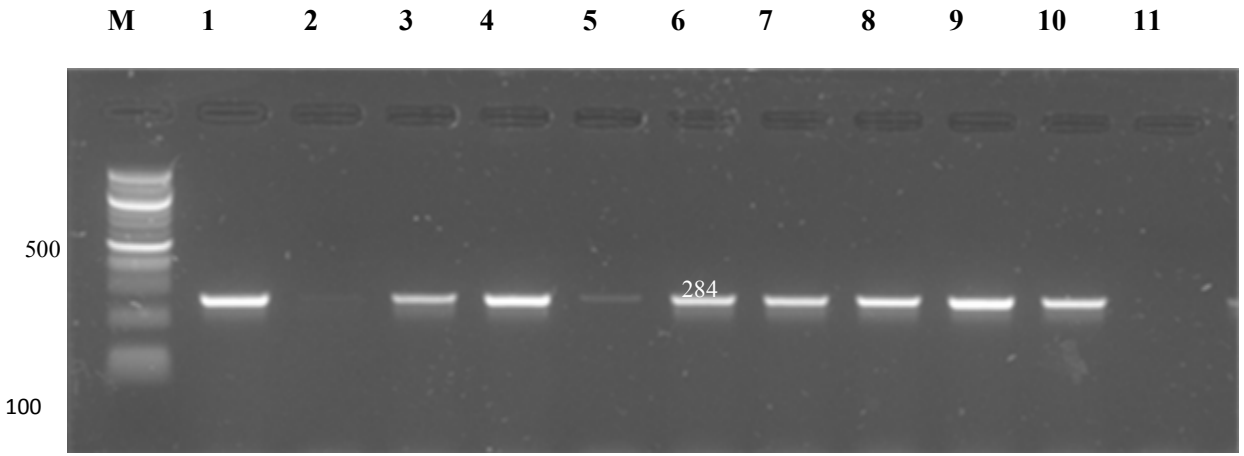

Figure 3.1: Showing representative invA gene (284 bp) from Salmonella isolates. Lane M: 100 bp marker, lane 1-9: test samples, lane 10: positive control, lane 11: negative control.

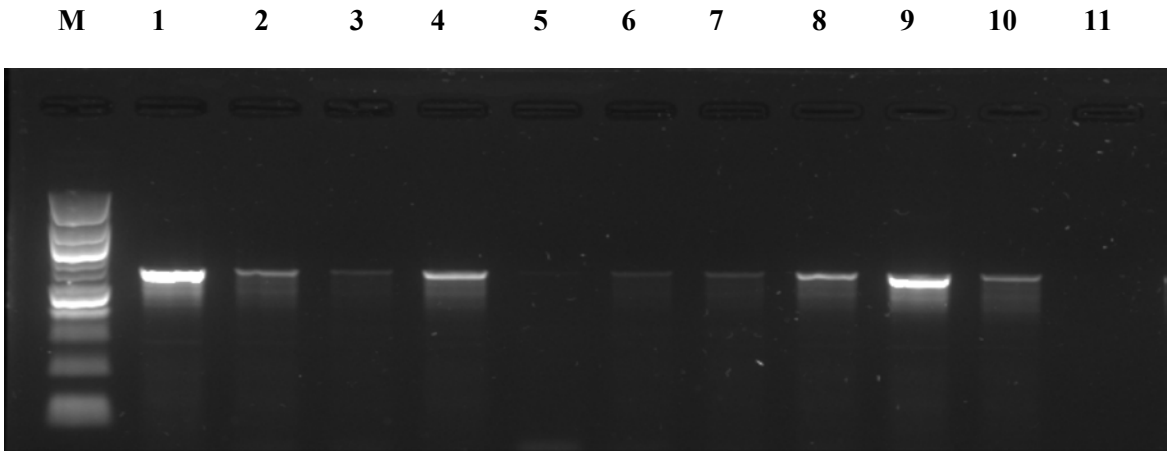

Figure 3.2: Showing representative IroB gene (606 bp) from Salmonella isolates. Lane M: 100 bp marker, lane 1-9: test samples, lane 10: positive control, lane 11: negative control.

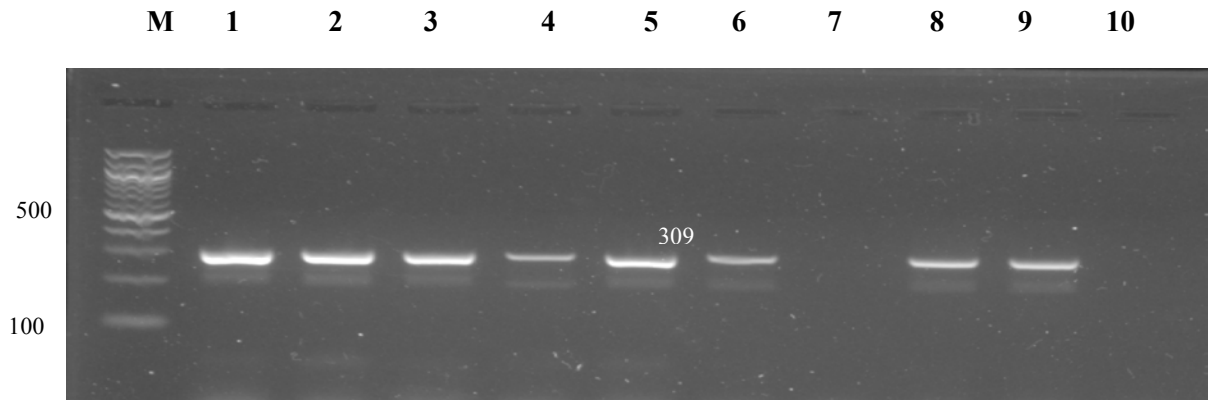

Figure 3.3: Showing representative *spiC* gene (309bp) from Salmonella isolates. Lane M: 100bp marker, lane 1-9: test samples, lane 10: negative control.

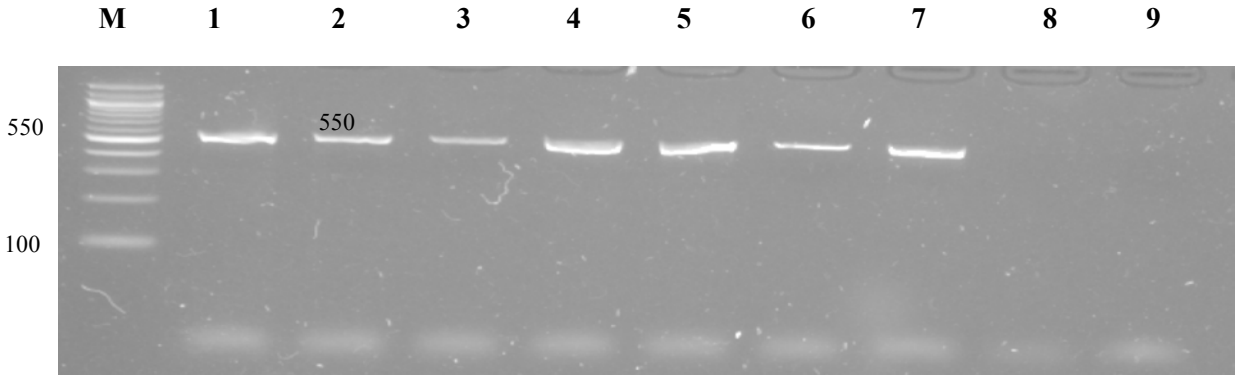

Figure 3.4: Showing representative misL gene (550bp) from Salmonella isolates. Lane M: 100bp marker, lane 1-8: test samples, lane 9: negative control.

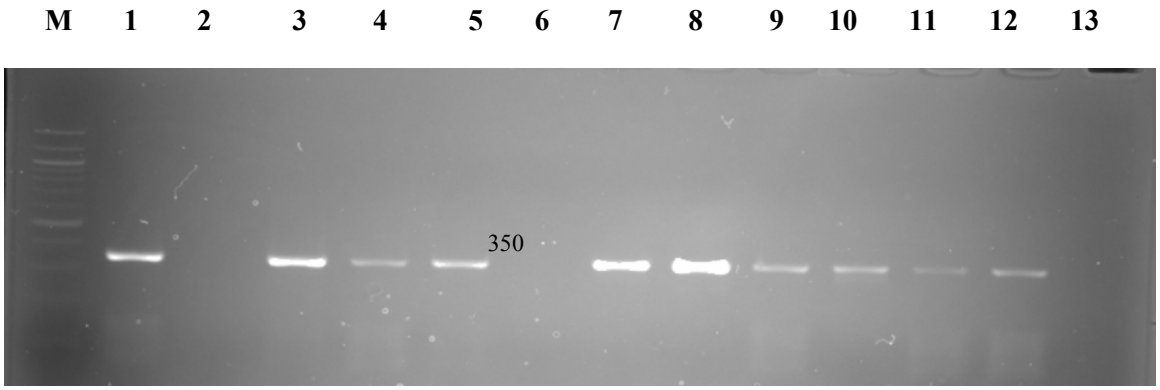

Figure 3.5: Showing representative orfL gene (350bp) from Salmonella isolates. Lane M: 100bp marker, lane 1-12: test samples, lane 13: negative control.

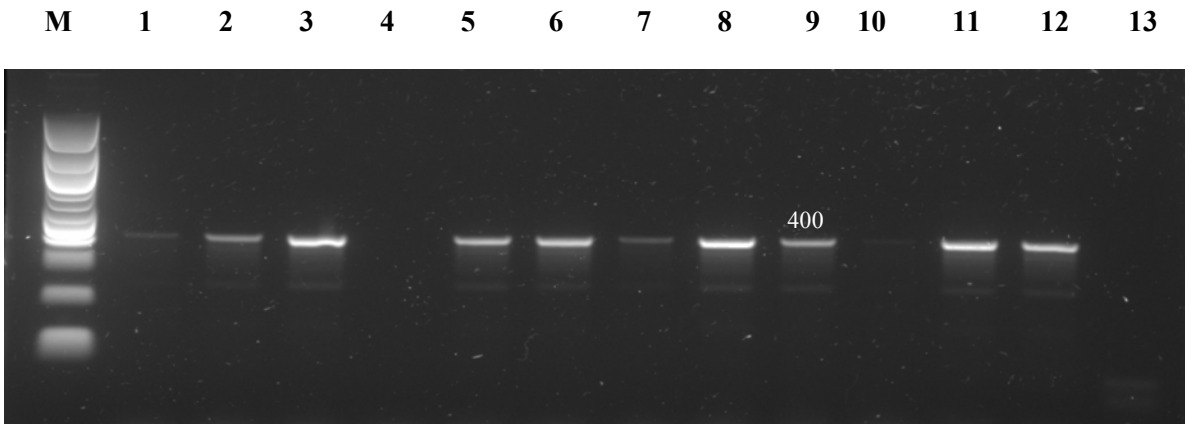

Figure 3.6: Showing representative pipD gene (400bp) from Salmonella isolates. Lane M: 100bp marker, lane 1-12: test samples, lane 13: negative control.

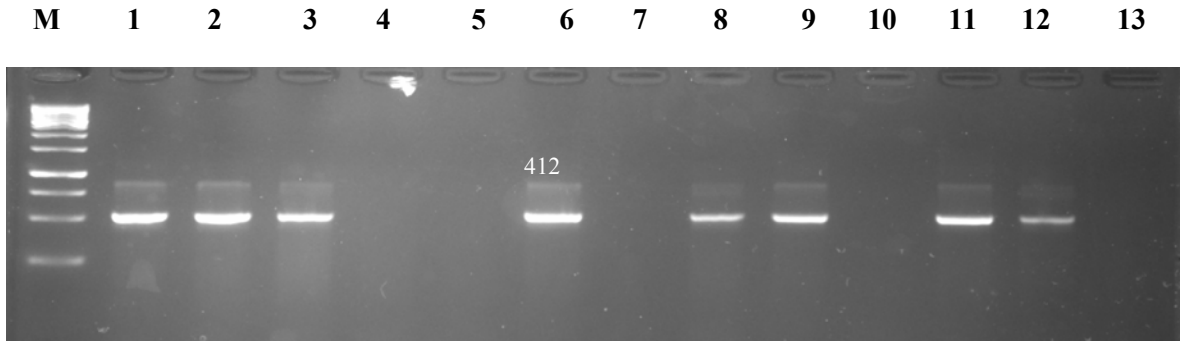

Figure 3.7: Showing representative pse-1 gene (412bp) from Salmonella isolates. Lane M: 250 bp marker, lane 1-12: test samples, lane 13: negative control.

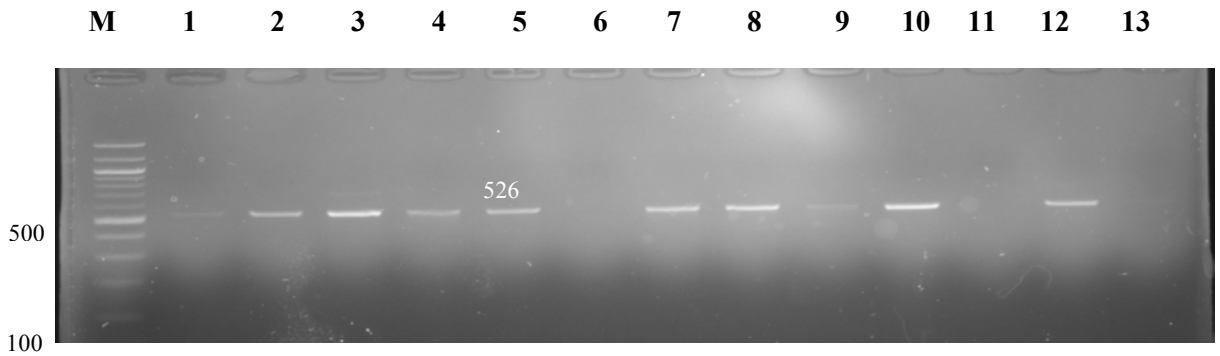

Figure 3 8: Showing representative anti (3") la gene (526 bp) from Salmonella isolates. Lane M: 100 bp marker, lane 1-12: test samples, lane 13: negative control.

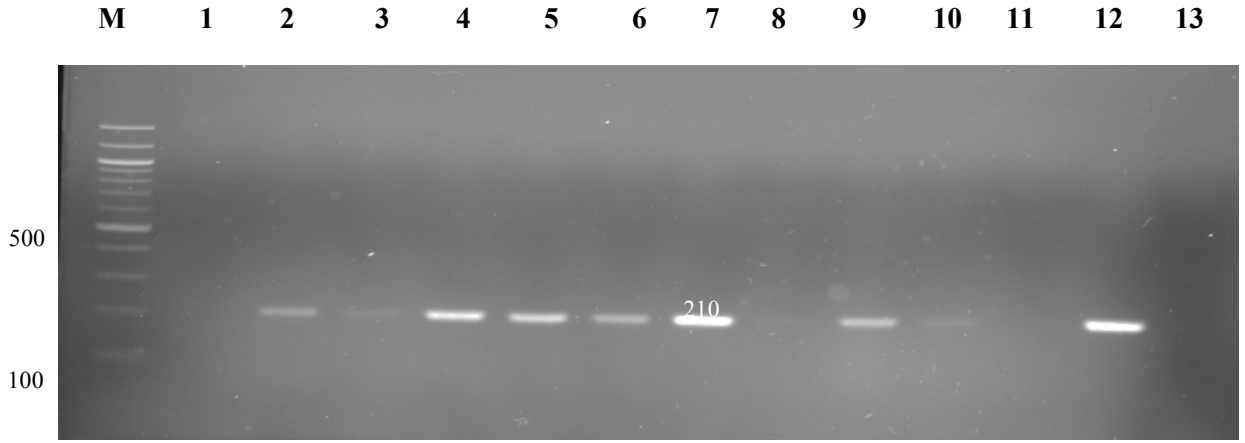

Figure 3.9: Showing representative tet A gene (210 bp) from Salmonella isolates. Lane M: 100 bp marker, lane 1-12: test samples, lane 13: negative control.

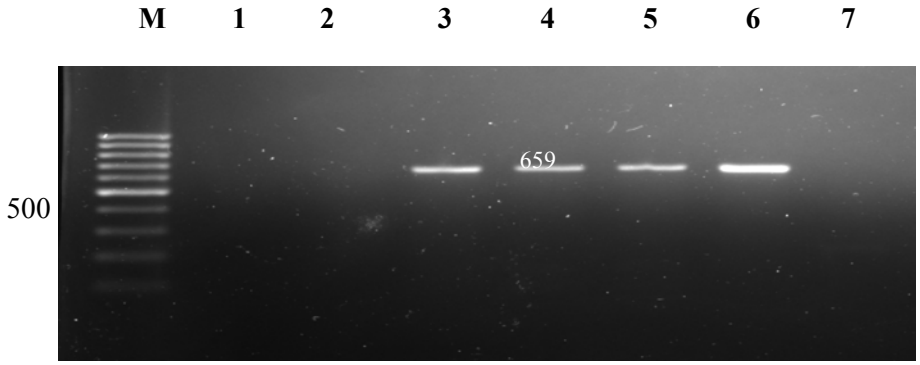

Figure 3.10: Showing representative tet B gene (659 bp) from Salmonella isolates. Lane M: 100 bp marker, lane 1-6: test samples, lane 7: negative control.

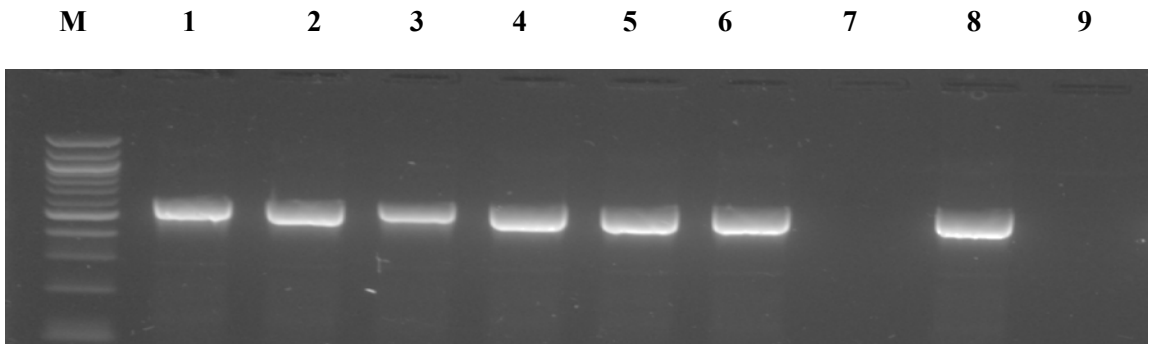

Figure 3. 11: Showing representative Sul 1 gene (350bp) from Salmonella isolates. Lane M:50 bp marker, lane 1-8: test samples, lane 9: negative control.

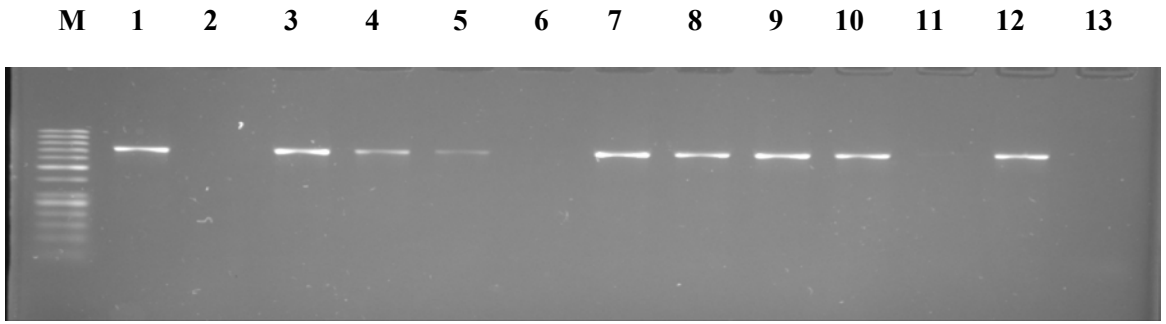

Figure 3.12: Showing representative *Sul 2* gene (720 bp) from *Salmonella* isolates. Lane M: 100 bp marker, lane 1-12: test samples, lane 13: negative

3.4. Discussion

The injudicious use of antimicrobials leads to resistance in various bacteria such as Salmonella, and thus antibiotic resistance in foodborne bacterial enteric pathogens is an almost inevitable consequence of the irrational use of antimicrobial drugs in animal production systems (Newell et al., 2012). Findings from the current study demonstrated the presence of Salmonella spp. in broiler chicken isolates at farm level. Out of 200 samples tested for Salmonella spp. only 102 (51%) samples were tested positive using PCR, amplifying the *invA* gene. These results were almost in agreement with results presented in previous studies by (Antunes et al., 2003; Capita et al., 2007; Chuanchuen & Padungtod 2009; Hao Van et al., 2007) which reported *Salmonella* spp. prevalence as 57%, 49%, 60% and 53.3% respectively. Cortez et al. (2006) conducted a study on identification of *Salmonella* spp. isolates from chicken abattoirs and reported that out of 288 samples collected 52 (18%) samples were tested positive. These results are relatively low and contrary to results obtained from the current study. Moreover, similar previous studies reported by Dogru et al. (2010); Kaushik et al. (2014); Van Nierop et al. (2005) and Zewdua nd Cornelius (2009) also reported very low *Salmonella* spp. prevalence 18%, 23.7%, 13.9% and 8% respectively compared to the current study. Information from literature provides proof that broiler chickens are potential carriers of *Salmonella* spp. However, there are various factors contributing to high prevalence of Salmonella isolates detected in the present study. Feed, housing and hygiene status of the farms where the chickens were reared could be some of the factors. Presence of *Salmonella* species at farm level is a serious issue because it shows that there is a potential for the pathogens to disseminate from the farms to communities. This is very worrisome because the majority of South Africans depend on food sold in informal retail outlets where hygienic conditions are of questionable standards. These conditions promote the accumulation and proliferation of pathogens posing a danger to consumers. The presence of Salmonella spp. in chicken meat is quite serious because South Africa imports a significant amount of poultry products from other countries such as Brazil, China and USA. Consumers are therefore, at risk of contracting salmonellosis from either local or imported poultry products.

It is worth noting that the greatest attribute *Salmonella* uses to survive in a host cell is pathogenicity. Various studies have tried to understand the significance of pathogenicity in a pathogen- host interaction and most conclusions drawn stated that pathogenicity is indeed significant for the pathogen to survive and proliferate. The significance of pathogenicity

helps Salmonella in invading and destroying epithelial cells in the host intestines (Uchiya and Nikai, 2008) and then propagating to other cell lines to colonize. All this happens as a result of the presence of genetic determinants responsible for virulence in Salmonella. spp. In the current study the prevalence of four virulence genes was established. The virulence genes were selected on the basis of their functions and danger toward chickens and humans. All genes detected belonged to different Salmonella pathogenicity islands (SP1). It was not part of the scope of the study to detect these islands; however, the information was sourced from literature. *Salmonella* pathogenicity islands SP1-2, SP1-5, SP1-3 and SP1-4 encodes for *spiC*, *pipD, misL* and *orfL* genes respectively.

The function of *spiC* gene is to interact with intercellular membrane trafficking in such a manner as altering it, hindering the correct cellular functioning (Uchiya and Nikai, 2008). Results obtained from South African broiler chickens present *spiC* gene as the most prevalent virulent gene detected compared to all the four genes that were screened. The prevalence rates reported were 47%, 35%, 2%, and 20.6% all corresponding to the following genes *spiC, pipD, misL and orfL* respectively. Since these genes were detected on isolates obtained at a farm level on carcass of health chickens, the emphasis made by Skyberg et al. (2006) which stipulated that there is a possibility for healthy chickens to be carriers of pathogenic Salmonella and still not show any signs of sickness. Moreover, this information demonstrates the risk of chicken meat toward consumers and implicating possible dissemination of virulence genes. The presence of the same virulence genes in human clinical samples demonstrates the dissemination and distribution of virulence genes although the origin of the Salmonella that infected patients was not known. Furthermore, there was no background information obtained from patients in terms of what they had ingested or uncounted that had resulted in them being infected by Salmonella. Salmonella isolates detected from Brazilian imported chicken meat also demonstrated the presence of all virulence genes screened during the study, illustrating *spiC* and *pipD* genes as the most prevalent genes with prevalence rate of 83% and 87% respectively. Furthermore, the genes with least prevalence rates were *orfL* (25%) and *misL* (29%), both these genes have been reported to be responsible for the survival of Salmonella in host cells namely macrophages. Virulence genes have also been detected in previous studies from Brazil (Castilla et al., 2006; Dias de Olivieira et al., 2003); Borges et al,. 2013), West Africa (Dione et al., 2011), Colombia (Sánchez-Jiménez et al., 2010) and England (Hughes et al.,. 2008). Therefore, detection of virulence genes from chicken meat imported from other countries and then sold in South Africa could be an indication that there

could be a transfer of Salmonella pathogenic strains from other countries to South Africa. Although virulence genes are common in local Salmonella strains, but receiving foreign strains via different mechanisms worsen the situation by increasing the prevalence of genes which encodes for pathogenicity. Consequently genetic diversity of Salmonella strains in South Africa is increased.

Antimicrobial resistance is a global public health problem. The increasing antimicrobial resistance in *Salmonella.* spp. is a forward irreversible reaction, but it can be reduced if certain precautions are followed worldwide. Salmonella spp. obtained were subjected to eleven commonly used antibiotics and our findings demonstrate that Brazilian chicken isolates and South African human isolates were 100% resistant to bacitracin. It was therefore, not surprising to note that 97% of South African chicken isolates were also resistant to bacitracin. This antibiotic is one of the antimicrobial agents used as a growth promoter and also to prevent necrotic enteritis in poultry (Si et al., 2007). Since it is extensively used, there is high likelihood for pathogens to develop high selection pressure against this drug. However, there could be many unforeseen factors behind the bacitracin resistance observed in the current study. Moreover, the use of this drug as an animal feed addictive was banned by European Union in 1999 (Cosewell et al., 2003). Regardless of the European prohibition of bacitracin use, there is no legislation prohibiting its use in South Africa. The worrying part is that any farmer could potentially purchase this antibiotic without a veterinary prescription.

Tetracycline is also a commonly used antimicrobial agent in human and animal medicine because it is cheap and easily accessible. Tetracycline resistance has been reported worldwide, and it comprises three types of resistance mechanisms namely tetracycline efflux, tetracycline modification and ribosomal protection (Roberts, 2005). During the current study Salmonella isolates from Brazilian chicken isolates, South African chicken isolates and South African human isolates exhibited resistance to tetracycline yielding prevalence rates of 83%, 93% and 10% respectively. All two antimicrobial resistance genes name *tetA and tetB* known to confer resistance toward tetracycline were detected in some isolates that exhibited resistance. The prevalence of *tetA* gene observed was high compared to *tet B*, in all groups of isolates screened. This was quite similar to previous studies which reported same pattern (Chuanchuen and Padungtod, 2009; Miko et al., 2005). Not all confirmed *Salmonella* isolates were harbouring *tetA and tetB* genes leading to prevalence of the genes being low compared to prevalence of *Salmonella* resistance on tetracycline. These results imply that there might be other antimicrobial genes conferring resistance that were not detected in the study, as there

are other determinants that confer resistance on tetracycline namely *tetC, tetD, tetR, tet M* and several others. However, in the human isolates the prevalence for resistance was very low but the two genes were detected even in the isolates which were susceptible to tetracycline.

Ampicillins and amoxicillin are amongst the drugs of choice for treating salmonellosis (de Toro et al. 2011). In the current study, Salmonella isolates from Brazilian chickens demonstrated high resistance of these two drugs compared to other isolates. A gene conferring resistance to β lactamase namely pse-1 gene was detected in most of salmonella isolates that were found resistance to ampicillin and amoxicillin. Llanes et al. (1999) reported that resistance of β-lactam is due to production of pse-1 enzyme. According to Glenn et al. (2011) Salmonella spp. isolated from food producing animals has be reported to entail pse-1 gene and it is one of the most prevalent β lactamase.

The current study demonstrated a high prevalence rate of Salmonella isolates which entail pse-1 gene compared to similar studies by (Baca et al. 2012; Chuanchuen and Padungtod, 2009) who reported very low prevalence rates of 0% and 5% respectively. South African studies have detected the presence of pse-1 gene in aquatic systems and in livestock production (Igbinosa, 201;, Igbinosa and Okoh, 2012). Although studies related to the mapping pse-1 gene have been conducted, there still exists a paucity of information on the prevalence of this gene in South Africa. More information on such genes can contribute to the solution of developing new drugs. Since high prevalence rates of pse-1 gene were observed, this implies that presence of β lactamase in foodborne pathogens is increasing. Batchelor et al. (2005) speculated that the increasing presence of β lactamase in pathogenic bacteria limits therapeutic use of antimicrobial agents. *Ant (3'')-la* gene is one of aminoglycoside resistance determinants. It has been detected in a number of bacterial pathogens but information of gene prevalence in Salmonella spp. is limited especial in Africa where little has been done. In the current study it was detected in some of the gentamicin resistant Salmonella isolates. Moreover genes conferring resistance to sulfamethoxazole (*sul 1 and sul 2*) were also detected in most Salmonella isolates which exhibited resistance to Trimethoprim-sulfamethoxazole. Results showed that some isolates were even harbouring both genes.

Multi drug resistance is the increasing problem that has been reported in both animal and human medicine. Salmonella isolates used in the present study illustrated high rate of multidrug resistance. Results in table 6, present patterns illustrating multi drug resistance and it can be confirmed if an isolate was resistant to more than two antibiotics. There may be

several reasons to possibly explain such outcomes but the main one could be due to lack of compliance with legislation governing the amount and type of antimicrobial agents used in South African poultry industry and also in human medicine. Multidrug resistance has a bad impact on therapy in both animal and human medicine. Moreover, various studies has proven that infections caused by multidrug resistant Salmonella strains are more dangerous than the infections caused by susceptible strains, since they extensively delay therapy placing patients' lives at risk (Martin et al., 2004; Varma et al., 2005). The presence of multidrug *Salmonella* strains in chickens and also in humans has great implications to public health systems as well as the economy as a whole.

Overall, results obtained from the study demonstrate that the detected *Salmonella* strains harboured both virulence and antimicrobial resistance genes. The current findings indicate that these strains are not good for the health and welfare of chickens and humans because there is potential for random dissemination of these genes. This can also serve as a catalyst enhancing increases in antimicrobial resistance. Since some developed and developing countries have prohibited the utilization of some antimicrobial agents as feed additives in animal husbandry especially poultry, South Africa should take note of what has been happening in other countries with regards to regulation of antimicrobial use and endeavour to prevent the escalating antibiotic resistance problem.

3.5. Conclusion

In conclusion, all the twelve genes examined in this study were successfully amplified in the *Salmonella* spp. isolated from different origins. These findings indicate that the selective pressure caused by the variety of antibiotics administered therapeutically in veterinary practice and poultry production systems for growth promotion and prophylaxis has resulted in an increase genes conferring resistance to Salmonella spp. It is difficult to make comparisons between Salmonella surveillance surveys conducted in different countries as the prevalence of *Salmonella* spp. varies regionally and isolation rates depend upon the country, sample plan, and methodology used. The data from this study indicates the dissemination of antimicrobial resistance genes in Salmonella spp. isolated from broiler chickens at the abattoir level. The emergence and dissemination of antimicrobial resistant Salmonella spp. in food animals has major public health impact especially for large scale suppliers who export their products both regionally and internationally thus foodborne salmonellosis should

constantly be monitored considering the escalation in drug resistant Salmonella spp. bacteria. Future work in the area of this study should include organism specificity by serotyping positive Salmonella spp. samples in order to determine which serovars of Salmonella is most prevalent in broiler chickens and phylogenetic analyses should also provide interesting insight into determining how closely related the positive Salmonella spp. samples are to each other.

2.6. Acknowledgements

The authors would like to acknowledge the College of Agriculture, Engineering and Science as well as the School of Life Sciences at University of KwaZulu-Natal (Westville campus) for financial support. National Health Laboratories Services of South Africa for providing with isolates from Brazilian broiler chickens and from human clinical isolates. Lastly we would like to express our most sincere gratitude to the various poultry slaughterhouses within KwaZulu-Natal province in South Africa who for confidentiality reasons remain anonymous for providing with chicken samples for this study.

3.7. Statement on animal rights

Animal studies have been approved by the appropriate ethics committee of the University of KwaZulu-Natal (Reference: 012/15/Animal) therefore, they have been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments.

3.8. Conflict of interests statements

There is no conflict of interest from all the parties involved in this publication and all parties gave their consent to publish this work.

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Chapter 4

Genetic characterization of antimicrobial resistance and virulence genes in *Staphylococcus aureus* isolated from commercial broiler chickens in Durban

Abstract

Antimicrobial resistant S*taphylococcus aureus* in human and veterinary medicine is serious problem worldwide. The aim of the study was to investigate the presence of *Staphylococcus aureus* in broiler chicken samples, and to further investigate antimicrobial susceptibility together with distribution of genetics determinants conferring resistance and virulence. A total of 194 samples were collected aseptically from broiler chicken slaughter houses and retail outlets around Durban. Microbiological (enrichment and plating on mannitol salt agar) and molecular methods were used to detect the *S. aureus* as well as its resistance and virulence associated genes. The Polymerase chain reaction was used to confirm the organism by amplifying the *nuc* gene. Out of 194 samples that were tested, 104 (54%) of them were confirmed positive for S*taphylococcus aureus.* The disk diffusion technique was used to investigate antimicrobial susceptibility profiles of isolates to 10 antimicrobial agents namely ampicillin, chloramphenicol, gentamycin, erythromycin, cefoxitin, kanamycin, streptomycin, tetracycline, vancomycin and trimethoprim. Findings showed that *Staphylococcus aureus* strains of abattoir origin had the highest level of resistance observed involving tetracycline, 50 % of the isolates were resistant to this drug. This was followed by ampicillin, vancomycin, cefoxitin, trimethoprim erythromycin and streptomycin with resistance rates of 41%, 39%, 38%, 37%, 36% and 29% respectively. *Staphylococcus aureus* strains of retail origin, high prevalence rates of antimicrobial resistance were observed on antimicrobial agents such as tetracycline (100%), cefoxitin (92%), erythromycin (83%), Streptomycin (83%), and kanamycin (67%). Overall results showed multi-drug resistance; isolates (100%) were resistant to two or more antimicrobial agents. Out of 4 virulence genes screened only two were detected (*coa and spa*) and their prevalence were very low. All antimicrobial resistance genes screened were detected (*mecA, BlaZ, tetK*), but their prevalence was not corresponding with the antimicrobial susceptibility results obtained

Keywords: *Staphylococcus aureus*; virulence; antimicrobial resistance; methicillin; broiler chicken

4.1. Introduction

Staphylococcus aureus (*S. aureus*) is an opportunistic bacterium which is part of the normal commensal flora in humans and mucous membranes of livestock, it is considered as the most pathogenic species compared to other organism of the same genus (Quinn and Markey, 2003). Moreover, this microorganism is considered as a significant cause of a wide range of avian diseases, namely arthritis, staphylococcal septicaemia, synovitis, omphalitis, infection of the yolk sac and several others (Mead and Dodd, 1990; Smyth and McNamee, 2001). These staphylococcal infectious diseases of chickens are an economic threat and they are regarded as global burden (Lowdera et al., 2009). The presence of *S. aureus* in the poultry chain has the potential to contaminate chicken carcasses during slaughter at the abattoirs since in most cases it is asymptomatic meaning that the flock can be affected without the farmer and the veterinarian knowing (Olivier et al., 1996; Schaumburg et al., 2013). This implies that there are high chances for contaminated chicken meat and it products to be transported to retail outlets and subsequently to the consumer table. The distribution of *Staphylococcus aureus* in contaminated chicken meat products is a serious matter for consumers, since this bacterium is known to produce thermostable enterotoxins which cause staphylococcal food poison in humans (Mureg et al., 1994; Balaban and Resole, 2000). This results in a wide range of infections such as gastroenteritis, heat shock like syndrome, skin infections, respiratory infections, urinary tract infections, autoimmune diseases and several others (Balaban, and Rasooly, 2000; Larsen et al., 2000). These diseases range from minor discomfort to death.

Staphylococcu aureus (S.aureus) virulence is very complex and depends on an array of virulence genes. Virulence genes involved in this microorganism are clustered under two categories namely cell-surface-associated (adherence) and secreted (exotoxins) factors (Diep and Otto, 2008) S. *aureus* achieves colonization of the host through production of various exoproteins (Salasia et al., 2004). A typical example of a well-known exoprotein is protein A. This protein acts as an immunological disguise and is considered to be important virulence factor (Agius, et al 2007). Furthermore, protein A encodes for the *spa* gene which is mostly used for typing of the organism. Coagulase *(coa)* gene is another example of *Staphylococcus aureus* virulence gene which is regarded as important since it plays an essential role in the alliance with other genes in order to survive inside the host cells and to invade a variety of important immune system cells in the host. Most virulence genes in *Staphyloccocus aureus* are known to be associated with staphylococcal food poisoning (Balaban, and Rasooly,

2001). Livestock associated antimicrobial resistance of *Staphylococcus aureus* towards several antimicrobial agents has been reported (Aarestrup, 1999; Ateba et al., 2010; Hanson et al., 2011). There is a potential for such antimicrobial resistance to be passed on from food producing animals to humans through food and direct contact with contaminated carcasses.

In South Africa there is limited information available regarding the presence and genetic characterization of *Staphylococcus aureus* in chicken meat and it products. Therefore, this study is aimed to investigate the presence of *Staphylococcus aureus* in chicken samples and to further assess the detected isolates by screening for genetic determinants carried by this bacterium encoding for virulence and resistance.

4.2. Materials and methods

4.2.1. Samples collection

Broiler chicken samples (caecum, feces and retail meat) were collected from poultry slaughterhouses and retail outlets within the Durban metropolitan area in KwaZulu-Natal province of South Africa. Abattoir samples were collected on the days of slaughter March and October 2014, in batches of 25 per month. A total of 200 samples were randomly collected over the eight months period, however 114 samples were used for the current study. A total of 30 samples were purchased from 10 retail outlets (3 each). Moreover, fifty fecal samples were collected at the market where different informal entrepreneurs are commercially selling live broiler birds. All samples were aseptically collected in plastic screw top tubes containing 45 ml of 0.1% w/v peptone-water and stored on ice until transported back to the University of Kwa-Zulu Natal (Westville Campus).

4.2.2. Detection of Staphylococcus aureus

Firstly, enrichment was conducted by taking 10 ml of rinse peptone-water from the collected samples into clean sterile test tubes and incubated at 37°C for 24hrs. After incubation 0.1ml aliquots from the peptone-water samples were inoculated into tubes containing 10ml of Brain Heart Infusion broth (BHI) and incubated at 37°C for 24hrs.

After enrichment, a loopful of the broth culture was streaked on plates a *Staphylococcus aureus* selective medium namely Mannitol salt agar and incubated at 37 °C for 24 hours. Typical phenotypic characteristics of yellow colonies with yellow zones were regarded as positive *Staphylococcus aureus.* Suspected *Staphylococcus aureus* colonies were picked and inoculated on BHI broth and incubated while shaking at 37 °C for 24 hours. The

resulting culture was used for DNA extraction and some was used for antimicrobial susceptibility tests. The remaining culture was used for 60 % glycerol stocks which was then stored at -80 °C for future purposes.

4.2.3. DNA extraction

Genomic DNA of all *Staphylococcus aureus* isolates was extracted from the culture using ZymoResearch Fungal and Bacterial Genomic DNA MiniPrep™ kit following manufacturer's instructions. A positive *Staphylococcus aureus* control was prepared by isolating genomic DNA from a reference strain of known *Staphylococcus aureus* broth culture. After DNA extraction NanoDrop Spectrophotometer was used to check the concentration and quality of the isolated DNA and extracted DNA was then stored at -20°C until use for molecular confirmation of the species and screening of virulence and antimicrobial resistance genes.

4.2.4. Molecular confirmation of *Staphylococcus aureus*

Polymerase chain reaction (PCR) was used to amplify the *nuc* gene for the confirmation detected *Staphylococcus aureus* is isolates. The *nuc* gene primers used were previously described in literature by Brakstad et al. (1992) (Table 4.1). The PCR reaction was carried out in a total volume of 25 µl containing12.5 µl Green Taq PCR Master, 1 µl *nuc* primer (forward), 1 µl *nuc* primer (reverse), 4 µl of template DNA and 6.5 µl dH_2O. Amplification was carried out in thermo-cycler using 34 cycles consisting of denaturation for 30 seconds at 95 ^{o}C, annealing for 30 seconds at 57 ^{o}C, extension for 1 minute at 72 ^{o}C and final extension for 5 minutes at 72 ^{o}C. PCR products were run on a 1.5 % agarose gel using electrophoresis, stained with gelred at 70 Volts for 60 minutes and visualized under UV light using a gel documentation system (Bio ChemiDoc™ MP imaging system).

Table 4.1: Sequences of oligonucleotides primers used to target genetic determinants responsible for species confirmation, virulence and resistance in *Staphylococcus aureus*

Target gene	Primer sequence (5' →3')	Product size (bp)	References
nuc	F: GCGATTGATGGTGATACGGTT R:AGCCAAGCCTTGACGAACTA AAGC	270	Brakstad et al., 1992
coa	F: CGA GAC CAA GAT TCA ACA AG R: AAA GAA AAC CAC TCA CAT CA	730	Aslantas et al., 2007
spa	F: CAA GCA CCA AAA GAG GAA R: CAC CAG GTT TAA CGA CAT	320	Fre´nay et al., 1996
sea	F: GCA GGG AAC AGC TTT AGGC R: GTT CTG TAG AAG TAT GAAACA CG	521	Monday et al., 1999
see	F: TAC CAA TTA ACT TGT GGA TAG AC R: CTC TTT GCA CCT TAC CGCA	171	Monday et al., 1999
mecA	F: AAAATCGATGGTAAAGGTTGGC R: AGTTCTGCAGTACCGGATTTGC	532	Strommenger et al., 2003
BlaZ	F: ACTTCAACACCTGCTGCTTTC R: TAGGTTCAGATTGGCCCTTAG	240	Martineau et al., 2000
tet K	F: TTAGGTGAAGGGTTAGGTCC R: GCAAACTCATTCCAGAAGCA	718	Aarestrup et al., 2000

4.2.5. Antimicrobial susceptibility testing

Antimicrobial resistance of 146 *Staphylococcus aureus* isolates were tested against 10 antimicrobial agents using the Kirby-Bauer disc diffusion method on Mueller Hinton Agar following the guidelines of the Clinical and Laboratory standards Institute (CLSI)(CLSI, 2013). The antimicrobials selected were those commonly used in poultry industry and these being ampicillin (25µg), chloramphenicol (30µg), erythromycin (30µg), cefoxitin (30µg), gentamicin (30µg), kanamycin (30µg), streptomycin (25µg), tetracycline (30µg), trimethoprim (5µg) and vancomycin (30µg). The Oxoid antibiotic discs were impregnated

with the concentrations of each antibiotic as mentioned above. Firstly, Mueller Hinton Agar was inoculated with 0.1ml of nutrient broth samples, which had been inoculated with a loopful of glycerol stocks of positive samples then incubated at 37°C for 24 hours. With the use of a swap the culture was spread on the agar for even distribution of *Staphylococcus aureus*, thereafter, discs impregnated with antibiotics were evenly placed on plates and the plates incubated at 37°C for 24 hours. The inhibition zones were measured and scored as sensitive (S), intermediate susceptibility (I) or resistant (R) according to the CLSI recommendations. *Staphylococcus aureus* was used as a reference strain for antibiotic disc control.

4.2.6. Screening of virulence and antimicrobial resistance genes

Screening of virulence and antimicrobial resistance genes (*coa, spa, sea, see, mecA, Blaz, tetK*) was carried out using PCR with the use of oligonucleotide primers which were previously described in literature by the authors declared in Table 4.1. The reactions were performed in final volume of 25µl each made by 12, 5µl Green Taq PCR Master, 1 µl primer (forward), 1 µl primer (reverse), 4 µl of template DNA and 6.5 µl dH_2O. PCR conditions described by the originally generators of primers were used without any amendments. After the reactions were finished, 7µl of the products were analysed by 1.5 % gel electrophoresis technique using 1X TBE as a medium buffer. The pictures were then taken using UV light gel documentation system called Bio ChemiDoc^{TM} MP imaging system.

4.3. Results

4.3.1. Species conformation

Out of 194 broiler chicken samples, only 104 (54%). of them were tested positive for *Staphylococcus aureus*. The fifty four percent consisted of samples from origins which are 33% caecum samples from the abattoirs, 6.2% different organs from retail outlets and 15% faecal samples from the EThekwini market. Figure 4.1 shows a gel pic with 270bp PCR amplicons representing the region of the *nuc* gene which was amplified on *Staphylococcus aureus* positive isolates.

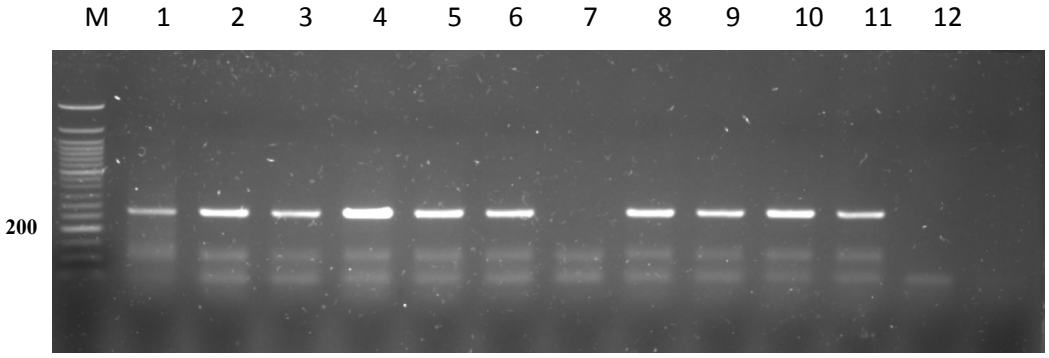

Figure 4.1: Agarose (1.5%) gel electrophoresis of nuc gene (270bp). Lane M is 50bp DNA ladder, lane 1 to 10 is test samples, lane 11 is a positive control and lane 12 is a negative control

4.3.2. Antimicrobial susceptibility profiles

Ten antimicrobial agents previously described were used to investigate resistance profiles of 104 *Staphylococcus aureus* positive isolates. Resistance to antimicrobial agents used was observed and the results are illustrated on Table 4.2. For *Staphylococcus aureus* strains of abattoir origin had the highest level of resistance observed involving tetracycline, 50 % of the isolates were resistant to this drug. This was followed by ampicillin, vancomycin, cefoxitin, trimethoprim erythromycin and streptomycin with resistance rates of 41%, 39%, 38%, 37%, 36% and 29% respectively. The low level of resistance observed involved gentamycin with only 10% of the isolates being resistant to this drug. In *Staphylococcus aureus* strains of retail origin, high prevalence rates of antimicrobial resistance were observed on antimicrobial agents such as tetracycline (100%), cefoxitin (92%), erythromycin (83%), Streptomycin (83%), and kanamycin (67%). Low levels of resistance were observed for gentamycin (25%) and no isolates exhibited resistance to either ampicillin or vancomycin. Lastly *Staphylococcus aureus* strains isolated from faecal samples were highly resistant to kanamycin (79.3%), cefoxitin (76%), tetracycline (69%), erythromycin (62.1%), streptomycin (62%), trimethoprim (58.6%), chloramphenicol (58.3%) and gentamycin (55%). And low rates of resistance were observed for ampicillin (27.6%) and vancomycin (14%).

Overall results observed showed that all 104 *Staphylococcus aureus* strains tested were resistance to 3 or more antimicrobial agents used. Table 4.3 shows the antimicrobial resistance patterns of *Staphylococcus aureus* isolates illustrating multiple-drug resistance.

Table 4.2: Prevalence rates for antimicrobial susceptibility tests on *Staphylococcus aureus* isolated from broiler chicken samples of different origins

	Abattoir samples(n=63)			**Retail samples (n=12)**			**Faecal samples(n=29)**		
	No of isolates (%)								
Antibiotics	R	I	S	R	I	S	R	I	S
AMP	41(65.1)	1(1.6)	21(33.3)	0	0	12(100)	8(27.6)	4(14)	17(58.6)
C	22(34.9)	4(6.4)	37(58.7)	5(41.7)	0	20(69)	7(58.3)	1(3.4)	8(27.6)
CN	10(15.9)	2(3.2)	51(81)	3(25)	1(8.1)	8(67)	16(55)	4(14)	9(31)
E	36(57,1)	15(23.8)	12(19)	10(83)	2(17)	0	18(62.1)	2(6.9)	9(31)
FOX	38(60.3)	2(3.3)	23(36.5)	11(92)	0	1(8)	22(76)	1(3.4)	6(20.7)
K	21(33.3)	0	42(66.7)	8(67)	0	4(33)	23(79.3)	2(6.9)	4(13.8)
S	29(46)	2(3.2)	32(50.8)	10(83)	0	2(17)	18(62)	4(14)	7(24)
TE	50(79.4)	4(6.4)	9(14.3)	12(100)	0	0	20(69)	1(3.4)	8(27.6)
VA	39(61.9)	4(6.3)	20(31.8)	0	0	12(100)	4(14)	3(10.1)	22(75.9)
W	37(58.7)	3(4.8)	23(36.5)	9(75)	0	3(25)	17(58.6)	0	12(41.4)

AMP- Ampicillin, C- Chloramphenicol, CN-Gentamycin, E-Erythromycin, FOX- Cefoxitin, K- Kanamycin, S-Streptomycin, TE-Tetracycline, VA- Vancomycin, W-Trimethoprim

Table 4.3: Resistance patterns of Staphylococcus aureus isolates

Antimicrobial resistance patterns	No. of isolates (%)		
	Abattoir samples	Retail samples	Faecal samples
FOX, AMP	34(54)	0	7(24.1)
FOX, S, TE	23(36.5)	9(75)	15(51.7)
FOX, AMP, K	12(19)	0	7(24.1)
FOX, C, CN	8(13)	3(25)	10(34.5)
FOX, K, TE	16(25.4)	7(58.3)	17(59)
FOX, W, S	20(32)	7(58.3)	12(41.4)
FOX, TE, VA	30(48)	0	4(14)
FOX, CN, TE, K	8(13)	3(25)	11(38)
FOX, S, W, E	16(25)	6(50)	11(38)
FOX, E, S, VA	14(22.2)	0	4(14)
FOX, K, CN, TE	8(13)	3(25)	11(38)
FOX, TE, E, C	16(25)	5(41.7)	12(41.4)
FOX, W, TE, K, S	11(17.5)	5(41.7)	11(38)
FOX, K, W, TE, E	12(19)	6(50)	11(38)
FOX, AMP, S, K, C	11(17.5)	0	7(24.1)
FOX, C, VA, CN, TE	6(9.5)	0	3(10.3)
FOX, TE, K, E, C	12(19)	4(33.3)	12(41.4)
FOX, W, CN,TE, VA, E	5(8)	0	4(14)
FOX, VA, E, C,TE, W	12(19)	0	4(14)
FOX, E, S, VA, TE, K	9(14.3)	0	4(14)

AMP-Ampicillin, C-Chloramphenicol, CN-Gentamycin, E-Erythromycin, FOX-Cefoxitin, K-Kanamycin, S-Streptomycin, TE-Tetracycline, VA- Vancomycin, W-Trimethoprim

4.3.3. Presence of virulence and antimicrobial resistance genes

Seven genetic determinants were screened from all the isolates. Among the seven genetic determinants, 4 of them were encoding for virulence (*spa, coa, sea* and *see)* and 3 of them were antimicrobial resistance gene (*mecA, BlaZ, and tetk*). Antimicrobial resistance genes were detected on all 104 *Staphylococcus aureus* isolates, regardless of antimicrobial susceptibility outcomes. The prevalence rates of the genes are illustrated on figure 4.2. Moreover, Figure 4.3-4.6 are images exhibiting the PCR amplicons of the genes that were detected during the study.

Out of 4 virulence genes screened from all the isolates only 2 (*spa, coa*) were detected. Isolates detected from the abattoir, retail and faecal samples the prevalence rates observed for *spa* gene were 11%, 8% and 52% respectively. The *coa* gene the prevalence observed were

All 3 antimicrobial resistance genes screened from 104 *Staphylococcus aureus* isolates were detected. With regards to the gene encoding for methicillin resistance (*mecA*) prevalence rates were 56%, 53% and 21% corresponding to isolates detected from abattoir, retail and faecal samples respectively. A beta lactamase gene (*BlaZ*) was detected from 4.8% of isolates of abattoir origin, 50% of isolates of retail origin and 10.3% of isolates from faecal samples. Lastly the prevalence rates for *tetK* gene encoding for tetracycline resistance observed were 37%, 17% and 24% corresponding to abattoir, retail and faecal samples respectively

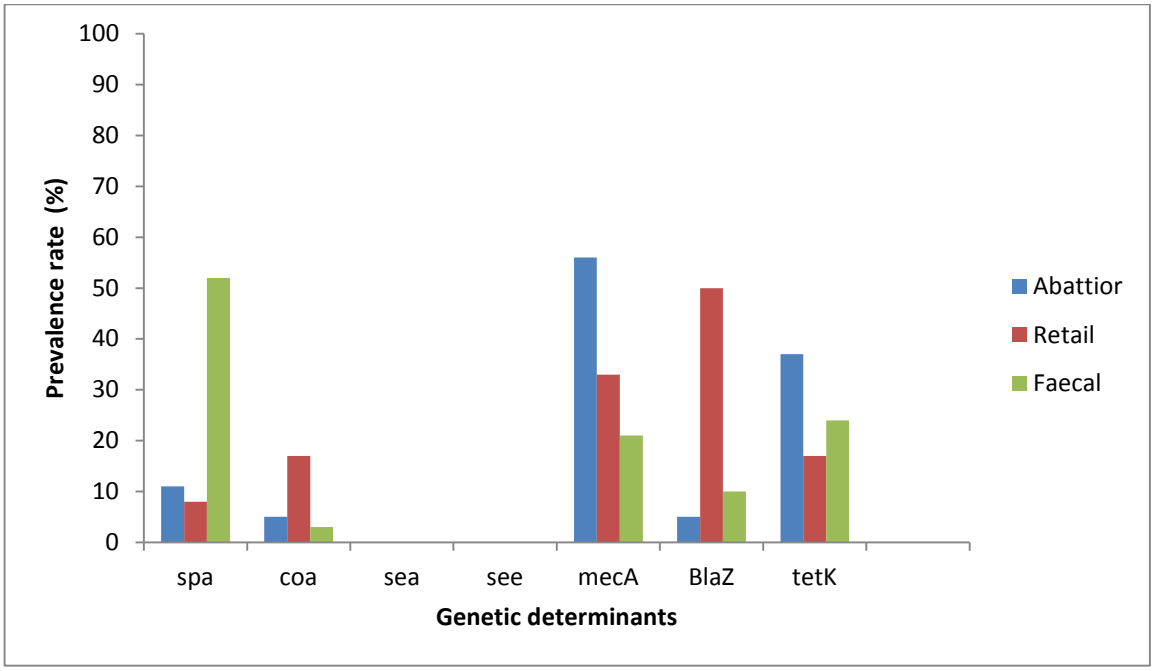

Figure 4.2: Prevalence rates of genetic determinants encoding for virulence and resistance *Staphylococcus aureus* isolates. The key words symbolise the sample origin where *Staphylococcus aureus* isolates were isolated on.

Prevalence rates of genetic determinants encoding for virulence and resistance *Staphylococcus aureus* isolates. The key words symbolise the sample origin where *Staphylococcus aureus* isolates were isolated on.

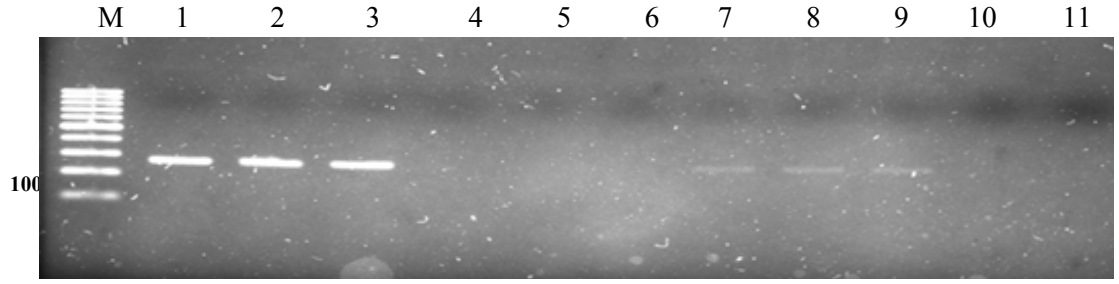

Figure 4.3: Agarose (1.5%) gel electrophoresis image showing 320 bp PCR amplicons of the spa gene isolated on *Staphylococcus aureus* detected from broiler chicken samples. Lane M is 100 bp DNA ladder, lane 1 to 10 are test samples, lane 11 is a negative control

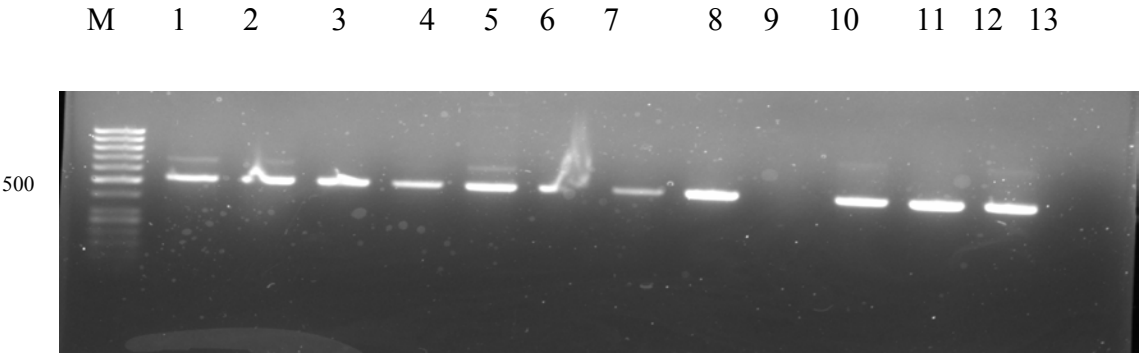

Figure 4.4: Agarose (1.5%) gel electrophoresis of mecA gene (532pb). Lane M is 50bp DNA ladder, lane 1 to 13 are test samples

.

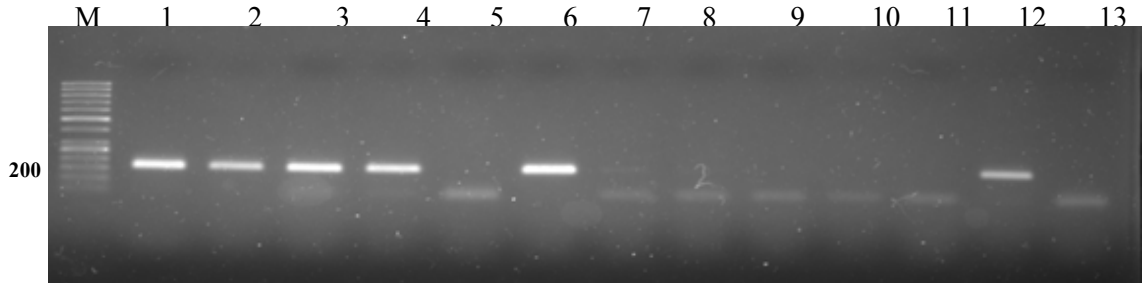

Figure 4.5: Agarose (1.5%) gel electrophoresis of BlaZ gene (240bp). Lane M is 50bp DNA ladder, lane 1 to 13 are test samples

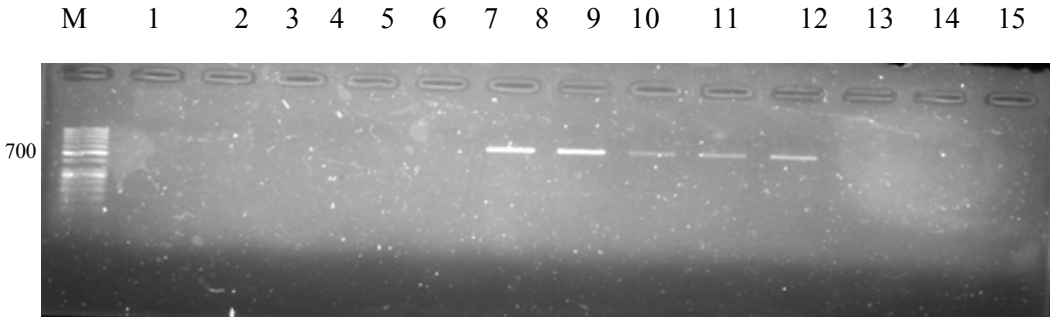

Figure 4.6: Agarose (1.5%) gel electrophoresis of tet K gene (718bp). Lane M is 50bp DNA ladder, lane 1 to 13 are test samples

4.4. Discussion

The increasing reports on antimicrobial resistance of *Staphylococcus aureus* associated with food producing animals such as poultry has been a driving force for surveillance studies focusing on detection and assessment of antimicrobial resistance profiles of this microorganism (Aarestrup et al., 1998; Aarestrup et al., 2000; Gundogan et al., 2005; Nemati et al., 2008). Most reports have been reporting that there is a continuous increase of incidence of antimicrobial resistance in *Staphylococcus aureus* however; most researchers have speculated that this increase is a result of unwarranted use of antimicrobial agents in veterinary and human medicine (Aarestrup, 1999; Teuber, 2001). Thus creating selection pressure which can be disseminated to the environment through various factors (such direct contact and food chain) and has a potential to pose threats to animals and humans.

The aim of the study was to investigate the presence of *Staphylococcus aureus* on broiler chicken samples and to further asses the detected isolates by screening for genetic determinants which encode for virulence and antimicrobial resistance. Findings observed after the detection of the pathogen on chicken samples demonstrated that out of 194 broiler chicken samples tested, only 104 (54%) of them were confirmed positive for S*taphylococcus aureus* basing on the presence of the *nuc* gene. These results are almost in agreement with findings from similar studies by Kitai et al. (2005) and Shareef, et al. (2012) which yielded prevalence rates of 44% and 47% respectively however, they are also relatively high compared to findings from similar studies by. Hanson et al. (2011), Momtaz et al. (2013) with contamination prevalence rates of 17.8% and 28.05% respectively. Furthermore, these findings are relatively low compared to finding obtained by Islam et al. (2014) where 95% of chicken samples used for the study were contaminated by S*taphylococcus aureus.* The presence of *Staphylococcus aureus* in chicken meat at an abattoir level presents a possibility for this pathogen to be disseminated to community through slaughter house workers and through transportation of contaminated to retail outlets. Moreover, on the current study, *Staphylococcus aureus* was detected from chicken samples collected at a retail level and from faecal samples collected from the market where there are a lot people moving around every day. This means there is a potential for consumers to obtain the pathogen through ingesting contaminated broiler chicken meat from retail and also through exposure to contaminated environment since faecal samples were found contaminated. Faecal samples are also regarded as major vehicle for the dissemination of pathogens from avian species.

Among the 4 virulence genes screened only two genes were detected, *spa* and *coa* gene. The prevalence rates of these two genes observed were much high compared to findings from a similar study by Bunnoeng et al. (2014) where 0% and 2.5% was observed for *coa* and *spa* gene respectively. However, the findings for the sea gene from Bunnoeng et al. (2014) study are in concordance with our current study where no enterotoxin genes were observed. Both the *coa* and *spa* genes are very important for *Staphylococcus aureus* and they can be used for research purposes to investigated diversity of this organism since they are polymorphic (Vintov et al., 2003). The *coa* gene is a virulence gene that is also used to determine the coagulate status of *Staphylococcus aureus* isolates. Relative low prevalence rates of *coa* gene were detected on *Staphylococcus aureus* isolates therefore, it can be concluded that detected isolates were highly coagulase negative. Coagulase negative *Staphylococcus aureus* is non-pathogenic, but it does harbour some virulence genes at a low rate. Therefore, this

information can be used as an explanation for the low prevalence rate of virulence genes obtained in the current study since most of isolates detected lacked *coa* gene.

The availability and easy accessibility of antimicrobial agents have been a catalyst for an extensive use of drugs in poultry industry, to promote growth and to treat infections caused by various bacterial pathogens. Aarestrup. (2005) reported that extensive use of antimicrobial agents both in small and in large quantities is problematic health wise and economically wise, since it creates selection pressure for antimicrobial resistance. That is why it is crucial to monitor antimicrobial resistance profiles of bacteria isolated from livestock and humans, so that the information can be used to inform public health officials for them to enforce prudent use of antimicrobial agents in human and veterinary medicine (Cummings et al., 2013). Concerning the current study, *Staphylococcus aureus* isolates detected were highly resistant to tetracycline, ampicillin, cefoxitin, trimethoprim and erythromycin, but mostly susceptible to gentamycin and chloramphenicol (Table 4.2) and multiple drug resistance was also observed almost on all the isolates (Table 4.3). These results correspond with finding by Momtaz et al. (2013); Islam et al. (2014) and Ugwu et al. (2015). In all previous studies used as comparatives above, tetracycline resistance was most prevalent compared to resistance of other antimicrobial agents, same scenario was observed for a current study. This confirms information provided by Huys et al. (2005), regarding tetracycline resistance as one of the most frequently occurring resistance phenotypes on *Staphylococcus aureus* isolated from farming, processing and storage environment of poultry. Tetracycline is widely used in poultry industry worldwide, because it is relative cheap and it has less side effective (Chopra and Roberts, 2001). Extensive use of tetracycline might be the reason behind high prevalence of resistant *Staphylococcus aureus* isolates associated with chicken samples. During the study, a gene (*tetK*) encoding for tetracycline resistance was screened from all the samples, and the prevalence rates (Figure 4.2) of *Staphylococcus aureus* isolates harbouring this gene were very low compared to rate of isolates which exhibited tetracycline resistance during antimicrobial agents susceptibility testing. Since there is a pool of genes encoding for tetracycline resistance it might happen that most genes which were responsible for the resistance in *Staphylococcus aureus* isolates of the current study were never screened.

Methicillin resistance *Staphylococcus aureus* (MRSA) is currently a major burden faced by the world. This type of resistance is considered as most important resistance of

Staphylococcus aureus in both human and veterinary medicine, since it has been implicated on high prevalence rate of mortality. In the current study MRSA was detected from abattoir samples (60.3%), retail samples (92%) and faecal samples (76%). The antimicrobial resistance gene responsible for conferring methicillin resistance was also detected, but not in all samples. The prevalence rates are presented on Figure 4.2. The *mecA* gene is regarded as major gene encoding for MRSA however, there are other genes namely *pbpB* (Pinho et al., 2001) and *murF* (Sobral et al., 2003) which have been recently discovered to play a role in MRSA. Febler et al. (2011) and Wulf and Voss (2008) reported that isolation of MRSA from livestock induced so much interest in most researchers since the impact it has on food chain and consumers uncertain. Febler et al. (2011) further reported that consequences of livestock associated *Staphylococcus aureus* are often fatal, since they create treatment complications which is normally accompanied by multi-drug resistance. So it is very importance to monitor and to combat the presence of MRSA on livestock especial poultry, since it is the most consumed protein globally with a potential to escalate widespread of MRSA among human beings.

In South Africa research similar to a current study is more focused on milk (Ateba, et al., 2010; Akindolire et al., 2015) compared to chicken meat, simple because *Staphylococcus aureus* is known to cause mastitis in cattle and it a challenge faced by the dairy industry since the economy is also affected. Most importantly it is considered as one of the most major sources of Staphylococcal infections in humans. A balance focus on investigations based on presence of *Staphylococcus aureus* and it antimicrobial agent susceptibility profile is crucial for all livestock across all provinces to close the gap of limited information, so that it can be used to mitigate in the high prevalence rates of organism on food chain. Availability of information can help enlighten South African Public health committee that unwarranted use of antimicrobial agents in livestock production is causing too much damage than good. In a country like South Africa where there is a high prevalence rate of population with compromised immune system, the issue of resistant bacteria associated with livestock need to be taken serious and addressed thoroughly as matter of protecting citizens' lives.

4.5. Conclusion

Staphylococcus aureus was detected from chicken samples collected from different origins including. The presence of Staphylococcus aureus in commercial meat can be attributed to a number of factors starting from the farm level propagating to retail level. It is crucial to

emphasis strong enforcement of good meat production and proper hygienic measures, in order to decrease meat contamination with foodborne pathogens such as Staphylococcus aureus. The detection of MRSA carriers is important for the prevention and follow-up of these infections

4.6. Statement of Animal Rights

Animal studies have been approved by the appropriate ethics committee of the University of KwaZulu-Natal (Reference: 012/15/Animal) therefore, they have been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments.

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Chapter 5

General Discussion and Conclusions

5.1. Summary

The overall study was focused on two main objectives. The first objective was to investigate the Prevalence of virulence and antimicrobial resistance genes in *Salmonella* spp. isolated from commercial chickens and human clinical isolates from South Africa and Brazil. The second objective was to genetically characterize antimicrobial resistance and virulence genes in *Staphylococcus aureus* isolated from commercial broiler chickens in Durban

Data obtained from the study showed that commercial broiler chicken samples collected around Durban are contaminated by zoonotic foodborne pathogens. Contamination prevalence rates observed were high (*Salmonella= 51%, Staphylococcus aureus*= 54%) and mostly in agreement with some previous studies associated with food safety which have also reported the presence of *Salmonella* spp. and *Staphylococcus aureus* in commercial broiler chickens (Kitai et al., 2005; Capita *et al*. 2007; Hao Van et al., 2007). This shows that there are high possibilities for this contamination to disseminate, since chicken meat is regarded as the main reservoir for zoonotic foodborne pathogens and they can be transmitted to humans mainly through food chain.

Detected isolates were mostly resistance to different antimicrobial agents and these antimicrobial agents are commonly used in human medicine for treatment of various infection. It is also evident that most of the isolates possessed multiple antimicrobial resistances. Development of multiple antimicrobial resistances among detected foodborne pathogens may be attributed to acquisition of antimicrobial resistance genes which emerge as a consequence of unwarranted use of antimicrobial agents in veterinary and human medicine. Molecular characterization of rapidly evolving zoonotic pathogens namely *Salmonella* spp. and *Staphylococcus aureus* was further focused on screening the virulence and antimicrobial resistance genes which pose a hazard to humans and animals health. Findings of the study at hand presented data indicating that most detected isolates were harbouring dangerous genetic determinants responsible for pathogenicity and antimicrobial resistance. Moreover, findings further demonstrated information which showed that the isolates that were detected from Brazilian imported meat also harboured virulence and antimicrobial resistance genes. This

provides evidence that new clones of foodborne pathogens are entering South Africa from different countries mainly through imports, since the country is extensively importing large quantities of broiler chicken meat and its related products. South Africa is a developing country with high prevalence rate of the population having compromised immune system and the country has its own burdens of antimicrobial resistance on most infections such as tuberculosis. Therefore, it is very questionable for such a country to import animal protein such as chicken meat which is contaminated by pathogenic and resistant bacteria and then sell it to the very same people with vulnerable immune systems and who are mostly victims of poverty. Moreover, it is also questionable for a developing country such as this, to still continue with the use of antimicrobial agents that were prohibited in most developed countries for poultry production.

5.3. Conclusion

The aims of the study was successfully achieved, since investigated foodborne pathogens namely *Salmonella* and *Staphylococcus aureus* were detected from broiler chicken samples collected within Durban, South Africa.

- Antimicrobial susceptibility profiles of *Salmonella* spp. and *Staphylococcus aureus* showed antimicrobial resistance of these microorganisms to a variety of antimicrobial agents. Additionally, multi-drug resistance of these two microorganisms was observed and the prevalence rates were almost 100%.
- For *Staphylococcus aureus*, the global well-known methicillin resistance burden was phenotypically and genotypically observed from samples isolated at an abattoir and retail level.
- Virulence and antimicrobial resistance genes were characterized from most detected isolates, including the isolates provided by NHLS which were detected from broiler chicken meat imported Brazil and from sick patients around Durban.

This data supports the notion that although there is limited information on the prevalence rates of foodborne pathogens in chicken meat and the presence of genetic determinants responsible for pathogenicity and antimicrobial resistance. However, collected samples were contaminated by resistant *Salmonella spp.* and *Staphylococcus aureus* harbouring virulence and antimicrobial resistance genes. Thus, has a potential to cause foodborne associated infections on consumers. Therefore, it is crucial for all stakeholders

involved in poultry production, food safety and department of health to work together to create strategies to mitigate the escalating issue of antimicrobial resistance.

5.4. Recommendations

Foodborne pathogens are capable of multiple adaptations for colonization, survival and replication. Therefore, it is crucial to study their ecological well-being extensively to ensure a full understanding of how to protect humans from bacterial infections. Additional to studying the bacterial ecological characteristics, more attention should be based on implementing and supporting studies which will investigate the emergence of antimicrobial resistance in foodborne pathogens residing on broiler chicken meat and other animal proteins such as beef, turkey and pork. Food safety officials need to educate people about hygiene when preparing food and the dangers involved if hygiene measures are not taken into consideration. Most importantly prudent use of antimicrobial agents in veterinary and human medicine needs to be taken as a matter of emergence and citizens need to be informed about this issue because most people are not aware about this escalating burden of antimicrobial resistance we are facing.

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