A SURVEY OF SELECTED PATHOGENIC BACTERIA IN CHICKENS FROM RURAL HOUSEHOLDS IN LIMPOPO PROVINCE

By

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RESEARCH DISSERTATION

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DECLARATION

I Madiwani Mohube Lizzy declare that the work titled: **A survey of selected pathogenic bacteria in chickens from rural households in Limpopo Province**, presented in this dissertation is my own work and all the sources that I have used or quoted have been indicated and acknowledged by means of complete references and that this work has not been submitted before for any other degree at this University or any other institution.

…… ……

Signature Date

DEDICATION

I dedicate my dissertation work to my loving parents, my father Maduana Lekgatlani Letters and my mother Maduana Moele Rebecca, for their support, encouragement and constant love that have sustained me throughout my life. My brothers Japhta and Katlego were always there for me and never left my side. My lovely daughter for her patience, without my presence as a mother I am sure was hard, but you encouraged me to work even more hard. This is for you.

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ACRONYMS AND ABBREVIATIONS

BC	Before Christ
BGA	Brilliant Green Agar
BPW	Buffered Peptone Water
CDC	Centres for Disease Control
CLSI	Clinical and Laboratory Standards Institute
DNA	Deoxyribonucleic acid
ISO	International Organization for Standardization
MALDI-TOF	Matrix-assisted Laser Desorption Ionization-Time of Flight
mL	Millilitre
MHA	Mueller Hinton Agar
mTT	Modified Tetrathionate
NA	Nutrient Agar
PCR	Polymerase Chain Reaction
RVs	Rappaport-Vassiliadis soy broth
UV	Ultraviolet
WHO	World Health Organization
XLD	Xylose Lysine Deoxycholate agar
µg	Microgram
µL	Microlitre
°C	Degrees Celsius

%	Percentage

ABSTRACT

Salmonella enterica serovar Gallinarum biovars Gallinarum, and Pullorum, *Pasteurella multocida* and *Escherichia coli* are among the most important pathogens in poultry and are the causal agents of fowl typhoid, pullorum disease, fowl cholera and collibacillosis in poultry. The present study was designed to identify and determine the distribution of these pathogens in household-raised chickens and their antibiotic and virulence profiles. For this purpose, 40 chickens were bought from household families at Ga-Dikgale, Ga-Molepo and Ga-Mphahlele in the Capricorn district of Limpopo Province and sacrificed for sampling. Tissues including breast meat, lungs, small and large intestines were harvested from each chicken. Bacteria associated with these samples were cultured in selective bacteriological media followed by biotyping using matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) for identification. Out of a total of 160 tissue samples evaluated, *E. coli* and *Salmonella* were detected in these tissues. Furthermore, determination of the pathogenic *E. coli* and *Salmonella* strains at species level using primer sets that target selected genes of interest in the polymerase chain reaction (PCR) assay was employed. The *invA* gene, a confirmatory gene for *Salmonella* species was detected in all the *Salmonella* isolates using PCR. For the pathogenic *E. coli*, *astA, eae, hlyA, fliC$_{H7}$, stxI* and the fimbrial genes (F6 and F41) were detected in some of the *E. coli* isolates recovered from the samples. Disk diffusion test was also performed to determine the antibiotic susceptibility of the bacteria. The results from the current samples reveals that there is a high distribution of *Salmonella* and pathogenic *E. coli* in these areas and therefore further epidemiological and identification studies are needed to determine these organisms at species level and investigate their pathogenicity. The antimicrobial susceptibly data generated from this study can be a valuable reference to veterinarians for treating bacterial diseases in poultry.

CHAPTER 1

1.0. Introduction

1.1. Background of the study

Domestic fowl (*Gallus gallus domesticus*) which is referred to as household-raised chicken in this study is reported to have originated and domesticated from the wild ancestor of jungle fowl known as *Gallus domesticus* from the South eastern Asia around year 2000-3000 BC. Four species of these jungle fowl were identified, namely; red jungle fowl (*Gallus gallus*), Ceylonese Jungle fowl (*Gallus lafayetti)*, gray jungle fowl (*Gallus sonnerati)* and the black or green jungle fowl (*Gallus varius*), which have spread around the world (Rotimi *et al*., 2016).

Household-raised chickens are capable of finding their own feed, are hardy as they have an adaptive ability to survive harsh environmental conditions, diseases, poor management, deficiency in diets and other external stresses (Apuno, Mbap and Ibrahim, 2011; Chebo, 2016). They depend and survive mostly on seeds of weeds, insects, and household wastes (Apuno, Mbap and Ibrahim, 2011).

Production of household-raised chickens plays an important role in securing the needs of livelihood for rural families that keep them as a source of animal protein in the form of meat and eggs and a source of income for ceremonial purposes (cultural and traditional celebrations), (Rotimi *et al*., 2016).

The poultry industry is amongst the largest food-producing industries in the world, and is made up of commercial farms while in developing countries it mostly consist of “backyard” (household-raised) poultry systems (Conan *et al.*, 2012). Chebo, (2016) reported that the total chicken population in Northern Ethiopia is estimated to be 44.89 million, 43.3 million (96.46%) of which are household chickens, thus emphasising the importance of rural chickens as a potential resource for the country.

Predators, lack of supplementary feed, poor housing, poor biosecurity measures, lack of guidance from veterinarians and the quality and cost of feed is considered to cause mortality in these poultry (Sambo *et al.*, 2015), with infectious diseases being the leading cause of mortality in household-raised chickens (Wang *et al*., 2013).

There are few reported research studies about these pathogenic bacteria (i.e *Salmonella* Gallinarum*, Salmonella* Pullorum*, Escherichia coli* (ETEC strains and *E. coli* O157:H7) and *Pasteurella multocida* that cause infections in household-raised chickens published in Limpopo Province. This is because most cases go unreported as the dead birds are either eaten by predators' such as dogs and cats or are thrown away, while in some other cases the sick birds are slaughtered for human consumption (Muhairwa *et al*., 2001).

Salmonella enterica serovar Gallinarum biovars Gallinarum and Pullorum (*Salmonella* Gallinarum and *Salmonella* Pullorum) are two poultry pathogens causing fowl typhoid and pullorum disease in poultry respectively (Barrow and Freitas Neto, 2011). They are responsible for a major economic loss to the poultry industry worldwide, producing high mortality in their acute form (Batista *et al.*, 2016).

Pasteurella multocida on the other hand is a bacterium that causes fowl cholera (FC), a contagious disease of poultry affecting many species of wild and domesticated birds including chicken, turkey, duck and geese with high mortality. There is scanty information available concerning the existence of this disease among household-raised chickens and studies have reported that household poultry are the carriers of *Pasteurella multocida* (Mbuthia *et al.*, 2008; Muhairwa, Mtambo and Christensen, 2001).

Furthermore, *Escherichia coli* is one of the common microbial flora of gastrointestinal tract of poultry, human and animals, but can however become pathogenic to all (Bélanger *et al.*, 2011).The main intestinal patho-types, reported according to observed clinical signs and expressed pathogenicity factors are: enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enteroaggregative *E. coli* (EAEC), enterohemorrhagic *E. coli* (EHEC), (Shiga toxin-producing *E. coli* (STEC)) and enteroinvasive *E. coli* (EIEC).

1.2. Aim of the study:

To establish the distribution of selected pathogenic bacteria, i.e. *S.* Gallinarum*, S.* Pullorum*, E. coli* (ETEC strains and *E. coli* O157:H7) and *P. multocida* in household-raised chickens from rural households in Limpopo Province.

1.3. Objectives of the study:

The specific objectives of the research were to:

i. Isolate and identify potentially pathogenic bacteria from various parts of the chickens.
ii. Investigate the antibiotic resistance of the isolated bacteria.
iii. Investigate the virulence profiles of the isolated bacteria.

1.4. Study rationale

1.4.1. Problem statement

Chicken is a nutritious, healthy food containing less fat and cholesterol as compared to other meats (Bhaisare *et al.*, 2014). Bacterial infections of chickens are of concern in the poultry industry with regards to both animal health and productivity (Wigley, 2013). Some of the major pathogenic bacteria causing these bacterial infections include; *Salmonella* Gallinarum, *Salmonella* Pullorum*,* pathogenic *Escherichia coli* and *Pasteurella multocida.* Despite the negative impact of these pathogens on poultry productivity, there is limited research reporting on their impact in household-raised chickens in Limpopo Province. The general trend has been to survey these pathogens in abattoirs that slaughter commercially bred chickens. Hence a survey of the above-mentioned pathogens will provide information on the distribution and serotypes of these pathogens and give insight into the general health of chickens raised in rural households.

This research will provide information and a better understanding into the existence and distribution of the pathogenic bacteria in household-raised chickens. Failure to detect a potential disease outbreak in the early stages is due to household poultry owners having limited contact with veterinarians (Manning, Gole and Chousalkar, 2015). Therefore, knowledge of the distribution of the specified pathogens will alert veterinarians to be

prepared for early detection. Early detection of the disease in any locality can help reduce/eliminate the losses. In addition, the knowledge on the distribution will facilitate good management and sanitation practices designed to lower the number of these pathogenic bacteria in household-raised chickens. Determining the antibiotic resistance profiles of these pathogens will be useful in the event of an outbreak.

CHAPTER 2

2.0. Literature review

2.1. Description of household chickens

Household-raised chickens are poultry mostly owned by communities in rural areas as well as in other developing countries (Malatji *et al.*, 2016). These household-raised chickens play an essential role by contributing to the socio-economic and cultural lives of small-holder farmers, and provide a source of income for many poor families as well as landless and marginalized societies (Tarwireyi and Fanadzo, 2013; Malatji *et al.*, 2016). Household-raised chickens can be used as an expression of gratitude or gift, and payment for services rendered (Kusina, Kusina and Mhlanga, 1999). They are famously known for their plumage colour that helps them to camouflage and protect themselves against predators (Padhi, 2016).

2.2. Global burden of diseases in poultry

Salmonellosis is reported to be one of the serious bacterial disease problems in the poultry industry in many areas of the world with reports of its isolation in majority of cases in poultry and poultry products than in any other animals (Agada *et al.*, 2014). *Salmonella* species are responsible for causing a variety of acute disease conditions such as respiratory distress, depression and chronic disease condition with loss of appetite and severe anaemia (Okwori *et al.*, 2013). Equally, fowl cholera is a deadly bacterial disease of domesticated and wild avian species caused by infection with *Pasteurella multocida*. It has a considerable economic importance due to its high mortality and occurs sporadically or enzootically in most countries wherever intensive poultry production occurs (Khaled *et al.*, 2016).

Avian collibacillosis is considered to be a serious infectious disease in birds of all ages with the most common form of the disease occurring among 2 to 10 weeks old chickens (Aggad *et al.*, 2010; Kabir, 2010). Collibacilosis is responsible for major economic losses

in the poultry industry globally through morbidity and mortality. It is caused by the pathogen *Escherichia coli* due to its association with various disease conditions, and occurs either as normal bacterial flora of the gastrointestinal tract or produces serious diarrheal diseases, as well as systemic diseases (Kabir, 2010; Himi *et al.*, 2015).

2.3. Pathogenic bacteria

2.3.1. *Salmonella* spp.

i. History and classification

The genus *Salmonella* was first discovered and named after Daniel Salmon a veterinary pathologist at the United States Department of Agriculture. He isolated the organism from pigs in 1885 and named it *Bacterium choleraesuis*, which is presently known as *Salmonella enterica* serovar choleraesuis (Kemal, 2014)**.**

Salmonella is a gram negative intracellular pathogenic bacteria, characterized by non-spore forming, facultative rods, belonging to the Enterobacteriaceae family and invades the host's mucus membrane (Fàbrega and Vila, 2013). Scientifically, *Salmonella* are classified and characterized under the following taxonomy (Kemal, 2014):

Domain: Bacteria

Phylum: Protobacteria

Class: Gamma Protobacteria

Order: Enterobacteriales

Family: *Enterobacteriaceae*

There are two species of *Salmonella*, namely *S. bongori* and *S. enterica*. *S. enterica* is divided into six subspecies including; II (*S. enterica* subsp. *salamae*), IIIa (*S. enterica* subsp. *arizonae*), IIIb (*S. enterica* subsp. *diarizonae*), IV (*S. enterica* subsp. *houtenae*) and IV (*S. enterica* subsp. *indica*). *S. bongori* are usually isolated from cold-blooded animals and the environment but barely from humans (Brenner *et al.*, 2000; Dunkley *et al.*, 2009).

About two thousand six hundred and ten (2610) *Salmonella* serovars have been described and these antigens are used to serotype *Salmonella* bacteria as proposed by Kauffmann-White according to their serologic identification; somatic O, flagella H and capsular Vi antigens and are differentiated based on their lipopolysaccharides and flagella antigens. Many of these serovars (60%) are in the subspecies Enterica, with the most common O-antigen serogroups being A, B, C1, D and E which cause 99% of infections in mammals and birds (Brenner *et al.*, 2000; Zaidi, Macías and Calva, 2006; Dunkley *et al.*, 2009; Kemal, 2014; Batista *et al.*, 2015).

Avian host specific salmonellae include *Salmonella* Gallinarum, *Salmonella* Pullorum and any other serotypes referred to as paratyphoid (PT) salmonellae causing fowl typhoid, pullorum disease and paratyphoid infections respectively (Jafari, Ghorbanpour and Jaideri, 2007; Rajagopal and Mini, 2013; Batista *et al.*, 2015).

ii. Clinical manifestations

Fowl typhoid caused by *Salmonella* Gallinarum infection is still the main disease of the poultry industry in many areas of the world. It causes acute disease conditions that include respiratory distress and depression, and mucoid yellow diarrhoea. In chronic disease condition loss of appetite and severe anaemia are observed in addition to the above mentioned. In chicks and poult, anorexia, diarrhoea, dehydration, weakness and high mortality can be observed and in mature fowls, both fowl typhoid and pullorum disease are expressed by anorexia, drop in egg production, increased mortality, reduced fertility and hatchability (Shivaprasad, 2000; Okwori *et al.*, 2013).

iii. Pathogenicity

Pathogenesis refers to both the processes of infection and the ability of an organism to cause a disease. The ability of *Salmonella* to cause an infection depend on invasive properties, and its ability to survive and multiply within the cells, especially those of macrophages (Kabir, 2010). Transmission occurs through a fecal-oral route and the site of infection is likely the gastrointestinal track (Foley *et al.*, 2013). An infection begins when food or water contaminated with *Salmonella* is ingested, followed by the passage of the bacterium through the alimentary system and its ability to withstand the low pH

environment of the stomach (Ruby *et al.*, 2012; Velge *et al.*, 2012; Foley *et al.*, 2013). The organism then enters the lumen of the gastrointestinal track organs such as the small intestine, colon and cecum (Foley *et al.*, 2013). The adherence process is triggered when *Salmonella* adhere and enter the cells lining the intestinal epithelium by using the enterocytes of M cells. Following the penetration of *Salmonella* into the intestinal epithelium, they secrete T3SS (a multiprotein responsible for bacterial invasion of host cells). T3SS is encoded by SPI-1, which confer virulence genes that are responsible for *Salmonella* invasion, adhesion and toxicity (Rosselin *et al.*, 2010). These steps are brought about by a subset of T3SS-1 effectors (SipA, SipC, SopB, SopE, SopE2). The SipA and SipC proteins directly bind actin and independently inhibit its depolymerization, while the SopB, SopE, and SopE2 proteins indirectly regulate actin activity by triggering Rho GTPases family (Rac1 and Cdc42) required for the initial rearrangements of cytoskeletal actin via the Arp2/3 complex, an eukaryotic factor involved in the actin networks. The cellular changes stimulated by *Salmonella* once inside the host cell are reversed by the bacteria itself by another effector protein of the T3SS-1-termed SptP. This indicate an activity counterbalancing those of SopE and SopE2 and inactivates Rho GTPases to enable the restoration of actin and the return of host cell to the normal state. The other T3SS-1 effectors contribute to a variety of post internalization processes such as host cell survival and modulation of the inflammatory response (Boumart, Velge and Wiedemann, 2014).

In contrast to this trigger mechanism, Rosellin *et al.* (2011) reported that *Salmonella* can also invade the host cell by a mechanism referred to as Zipper entry by Rck outer membrane protein, which stimulates an activation of host cell signalling pathways leading to local accumulation of actin, resulting in distinct membrane modification (Rosselin *et al.*, 2011). Within the lamina propria, *Salmonella* is taken up randomly by different phagocytes (macrophages, dendritic cells and polymorphonuclear cells) and spread rapidly through efferent lymph in mesenteric lymph nodes and the blood stream in the spleen and liver. It is also reported to be able to persist in the gall bladder and bone marrow (Kabir, 2010; Velge *et al.*, 2012).

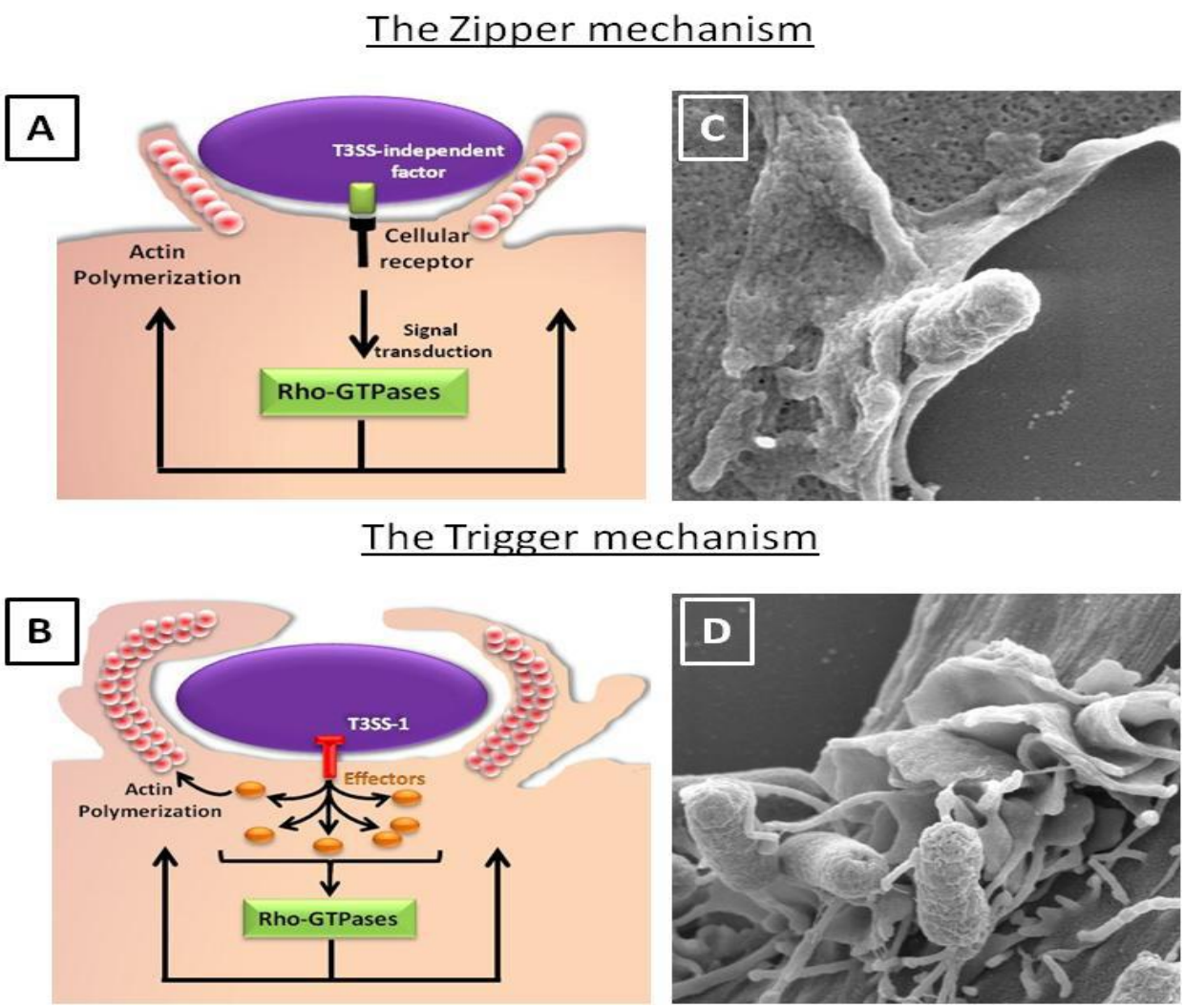


Figure 1: Illustration of Zipper and Trigger invasion mechanisms. (A) The Zipper process begins by an interaction between a host cell receptor and a bacterial surface protein which allows the activation of Rho GTPases and actin polymerization at the entry site. (B) During the Trigger mechanism, bacterial effectors target Rho GTPases, which are directly translocated into host cell through a type–three secretion system, resulting in actin polymerization and internalization. Electron scanning microscopy images showing (C) *S.* Enteritidis invading fibroblasts through a Zipper process which is described by weak membrane rearrangements and (D) via a Trigger process which is characterized by intense membrane rearrangements (Velge *et al.*, 2012).

iv. Transmission

Salmonellosis results from a spread emanating from the contaminated environment which serves as the main source of infection since the organism can survive in the environment for a long period of time (Pui *et al.*, 2011). However, Bouchrif *et al*. (2009) reported that in many instances, poultry, eggs, dairy products, beef and pork are usually the main vehicles of salmonellosis. Fruits and vegetables have also been reported as vehicles of the organism whereby contamination can occur at multiple steps along the food chain. Salmonellosis in poultry particularly fowl typhoid and pullorum disease can be transmitted by diverse types of methods. These methods include: direct transmission, whereby animals such as swine, cows and chickens play a role as a risk factor for infection through the oral route, since *Salmonella* naturally begins from the contaminated environment, feed, water and litter. Mechanical transmission occurs when *Salmonella* is transmitted through vectors such as rats, flies, insects and wild birds whereby the organism can be shed in infected droplets. Horizontal transmission occurs through the respiratory and oral routes. Studies also show that transmission through this route can occur when *Salmonella*-contaminated and *Salmonella*-free eggs are incubated together during hatching. Vertical (transovarian) transmission, on the other hand occurs when infected carrier birds pass the organism to their offspring in eggs. The reproductive organs in adult carriers are the preferred sites of transmission by this route and often lead to the infection of ovarian follicles resulting in the disease occurring in young chicks or penetrating the egg shell after the egg has been laid. These egg transmissions may occur due to contamination of the ovum after the process of ovulation or localization of the bacteria in the ova before ovulation (Shivaprasad, 2000; Proux *et al.*, 2002; Kabir, 2010; Pui *et al.*, 2011).

Infection in newly hatched chicks by nasal and cloacal route is represented as the main route of transmission, whereby chicks are infected early by either from an infected ovary, oviduct or from the infected eggs during the passage through the cloacal or by faeces from infected carrier hens (Kabir, 2010; Okwori *et al.*, 2013). Other possible modes of transmission include shell penetration, contact transmission either in the hatchery,

brooder, cages or floor, during cannibalism of infected birds, egg eating, and through wounds on the skin (Shivaprasad, 2000; Pui *et al.*, 2011).

v. Epidemiology *Salmonella* Gallinarum *and Salmonella* Pullorum

Salmonella Gallinarum and *Salmonella* Pullorum have been eradicated in commercial poultry productions in the developed world such as North America, Western Europe and other countries such as Australia and Japan, whereas , they are still prevalent in the poultry industry in developing world of Africa, Asia and Central and South America (Kang *et al.*, 2010, 2011, 2012). Reports show that fowl typhoid caused by *Salmonella* Gallinarum has successfully been eliminated in Australia and most European countries and North America, as a results of improved surveillance and slaughter practices including an effective control policy such as the National Poultry Improvement Plan of the United States (Kwon *et al.*, 2010). In Africa, however, *Salmonella* Gallinarum has been reported in many countries including Tanzania, Uganda, Nigeria, Zambia, Senegal and Morocco (Agbaje *et al.*, 2010; Okwori *et al.*, 2013).

Outbreaks in an integrated broiler operation were also described in 1990 in the United States, whereby a grandparent male-line breeding flock contaminated 19 parent flocks and 261 roaster flocks in 5 Southern states (Proux *et al.*, 2002). Similar problem occurred in Germany in 1992 and in Denmark during the same period, possibly due to cross contamination during transportation of hens at the end of the laying period (Proux *et al.*, 2002).

vi. Detection and diagnosis

Detection of fowl typhoid and pullorum disease can be tentatively diagnosed made based on flock history, clinical signs, mortality and lesions (Okwori *et al.*, 2007; Kang *et al.*, 2011). Conclusive diagnosis of these diseases is determined by isolation and identification of the organism, followed by serological tests in order to show the presence and through survey of the distribution of these infections (Agbaje *et al.*, 2010; Kang *et al.*, 2011). Isolation of *Salmonella* involves a number of methodological steps using ISO

6579:2002 standard method, which is made up of a pre-enrichment of samples in buffered peptone water (BPW) followed by a selective enrichment in Rappaporte Vassiliadis (RVS) and Muller-Kauffmann Tetrathionate-Novobiocin (MKTTn) (Sparbier *et al.*, 2012; ISO, 2002). Identification is differential and is based on their biochemical properties (Kang *et al.*, 2011). These biochemical properties are determined by isolating presumptive *Salmonella* colonies on plating media, and then incubating in triple sugar iron agar (TSI) using glucose-fermentation and lysine iron agar (LIA) which use lysine decarboxylase reactions for screening *Salmonella spp.*, followed by urease test and additional tests for cultures that do not produce urease (Lee, 2014). This is followed by serological identification tests (Lee, 2014). However serological reports are, highly dependent on rapid screening by plate agglutination test without confirmation of positive reactions by more specific agglutination test (Kumar *et al.*, 2014). In general, these methods are accurate, but time consuming and require thorough labour. However, in recent years, matrix-assisted laser desorption ionization– time-of-flight mass spectrometry (MALDI-TOF MS), which is designed to analyse peptides and proteins, has emerged as a potential tool and has been employed for the identification and diagnosis of microorganisms within minutes (Sparbier *et al.*, 2012; Singhal, 2015).

Several techniques have been developed for the improvement in the detection of *Salmonella* serovars, such as enzyme-linked immunosorbent assay (Kabir, 2010; Kang *et al.*, 2011). However, there has been debates in interpretation of results, and the low sensitivity and specificity of these methods, which require improvement and modification (Salehi, Mahzounieh and Saeedzadeh, 2005; Shanmugasamy, Velayutham and Rajeswar, 2011). Molecular methods are shown to be fast, highly sensitive and very specific (Mirzaie, Hassanzadeh and Ashrafi, 2010). Shanmugasamy *et al*. (2011) reported that in-vitro application of DNA using PCR methods is a powerful instrument for microbiological diagnosis of these organism in environmental, food and faecal samples. Several genes particularly the *InvA* gene of *Salmonella*, contains sequences that are specific to this genus and has proved to be appropriate PCR target with potential diagnostic application (Amini *et al.*, 2010).

2.3.2. *Pasteurella multocida*

i. History and classification

Pasteurella multocida (*P. multocida*) was first discovered in the blood of birds in 1877 (Weber *et al*., 1984) and in 1881, Louis Pasteur identified it as the causative agent of fowl cholera. Since then, it has been described as the causative agent of many other serious diseases in many different hosts (Harper, Boyce and Adler, 2006).

P. multocida is a facultative anaerobic, Gram-negative, non-motile, cocco-bacillus, capsulated and non-spore forming bacterium occurring singly, in pairs or sometimes as chains or filaments belonging to the Pasteurellaceae family (Ashraf *et al.*, 2011; Kim *et al.*, 2011; Ievy *et al.*, 2013; Akhtar *et al.*, 2016). *P. multocida* is classified serologically into four capsular serogroups, namely serogroup A, B, D, and F and somatic serotypes (1-16) based on their lipopolysaccharide antigens. All somatic serotypes have been isolated from birds and among them , serotypes, A:1, A:3 and A:4 are frequently reported as the causative agent of most fowl cholera outbreaks in poultry (Kim *et al.*, 2011; Mohamed, Mohamed and Ahmed, 2012; Varga, Volokhov and Magyar, 2013; Panna *et al.*, 2015).

ii. Clinical manifestations

P. multocida infection occurs in three forms, namely peracute, acute and chronic infection (Huang, Lin and Wu, 2009). Kumar *et al*. (2017) reported that the disease often causes acute septicaemia with high mortality or chronic localized caseous inflammation with purulent exudative lesions in the joints, wattles, ovaries, brain, liver, spleen and lungs in chickens, turkeys and waterfowl.

Other signs and symptoms include depression, ruffled feathers, fever, and anorexia, mucous discharge from the mouth, diarrhoea and an increased respiratory rate (Akhtar *et al*., 2016).

Mohamed *et al. (*2012) reported that the gross lesions are extensive congestion, enlarge and necrotic foci on spleen and liver, petechial haemorrhage in cardiac muscle and necrotic parenchymatous hepatitis.

iii. Pathogenicity

It is clear that diseases caused by *P. multocida* impose an enormous economic concern on the livestock industry, and as such researches have attempted to understand the pathogenesis of this organism (Dziva *et al.*, 2008). The mechanism by which *P. multocida* invades and causes the disease (s*)* is variable and complex, depending on the host species, strain, variation within the strain or host, and conditions of contact between two birds (Panna *et al.*, 2015). The process is associated with various virulence factors, with the main ones identified as being capsule and polysaccharides (Tang *et al.*, 2009). The recognized virulence factors includes various adhesins (e.g., filamentous hemagglutinin, type 4 fimbriae, and Flp pilin), toxins (dermonecrotic toxin), siderophores (e.g. iron acquisition proteins), sialidases (which may increase bacterial virulence by exposing the key host receptors and/or reducing the effectiveness of host defences), and outer membrane proteins (e.g., OmpA, OmpH, Oma87, and PlpB) (Tang *et al.*, 2009). These virulence factors promote the colonization and invasion of the host, the avoidance or disturbance of host defence mechanisms, injury to host tissues, and/or stimulation of a noxious host inflammatory response (Harper, Boyce and Adler, 2006).

Generally, it is believed that *P. multocida* enters the host through the tissue of the respiratory tract in birds. The organism adheres to the turkey's air sac macrophages, and then is injected into the upper respiratory tract or trachea and can be identified in the internal organs between 6 and 12 hours post-inoculation (Harper, Boyce and Adler, 2006).

iv. Epidemiology

Fowl cholera (FC), caused by *P. multocida*, is a deadly disease in both domesticated and wild birds including chicken, turkey, duck and geese, causing high fatality and resulting in a serious economic losses globally (Kim *et al.*, 2011; Sarangi *et al.*, 2014). A large number of *P. multocida* strains were reported to have been isolated from outbreaks of fowl cholera in various agro-climatic or geographical regions in India (Pillai, 2013). Both healthy carrier birds within a flock and infected wild birds can act as sources of infection, with turkeys and waterfowl being the most affected. Death from fowl cholera in chickens usually occurs

in laying flocks, because birds of this age are more susceptible than younger chickens (Mbuthia *et al.*, 2008; Kim *et al.*, 2011). There are three subspecies within *P. multocida* that are recognized, namely; *P multocida* subsp. *multocida, P multocida* subsp. *septica,* and *P multocida* subsp. *gallicida* depending on results of total genomic DNA hybridization, carbohydrate fermentation, enzymatic activities and phylogenic gene sequence comparison studies (Varga, Volokhov and Magyar, 2013).

Literature on the epidemiology and the importance of fowl cholera caused by *P. multocida* in poultry in developing countries is limited with few reports that show the presence of this infection in household-raised chickens in countries such as Tanzania (Muhairwa, Mtambo and Christensen, 2001), Kenya (Mbuthia *et al.*, 2008) and Zimbabwe (Kelly *et al.*, 1994).

v. Detection and diagnosis

Normally, diagnosis of the disease in natural outbreaks depends mainly on conventional methodologies consisting of bacterial isolation and identification by serotyping and biochemical characterization, which shows the presence of variable serogroups in various geographical regions (Harper, Boyce and Adler, 2006). However, other researchers discovered that conventional characterization is not sensitive enough to identify and differentiate each strain involved in natural infections (Stahel, Hoop and Kuhnert, 2009; Varga, Volokhov and Magyar, 2013). Pillai, (2013) states that diagnosis of fowl cholera is based on clinical findings, pathology, culturing of organism and biochemical characteristics.

2.3.3. *Escherichia coli*

i. History and classification

Escherichia coli was first discovered by Theodor Escherich in 1885 after its isolation from the faeces of new-borns of human (Dash *et al*., 2012). They are Gram-negative, rod shaped, facultative anaerobes belonging to the family Enterobacteriaceae. They are normally found in the lower intestine of animals and humans and form part of the normal microbial flora of the gut (Shahzad *et al.*, 2013; Himi *et al.*, 2015). However, they can

become pathogenic when there is a nutritional imbalance. *E. coli* grow at optimum temperature of 37 °C, in a variety of laboratory media, with nutrient agar shown to be a better growth medium for these laboratory strains (Noor *et al*., 2013).

Colibacillosis in chickens is a local or systemic infection caused partially or entirely by *Escherichia coli* and has been identified as the main cause of infectious disease in poultry of all ages, leading to a number of disease manifestations such as yolk sac infection, respiratory tract infection, swollen head syndrome, septicaemia and enteritis (Kabir, 2010; Van der Westhuizen and Bragg, 2012; Saidi, Mafirakureva and Bc, 2013; Mbanga, 2015).

2.3.3.1. Pathotypes of *E. coli*

a) EPEC

Neter *et al*. (1955) was the first to use the term Enteropathogenic *E. coli* (EPEC) in 1955 to explain in detail the strains of *E. coli* epidemiologically associated with several outbreaks of infantile diarrhoea in the 1940s and 1950s. EPEC serotypes are the pathotypes of *E. coli* that belong to a group of bacteria known as attaching and effacing pathogens, capable of causing distinctive lesion on the surface of the intestinal epithelial cells (IECs) (Croxen *et al.*, 2013). EPEC are further divided into typical and atypical subtypes, with typical EPEC (tEPEC) having the large virulence plasmid known as adherence factor (EAF) plasmid (pEAF), which encodes the type V fimbriae called the bundle-forming pilus (BFP), while the atypical EPEC (aEPEC) lack this plasmid (Nataro and Kaper, 1998; Trabulsi, Keller and Gomes, 2002). Based on Multilocus enzyme electrophoresis analysis (MLEE), strains of EPEC (tEPEC) fall into 4 clonal lineages, namely; EPEC 1 to EPEC 4 , and have acquired the locus of enterocyte effacement (LEE) region pEAF independently (Lacher *et al.*, 2007; Hazen *et al.*, 2013). On the other hand, aEPEC, O151 serogroups are the most isolated, followed by O145, O26, O55, O111 and O119. Nonetheless, many of these strains are O/H-antigen non typeable (Croxen *et al.*, 2013).

In addition, it has been found that some aEPEC strains (35%) belong to the tEPEC lineages.Theoretically, these strains may have emerged from tEPEC strains that have lost pEAF in the host or in the environment (Gomes *et al.*, 2016).

The A/E lesion is the hallmark of the EPEC pathogenesis, characterized by effacement of brush border microvilli at the site of bacterial attachment (Croxen *et al.*, 2013; Gomes *et al.*, 2016). During pathogenesis, these intestinal brush border dissociates, followed by formation of pedestals that extend from the surface of the epithelium into the lumen (Croxen *et al.*, 2013). These pedestal-like structures are manufactured through a production of a conserved bacterial receptor protein, Tir, via a type III secretion system (T3SS) (Croxen *et al.*, 2013).

A three-stage model of EPEC pathogenesis was first reported in the 1990s by Donneberg and Kaper, namely adherence of host cells, in which the initial attachment of tEPEC to the surface of the host surface intestinal epithelium is mediated by Bundle-forming pilli, signal transduction and intimate attachment. A/E lesion formation occurs by the subversion of actin dynamics within host cells and is mediated by the association between intimin and the bacterial translocated intimin receptor, Tir (Donnenbergl and Kaper, 1992; Croxen *et al.*, 2013). Intimin is an outer membrane protein expressed by EHEC and EPEC, required for intimate attachment to the host cell and formation of the A/E lesions. Intimin is classified into a family of adhesin proteins that are able to bring about A/E lesions and are generally divided into five distinct subtypes (α, β, γ, ζ and ε) based on their divergent C-terminus domains (Hartland *et al.*, 2000; Yi *et al.*, 2010).

In addition, the N-terminus of intimin anchors the protein in the EPEC outer membrane, whereas the C-terminus extend from the EPEC's surface and binds to the Tir (Sepehri, 2015). The intimin-Tir association results in intimate adherence and pedestal formation beneath adherent bacteria and inhibits NF-$_{K}$B activity through tumour necrosis factor alpha (TNF-α) receptor-associated factors (Ruchaud-Sparagano *et al*., 2011). In addition to Tir, the EPEC genome contains six other LEE-encoded effector proteins (Map, EspI, EspG, EspZ, EspH and EspB) that are translocated into the cells, which plays physiological roles significant to A/E pathogen infection (Santos and Finlay, 2015). In addition to the LEE effectors, different non-LEE (NLE)-encoded effector genes (cif,

espI/nleA, nleB, nleC, nleD, nleE and nleH) have been reported. These genes are located outside the EPEC LEE region, in at least six chromosomal PAIs or in prophage elements (Vossenkämper, Macdonald and Marchès, 2011; Wong *et al.*, 2011; Santos and Finlay, 2015). These proteins (NLE) destroy the cytoskeleton and fight junctions of the host cell, and to block inflammatory response (Vossenkämper, Macdonald and Marchès, 2011; Wong *et al.*, 2011; Raymond *et al.*, 2013). However, Santos *et al*. (2011) reported that they are not essential for AE lesion formation but play a role in bacterial virulence.

b) EHEC

In recent years, a group of these pathogens has been shown to be more prevalent throughout the world, causing a serious gastrointestinal disease such as those that are incurable. The EHEC strains are a subset of Shigatoxin-producing *E. coli* (STEC) or verocytotoxic-producing *E. coli* including *E. coli* O157: H7 and non O157.They are responsible for human Hemorrhagic Colitis and sometimes fatal Haemolytic Uremic Syndrome (HUS) and animal diseases (Karmali, Gannon and Sargeant, 2010; Tahamtan, Hayati and Mm, 2010). Their capacity to produce one or more of the Shiga toxin (Stx) family cytotoxins, constitutes the main virulence attribute of this pathogroup of *E. coli* (Gomes *et al.*, 2016). They produce shiga-like toxins encoded by either stx1 or stx2 genes or its variants and the products of the locus of enterocyte effacement (LEE), the pathogenicity island, and often carry *eaeA* gene that encodes for the intimin (protein involved in the intimate adhesion of bacteria to enterocytes and production of attaching and effacing (AE)) lesion (Xia *et al.*, 2010). Other factors that play a role in virulence include EHEC hemolysin (encoded by EHEC *hly*A), which acts as a pore-forming cytolysin and causes damage to cells (Jamshidi *et al*., 2016; Paton and Paton, 1998).

STEC virulence genes play a vital role in causing diseases with the most dominant EHEC serotype being *E. coli* O157:H7, but, other serogroups such as 206, 0145 and 0103 (usually referred to as non–O157 EHEC) have been reported in some countries (Bardiau, Zalo and Ainil, 2010; Momtaz and Jamshidi, 2013).

Production of shiga toxins in combination with the heat-labile and heat-stable (ST) enterotoxins and colonization factors such as fimbriae and non-fimbrial adhesins play an

essential role in the initial step of *E. coli* to adhere, invade and persist inside the host (Kalita, Hu and Torres, 2014; Mohlatlole *et al.*, 2013).

c) EAEC

Enteroaggregative *E. coli* was identified from a case study conducted in 1987, when Nataro *et al*. (1987) differentiated “diffuse adherence” as the truly diffuse adherence (DA) and the AA pattern by comparing the adherence pattern from over 500 isolates.

Strains of EAEC are a diverse group of *E. coli* having about 90 serotypes, with the most common ones being O15: H18, O44:H18, O77:H18, O11:H12, O125 and O126 (Gomes *et al.*, 2016). The aggregative adherence (AA) pattern of these strains of EAEC to the intestinal cells is represented by “stacked-brick” formation of bacterial cells attached to the HEP-2 cells (Nataro and Kaper, 1998). Their activity results in colonization of the intestinal mucosa, primarily that of the colon, followed by secretion of enterotoxins (Weintraub, 2007). During pathogenesis, EAEC adhere to the intestinal mucosa by plasmid encoded aggregative adherence fimbria (AAF/III), increased production of mucus biofilm, mucosal toxicity due to inflammation and cytokine release (Boll *et al.*, 2017; Gomes *et al.*, 2016).

Recent reports, documented a division of EAEC strains into typical or atypical subgroups based on the presence or the absence of aggR (Gomes *et al.*, 2016). Strains expressing aggR are reported to have more pathogenic potential due to the presence of aggR regulon and consequently pAA virulence factors, hence the term “ Typical EAEC” (Morin *et al.*, 2013). Found in PAA 2 of EAEC O42 is the *aaP* gene encoding anti-aggregation protein called dispersin (Rodrigues *et al.*, 2016; Karam *et al.*, 2017). This protein is produced and associated with lipopolysaccharide, neutralising the negative charge on the surface bacterial cell resulting in AAF/II projection and subsequent anti-aggregation and dispersion along intestinal mucosa (Gomes *et al.*, 2016; Navarro-garcia and Elias, 2017). Although this protein is found in other pathotypes of *E .coli* and in commensal *E .coli*, there are other toxins that have been described in EAEC associated with cytotoxic or enterotoxic effects such as heat stable enterotoxin enteroaggregative *E. coli* known as heat-stable enterotoxin 1 (EAST-1) (Gomes *et al.*, 2016). This toxin activates adenylate

cyclase inducing increased cyclic GMP levels in enterocytes, generating a secretory response (Navarro-garcia and Elias, 2017). ShET 1, is a subunit toxin that induce intestinal cyclic AMP and cGMP-mediated secretion and is also encoded by *Shigella flexneri* and Pic.Mucinase is shared among many different pathogenic *E. coli* (Croxen *et al.*, 2013; Jensen *et al.*, 2014).

d) ETEC

This group of organisms produce enterotoxins, namely heat-stable (ST) and heat-labile (LT) toxins. Reports states that this type of strains colonize the small intestine using colonization factors (CFs) by invading the intestinal epithelial cell using g fimbrial adhesins that promote the attachment of bacteria to the surface of the epithelium and by production of toxins that cause diarrhoeal diseases in both farm animals and humans (Zhang *et al.*, 2007).

The LT triggers adenylate cyclase resulting in an increase in levels of intracellular cyclic AMP (cAMP) followed by stimulation of chloride secretion leading to diarrhoea. The ST binds guanilate cyclase C and triggers an increase in intracellular cyclic guanosine monophosphate (cGMP) levels and chloride secretion, resulting in dehydrating diarrhoea (Read *et al.*, 2014; Kartsev *et al.*, 2015).

ii. Clinical manifestations

Avian colibacillosis, caused by *E .coli* is the primary cause of a large economic loss in poultry production, resulting in low performances, weight loss, delayed onset of egg production and mortality (Aggad *et al.*, 2010). It also causes a diverse types of disease manifestations such as yolk sac infection, omphalitis, respiratory tract infection, swollen head syndrome (SHS), septicemia, polyserositis, coligranuloma, enteritis, cellulitis and salpingitis (Kabir, 2010).

iii. Pathogenicity of *E. coli*

The mechanism by which infection is caused by avian pathogenic *E. coli* has not been elucidated. However, a cross- sectional study of wild birds in Northern England has been conducted to describe the distribution of *E. coli* containing gene that encodes Shiga toxins (stx1 and sxt2) and intimin (*eae*), which are important virulence determinants of STEC

associated with human disease (Hughes *et al*., 2009). It has been reported that while wild birds were unlikely to be direct sources of STEC infections, they do represent a potential reservoir of virulence genes (Hughes *et al*., 2017). APEC causes a variety of diseases at different ages. Neonatal infection of chicks can take place horizontally, or vertically, from the environment or hen respectively. A laying hen with *E. coli*-induced oophorosis or salpingitis may infect the internal egg before shell formation. Eggshell can be contaminated with faeces during the passage of the egg through the cloaca and after laying. Before hatching, the yolk sac can be infected with APEC resulting in embryo mortality; the chick can also be infected during or shortly after hatching (Kabir, 2010) .

iv. Detection and diagnosis

The diagnosis of colibacillosis is achieved by isolating *E. coli* from cardiac blood and affected tissues, such as the liver, spleen or bone marrow (Abalaka *et al.*, 2017) . Selective media such as McConkey agar, Eosin Methylene Blue or Drigalki agar are used for isolation of *E. coli* and biochemical reactions such as indole test, fermentation of glucose test and urease test are used to show the presence of *E. coli* (Islam, Islam and Fakhruzzaman, 2014; Humam, 2016).

2.4 Antimicrobial resistance in chickens

The advent of antibiotics began with the discovery of penicillin by Sir Alexander Fleming in 1928 and were first prescribed to treat serious infections in the 1940s (Ventola, 2015). However, antimicrobial resistance became a major clinical problem by the 1950s (Spellberg and Gilbert, 2014). Difference in susceptibility to antibiotics against microorganisms has become a major factor in the drug of choice and success of treatment (Huang, Lin and Wu, 2009). The misuse of antibiotics in animal feed stocks for growth purposes also plays a role in increasing the spread of resistance in bacteria (Fair and Tor, 2014). This emergence and spread of antimicrobial resistance is a global concern for both humans and veterinary medicine (Rigobelo *et al.*, 2013; Usui *et al.*, 2014). The use of antimicrobials in chickens creates a selection pressures that favour the survival of antibiotic-resistant pathogens (Harsha *et al.*, 2011; Obi and Ike, 2015). They are mostly used for therapy of infections in both animals and humans, as well as prophylaxis and

growth promotion of food producing animals (Van Boeckel *et al*., 2015). However, many findings suggest that the inadequate selection, particularly at a low level for a long time and imprudent use of antimicrobials may lead to resistance in bacteria rendering the antibiotics ineffective, leading to failure of the treatment (Kilonzo-Nthenge *et al*., 2008; Singh *et al.*, 2010; Paulo, 2014). Previous studies reported a higher bacterial resistance to antibiotics in chickens than in cattle and pigs, which possibly indicates its high use in the poultry (Usui *et al.*, 2014). Recently there is an increasing concern regarding the emergence of multidrug resistant strains (Obi and Ike, 2015), as a result of the massive imprudent usage of antimicrobials developing resistance mechanisms (Giedraitienė *et al*., 2011)

Many developed countries such as Japan, the United States and Denmark, have national monitoring program for assessing bacterial susceptibility to antimicrobials among enteric bacteria isolated from healthy animals, which revealed a high distribution of antimicrobial resistance in food producing animals. Though studies have been conducted to manage the problem of antimicrobial resistance and the development thereof, there have been delayed efforts to develop solutions (Amadi *et al.*, 2015). In developing countries, very little information has been published regarding the existence of antimicrobial resistant bacteria in food producing animals (Usui *et al.*, 2014).

2.4.1 Mode of action of antibacterial agents

There are four key mechanisms of action employed by antibacterial drugs

- Bacterial cell wall synthesis.
- Bacterial deoxyribonucleic acid (DNA) synthesis.
- Bacterial protein synthesis.
- Folate synthesis.

Inhibition of bacterial cell wall synthesis; Lack of peptidoglycan in eukaryotic cells makes the wall of the bacterial cell an ideal target for antibiotic therapy. β-lactam antibiotics such as Penicillins and cephalosporins carbapenems, and monobactams, and the glycopeptides, including Vancomycin and Teicoplanin are examples of antibiotics that interrupt peptidoglycan layer synthesis (Den, 2018). These antibiotics enter the bacterial cell and bind to enzymes known as penicillin-binding proteins (PBPs) and block the

crosslinking of peptidoglycan units by inhibiting the peptide bond formation reaction that is catalysed by these enzymes (Cho *et al*., 2015). This result in the formation of a weak or morphological change of the cell wall, which swells and then lyse (Lai, Cho and Bernhardt, 2017). However, Vancomycin and teicoplanin interrupt cell wall synthesis, by binding to the terminal D-alanine residues of the nascent peptidoglycan chain, thereby preventing the cross-linking steps required for stable cell wall synthesis (Hughes *et al.*, 2017).

Inhibition of bacterial DNA synthesis: Plasmids are genetic structures that carry antibiotic resistance genes (Adamczuk *et al.*, 2015). The quinolones (such as ciprofloxacin) as well as drugs such as metronidazole, nitrofurantoin and rifampicin hinder the maintenance of chromosomal topology by preventing the action of two enzymes, namely DNA gyrase and topoisomerase IV at the DNA cleavage stage and thus block the strand re-joining (Aldred, Kerns and Osheroff, 2014). Rifampicin impedes the production of proteins that are important to bacterial cell structure and function. They accomplish this by inhibiting the enzyme required for the formation of messenger ribonucleic acid (mRNA) (Kohanski, Dwyer and Collins, 2010).

Inhibition of bacterial protein synthesis; Antibiotics that inhibit protein synthesis include the aminoglycosides (such as Gentamicin), which cause misreading of the code on mRNA, generating dysfunctional proteins in the bacteria (Hong *et al.*, 2015). Tetracyclines (such as oxytetracycline, doxycycline, minocycline and Tetracycline) interfere with protein synthesis by blocking a molecule known as transfer RNA to the ribosome, this subsequent blockage of the peptidyltransferase elongation reaction, eventually activates the dissociation of the peptidyl tRNA (Chopra and Reader, 2015). The macrolides (such as erythromycin and clarithromycin) bind to one of the ribosomal subunits (30S), whereas Chloramphenicol binds to the 50S subunit leading to inhibition of the ribosomal function (Liwa and Jaka, 2015).

Inhibition of folate synthesis; Trimethoprim and the sulphonamides such as sulfadiazine are antibiotics essential for the inhibition of folate synthesis (Grenni, Ancona and Barra

Caracciolo, 2018). These antibiotics interfere with folic acid metabolism in the microbial cell by competitively hindering the biosynthesis of tetrahydrofolate, which acts as a carrier of one-carbon fragments and is responsible for the synthesis of DNA, RNA and bacterial cell wall proteins (Liwa and Jaka, 2015).

Sulfonamides competitively prohibit the conversion of pteridine and *p*-aminobenzoic acid (PABA) to dihydrofolic acid by the enzyme pteridine synthetase. Trimethoprim act at a later stage of folic acid synthesis. It has a tremendous affinity for bacterial dihydrofolate reductase; when bound to this enzyme, it inhibits the synthesis of tetrahydrofolate (Liwa and Jaka, 2015; Kapoor *et al*., 2017).

Disruption of bacterial membrane structure; may be a fifth, although less well characterized, mechanism of action. However, it is assumed that polymyxins exert their inhibitory effects by promoting bacterial membrane permeability and damaging the physical integrity of the phospholipid bilayer of the inner membrane, resulting in leakage of bacterial contents (Huang and Yousef, 2014). The cyclic lipopeptide daptomycin apparently place its lipid tail into the bacterial cell membrane (Carpenter, 2004), causing membrane depolarization and ultimately death of the bacterium (Müller *et al.*, 2016).

2.4.2 Molecular mechanisms of antibiotic resistance

Resistance to antibiotics takes place in four diverse ways. It can happen through the modification of a drug target, which may develop through point mutations in selected genes leading to relatively rapid and facile resistance where such changes have minimal impact on microbe fitness (Wright and Wright, 2011). Another mechanism involves the acquisition of an efflux pumps by the bacterial that actively remove the antibacterial agent from the cell before it can reach its target site and apply its effect (Munita *et al.*, 2016).

In the molecular bypass strategy, the bacteria is capable of developing new targets (usually enzymes) that have the same effect as biochemical functions of the original target but are not inhibited by the antimicrobial molecule (Tanwar *et al.*, 2014). Wright (2011) showed the use of Vancomycin as an effective example, i.e. Vancomycin binds to the acyl-D-Alanine-D-Alanine terminus of the growing peptidoglycan component of the bacterial cell wall, which forms a non-covalent complex with acyl-D-Alanyl-D-Alanine

through a series of five hydrogen bonds. Resistance take place through substitution of acyl-D-Alanyl-D-Alanine with the isosteric depsipeptide acyl-D-Alanyl-D-Lactate by removing a single hydrogen bond and introduces an electronic repulsion that presents a productive binding of the antibiotic (Munita *et al.,* 2016).

Lastly, antibiotic activation or modification can be accomplished by three types of enzymes; β-lactamases, aminoglycoside modifying enzymes and Chloramphenicol acetyltransferase (Giedraitienė *et al*., 2011).This is one of the most successful mechanisms to survive the presence of antibiotics through production of enzymes that inactivate the drug by adding the chemical moieties to the compound or that destroy the molecule itself, making the antibiotic to be unable to interact with its target (Gupta and Birdi, 2017).

CHAPTER 3

3.0. Materials and methods

3.1 Materials

3.1.1 Reagents

Buffered Peptone Water (BPW), Rappaport Vassiliadis (RVs) broth, Tetrathionate broth (TT), Xylose Lysine Deoxycholate (XLD) agar, Brilliant Green Agar (BGA), Tryptose Blood agar (TBA), McConkey agar (MCC), Nutrient agar (NA), Tryptose water, Kovac's reagents, MALDI matrix hydroxycinnamic acid (HCCA), Deionised water, Primers, Antibiotics

3.1.2 Equipment's

Sterile 50 mL tubes, Laboratory blender (stomacher 200), Maldi-TOFF\TOFF, Heating block (mrc Dry Bath incubator), Microfuge® 16 centrifuge, Polymerase Chain Reaction (PCR), Agarose gel, PCR plates, Vortex mixer (Maxi Mix II thermolyte), magnetic stirrer.

3.2 Methods

3.2.1 Sample collection

Samples of household-raised chickens were collected from Capricorn district (Ga-Dikgale, Ga-Molepo and Ga-Mphahlele) in Limpopo Province. Up to five chickens from each household were purchased. Fourty (40) chickens from the villages were used in the study. Chickens were purchased from the households at an agreed price and sacrificed by stunning followed by decapitation as performed by the household owners and then transported to the University of Limpopo Animal unit where the specific tissues were removed and separately packed in sterile tubes and processed within 4 hours.

3.2.2 Tissue sample processing, culturing and isolation of bacteria

Upon arrival to the Biotechnology Unit laboratory, tissue samples were processed immediately. Bacteria from each tissue sample such as meat, lungs, small and large intestines, were pre-enriched by adding 25g of the tissue sample into 225 mL of Buffered Peptone Water (BPW) containing 0.225 mL of Tween 80, and homogenising in a laboratory blender (Stomacher 400) followed by incubation at 37 °C for 18 hours (Madoroba, Kapeta and Gelaw, 2016).

3.2.3 Culturing and isolation of *Salmonella* Gallinarum and *Salmonella* Pullorum

For the detection of *Salmonella* spp*.*, a modification of the ISO 6579 method was applied; after pre-enrichment of the sample tissues, selective enrichment was carried out in Rappaport-Vassiliadis (RV) broth and Tetrathionate (TT) broth. The enrichment was performed by adding 1 mL of the pre-enriched culture into 9 mL of RV broth and TT broth, followed by incubation at 42 °C for 24 hours and 37 °C for 24 hours respectively. The enriched cultures were selectively streaked on xylose lysine deoxycholate (XLD) agar and brilliant green agar (BGA) plates and then incubated at 37 °C for 24 hours. Small red translucent and or dome-shaped colonies, with a central black spot on XLD and pink colonies surrounded by a red medium on BGA were selected for subculturing.

3.2.4 Culturing and isolation of *Pasteurella multocida*

Pre-enriched cultures were streaked on Tryptose blood agar plates (containing sheep blood), followed by incubation for 24 hours at 37 °C. Large translucent colonies, greyish in color and mucoid in consistency from Tryptose blood agar plates were propagated in tryptophan broth and incubated at 37 °C for 8 hours (Kim *et al.*, 2011).

3.2.5 Culturing and isolation of *E. coli*

A loopful of the pre-enriched culture was platted onto MacConkey agar and incubated at 37 °C for 24 hours. Typical *E. coli* colonies, both lactose fermenters (indicated by bright pink halo and bile precipitant around the colonies) and non-lactose fermenters indicated by colourless or clear colonies were observed and then subcultured on nutrient agar followed by incubation at 37°C for 24 hours.

A loopful of an *E. coli* colony was inoculated into 9 mL of tryptose water and incubated at 37 °C for 24 hours followed by addition of a drop of Kovac's reagent to determine whether the organism present can split indole from tryptophan. This was shown by a pink to red ring in the reagent layer on top of the medium.

3.2.6 Identification of the isolated bacteria

Colonies suspected to be of a desired species were sub-cultured on nutrient agar plates, incubated overnight and then spotted on a matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) target plate. MALDI matrix hydroxycinnamic acid (HCCA) was added onto the spots. The spots were then subjected to MALDI-TOF mass spectrometry and the resulting mass spectra were queried against a Bruker Daltonics database of bacteria. The following formic acid extraction method was applied: 300 µL deionized water was pipetted into an Eppendorf tube, and to it 5mg from a single colony of biological material (isolated bacteria) was added. The solution was mixed thoroughly by vortexing. Nine hundred (900 µL) of ethanol (EtOH) was added and mixed thoroughly, followed by centrifugation at maximum speed (1≥13,000 rpm). The supernatant was decanted and carefully pipetted off all residual of EtOH without disturbing the pellet. The EtOH was allowed to dry at room temperature for 2 to 3 minutes, followed by addition of 70% formic acid (1 to 80 µL) and mixed very well by vortexing. Pure Acetonitrile (ACN) (1 to 80 µL) was added and mixed very carefully. The same volume was used as formic acid, followed by centrifugation for 2 minutes at maximum speed (1 ≥13,000 rpm), such that all the material was collected neatly in a pellet. One microliter (1 µL) of supernatant was pipetted onto a MALDI target plate and allowed to dry at room temperature. Then the entire spot was overlaid with 1 µL of HCCA solution within 1 hour and dried at room temperature.

3.2.7 Molecular characterization or identification of the organisms using PCR

DNA extraction was carried out by using Loop-fuls of bacterial colonies suspended in 200 µL sterile distilled water and boiled at 99 °C on a heating block for 20 min, cooled at room temperature and centrifuged for 5 minutes using a microcentrifuge at 13,000 rpm. Supernatants containing crude DNA were used as templates for PCR.

For DNA Amplification using multiplex PCR, identified organisms were genotyped at molecular level using PCR and appropriate primers for the target genes (Table 1A, Table

1B and table 2). The virulence genes of the isolates were detected by PCR using the following primer pairs: A058 and A01 (*SefA* gene) specifically for *S.* Gallinarum and for *S.* Pullorum, Fli15 and Typ04 (*Flic* gene) specifically for *Salmonella* Typhimurium and *Salmonella* Enteritidis and S139 and S141 *(invA* gene) for the confirmation of *Salmonella* (Oliveira *et al.*, 2002). ENT(*Sdf1* gene) specific for *Salmonella* Enteritidis, ViaB (*ViaB* gene) specific for *Salmonella Typhi* and Typh (*Spy* gene) specific for *Salmonella* Typhimurium (Al *et al.*, 2016). The following PCR conditions were used for the DNA amplification; initial denaturation for 30 seconds; 35 cycles of denaturation for 30 seconds at 95°C, annealing for 15 seconds at 55 °C and elongation for 1 minute at 72 °C; and final extension for 7 minutes at 68 °C.

The toxin (Stx1, Stx2, Stx2e, Sta and Stb), EAST-1, *hlyA*, $fliC_{H7}$, $rfbE_{O157}$ and fimbrial genes for *E. coli*; F4, F141, F6 and F18 (Madoroba *et al.*, 2009;Cai *et al*., 2003; Ngeleka *et al*., 2003), were detected using relevant primers (Table 1A and Table 1B). PCR conditions were as follows; initial denaturation for 3 minutes at 94 °C; 30 cycles of amplification with denaturation for 30 seconds at 90 °C, annealing for 30 seconds at 55 °C, primer extension for 1 minute at 72 °C; and final extension for 10 minutes at 72 °C (Madoroba *et al.*, 2009).

Table 1A**:** Primers used in multiplex PCR reactions for specific detection of toxin and fimbrial genes in pathogenic *E. coli*

Primer Target Gene	Forward(-F) and Reverse (-R) Primer Codes	Nucleotides Sequence (5'-3')	Size of Amplified DNA Fragment (bp)
astA	EAST-1-F	TCG GAT GCC ATC AAC ACA GT	125
	EAST-1-R	GTC GCG AGT GAC GGC TTT GTA G	
stxI	Stx1-F	ATT CGC TGA ATG TCATTC GCT	664
	Stx1-R	ACG CTT CCC AGA ATT GCA TTA	
stxII	Stx2-R	GAA TGA AGA AGA TGT TTA TAG	281
	Stx2 -F	CGG GGT TAT GCC TCA GTC ATT ATT AA	
stx2e	Stx2e-F	GAA TGA AGA AGA TGT TTA TAG CGG TTT TAT GGA ACG TAG GTA TTA CC	454
	Stx2e-R		

Table 1B**:** Primers used in multiplex PCR reactions for specific detection of toxin and fimbrial genes in pathogenic *E. coli*

eaeA	eaeAF eaeAR	CCACCTGCAGCAACAAGAGG CCACCTGCAGCAACAAGAGG	384
hlyA	hlyAF hlyAR	GCATCATCAAGCGTACGTTCC AATGAGCCAAGCTGGTTAAGCT	534
Est	Sta-F Sta-R	GGG TTG GCA ATT TTT ATT TCT GTA ATT ACA ACA AAG TTC ACA AGC AGT A	183
estII	STb-F STb-R	ATG TAA ATA CCT ACA ACG GGT GAT TAT TTG GGC GCC AAA GCA TGC TCC	360
F5	F5 (K99)-F F5 (K99)-R	CTG AAA AAA ACA CTG CTA GCT ATT CAT ATA AGT GAC TAA GAA GGA TGC	543
F6	F6 (987P)-F F6 (987P)-R	GTT ACT GCC AGT CTA TGC CAA GTG TCG GTG TAC CTG CTG AAC GAA TAG	463
F18	F18-1 F18-2	TGGTAACGTATCAGCAACTA ACTTACAGTGCTATTCGACG	313
F6	987P-1 987P-2	GTAACTCCACCGTTTGTATC AAGTTACTGCCAGTCTATGC	409
F4	K88-1 K88-2	GAATCTGTCCGAGAATATCA GTTGGTACAGGTCTTAATGG	499
F41	F41-1 F41-2	AGTATCTGGTTCAGTGATGG CCACTATAAGAGGTTGAAGC	612
$fliC_{H7}$	FLICH7-F FLICH7-R	GCGCTGTCGAGTTCTATCGAGC CAACGGTGACTTTATCGCCATTCC	625
$rfbE_{O157}$	RFBEO157-F RFBEO157-R	GGATGACAAATATCTGCGCTGC GGTGATTCCTTAATTCCTCTCTTTCC	213

Table 2: Primers used in multiplex PCR reactions for detection of *Salmonella*

Primer target gene	Forward(-F) and Reverse (-R) Primer Codes	Nucleotides Sequence (5'-3')	Size of Amplified DNA Fragment (bp)
Spy	TyphF TyphR	TTG TTC ACT TTT TAC CCC TGA A CCC TGA CAG CCG TTA GAT ATT	401
ViaB	ViaBF ViaBR	CAC GCA CCA TCA TTT CAC CG AAC AGG CTG TAG CGA TTT AGG	738
Sdf I	ENTF ENTR	TGT GTT TTA TCT GAT GCA AGA GG TGA ACT ACG TTC GTT CTTCTG G	304
fliC	Fli15 Typ04	CGGTGTTGCCCAGGTTGGTAAT ACTGGTAAAGATGGCT	620
sefA	A058 A01	GATACTGCTGAACGTAGAAGG GCGTAAATCAGCATCTGCAGTAGC	488

3.3 Electrophoresis analysis

Approximately 10µL of PCR products were electrophoresed through 2% ethidium bromide-stained agarose gel, followed by visualization under UV light using a transilluminator (GeneSnap Syngene). The characteristic amplification products were then identified based on their sizes estimated using a 100-bp plus DNA ladder.

3.4 Susceptibility of the bacteria to antibiotics

The bacteria isolated in this study were assessed for antibiotic susceptibility by the disc diffusion technique on Mueller-Hinton (MH) agar (Bauer *et al*., 1966). The isolates were spread on the agar and antibiotic discs impregnated with different antibiotics were placed on the agar surface followed by incubation of the plates overnight at 37 °C. This bioassay was carried out where an isolated bacterium was sub-cultured on Nutrient broth, incubated overnight at 37°C, followed by centrifugation at 3000 rpm for 20 minutes, the supernatant was discarded, and the pellet was used. The pellet was emulsified in 3mls of 0.9% physiological saline solution to make a suspension equivalent to 0.5 McFarland turbidity standards (adjusted to an OD of 0.1 at 600Nm). A fresh sterile swab was dipped into the suspension and spread evenly over the Muller Hinton agar. The inoculum was soaked in agar and was allowed to dry for 5-10 min. Antibiotics discs impregnated with different antibiotics were dispensed onto agar by use of forceps dipped in alcohol, flamed

and cooled, followed by incubation at 37 °C for 24 hours. The diameter of the zones of inhibition width (by measuring zones with a ruler) was measured and compared against a reference standard. The reference standards contain measurement ranges and with equivalent qualitative categories of susceptible, intermediately susceptible or resistant based on the diameter of the clearance zone. The basis of these categories is from recommendations by Clinical and Laboratory Standards Institute (CLSI) 2013. The following antimicrobials were used in the susceptibility assays: Ampicillin (10µg), Amoxicillin (10µg) Chloramphenicol (30µg), Streptomycin (10µg), Gentamycin (10µg), Tetracycline (30µg), Penicillin (10 units), Compound sulfonamides (300µg) and Vancomycin (3µg) (Tang *et al.*, 2009; Singh *et al.*, 2010; Chen *et al.*, 2014).

3.5 Ethical considerations

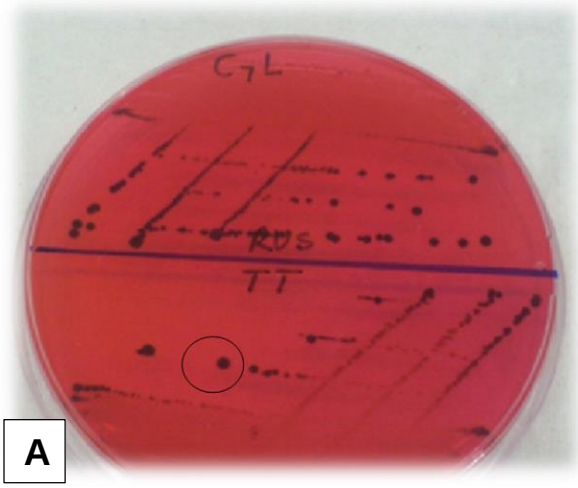
All procedures performed in this study involving animals required ethical clearance. In accordance with the ethical standards of the University of Limpopo, the proposal and application for ethical clearance were submitted to the Animal Research Ethics Committee (AREC) for ethical clearance and approved for continuation with the study.

CHAPTER 4

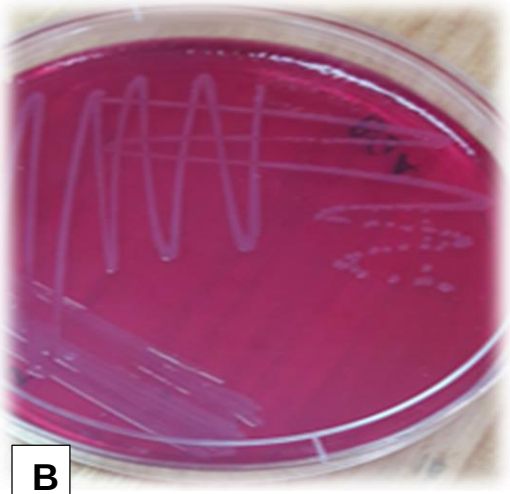
4.0 Results

4.1 Microbial analysis

Macroscopic/morphological appearance of *E. coli* and *Salmonella* on culture media is depicted in the figures below. The presence of *Salmonella* and *E. coli* in tissue samples was observed on XLD, BGA and McConkey agar. All presumptive colonies of *Salmonella spp.* appeared to be small sized, dome-shaped red colonies with a central black spot on XLD (Figure 2.1 Panel A) and pink colonies surrounded by a red medium on BGA (Figure 2.1 Panel B). Typical *E. coli* colonies, which are lactose fermenters, were indicated by bright pink halo and bile precipitant around the colonies on McConkey agar (Figure 2.1 Panel C).



A



B

Figure 2: A typical picture showing *Salmonella* species (from this study) growth appearance on XLD agar (A) and BGA agar (B) and *E. coli* on McConkey agar (C).

4.2 Identification of the isolated bacteria

Pure presumptive *E. coli* and *Salmonella* strains were confirmed by biotyping using a matrix-assisted laser desorption ionization-time of flight (MALDI-TOF). The results from this study showed that from a total of 160 tissue samples, *E. coli* strains were isolated, resulting in an overall proportion of 83% (Table 3). Positive isolates of *E. coli* were observed at 85% in breast meat, 83% in the lungs, 80% and 85% in small intestines and the large intestine respectively (Table 4). *Salmonella* was found in 3% of the chickens with none of this pathogen coming from breast meat and the small intestine, 2.5% in the lungs and 10% large intestines (Table 5).

Table 3**:** Distribution of isolated *E. coli* and *Salmonella* strains from household-raised chickens.

Isolated bacteria	Number of isolated bacteria	Percentage (%)
E. coli	133	83
Salmonella	5	3

Table 4: Distribution of *E. coli* isolates among the tissue samples.

Tissue sample	Number of *E. coli* isolates	Percentage (%)
Meat	34	85
Lung	33	83
Small intestine	32	80
Large intestine	34	85

Table 5: Distribution of *Salmonella* isolates among the tissue samples.

Tissue sample	Number of *Salmonella* isolates	Percentage (%)
Meat	0	0
Lung	1	2.5
Small intestine	0	0
Large intestine	4	10

4.3 Molecular characterization/genotyping of the isolates

All the *E. coli* isolates were tested for the presence of the following genes; *stxI, stxII, estI, astII, astA, hlyA, eae, fliC$_{H7}$ and* fimbrial genes; F18, F6, F4 and F41. Some of the *E. coli* isolates were positive for *astA*, estI*, eae, hlyA, fliC$_{H7}$* and fimbrial genes (F4 and F6) (Figure 3,4, and 5)

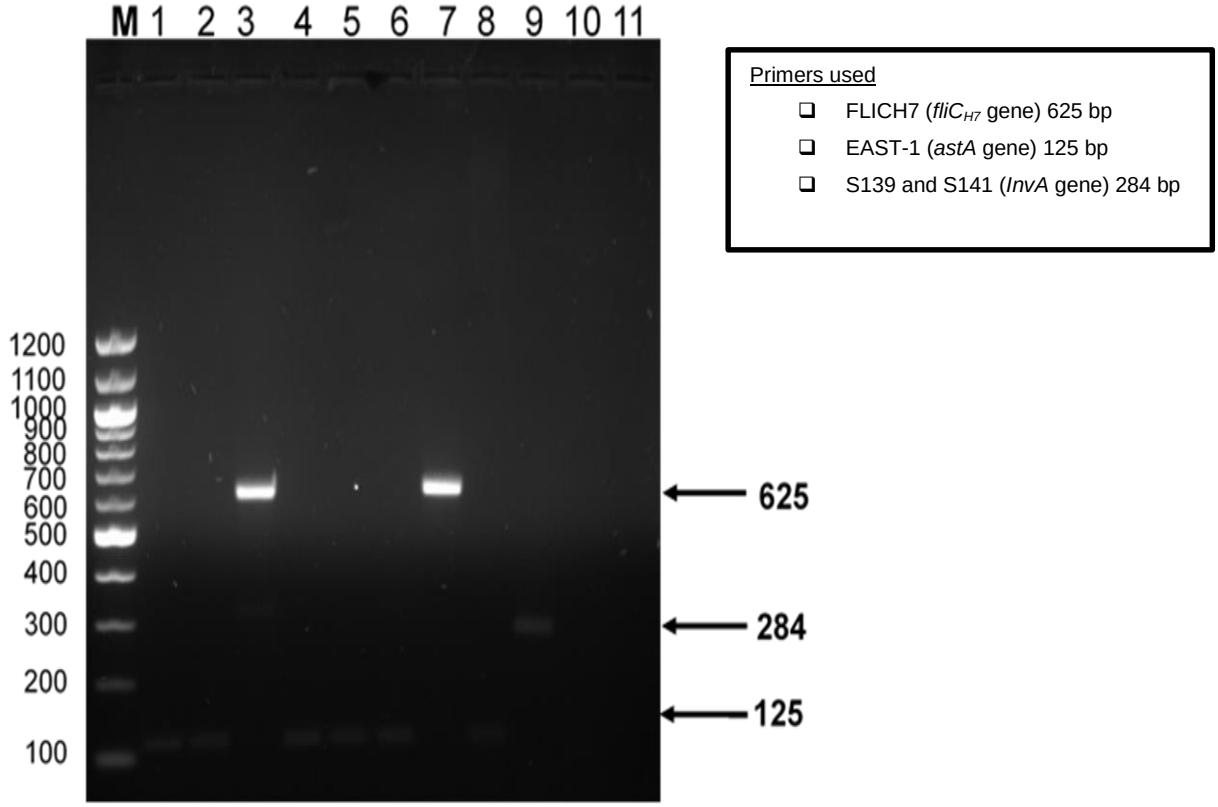


Figure 3**:** Agarose gel showing typical results from multiplex PCR. Lane M: 100 bp DNA ladder; lane 3 and lane 7: *E. coli* isolates (positive for *fliC$_{H7}$* gene with an amplicon size of 625 bp); lane 9: *Salmonella* isolate positive for the *invA* gene; lane 1, 2,4,5,6, and 8: *E. coli* isolates positive for the *astA* gene with an amplicon size of 125 bp, and Lane 10: negative control.

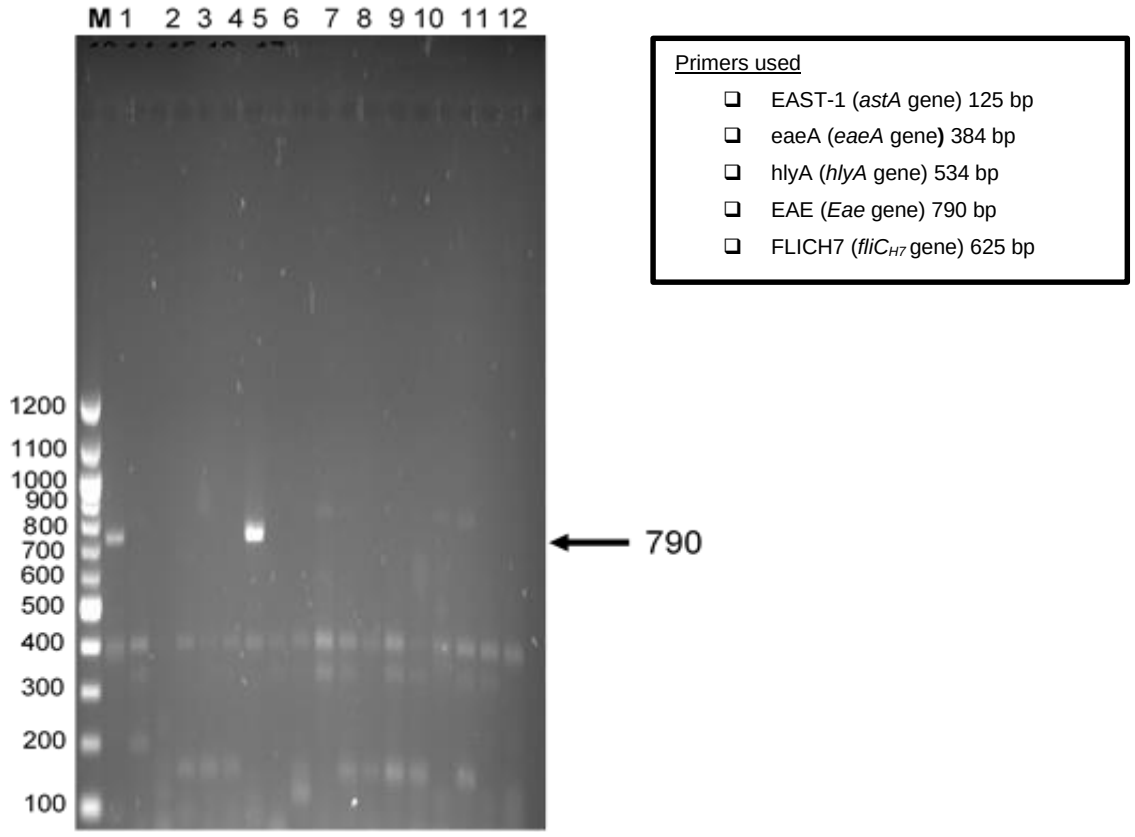


Figure 4**:** Agarose gel showing typical results from multiplex PCR. Lane M: 100 bp DNA ladder; lane 1 and lane 7: *E. coli* isolates (positive for *Eae* gene with an amplicon size of 790 bp); and Lane 17: negative control.

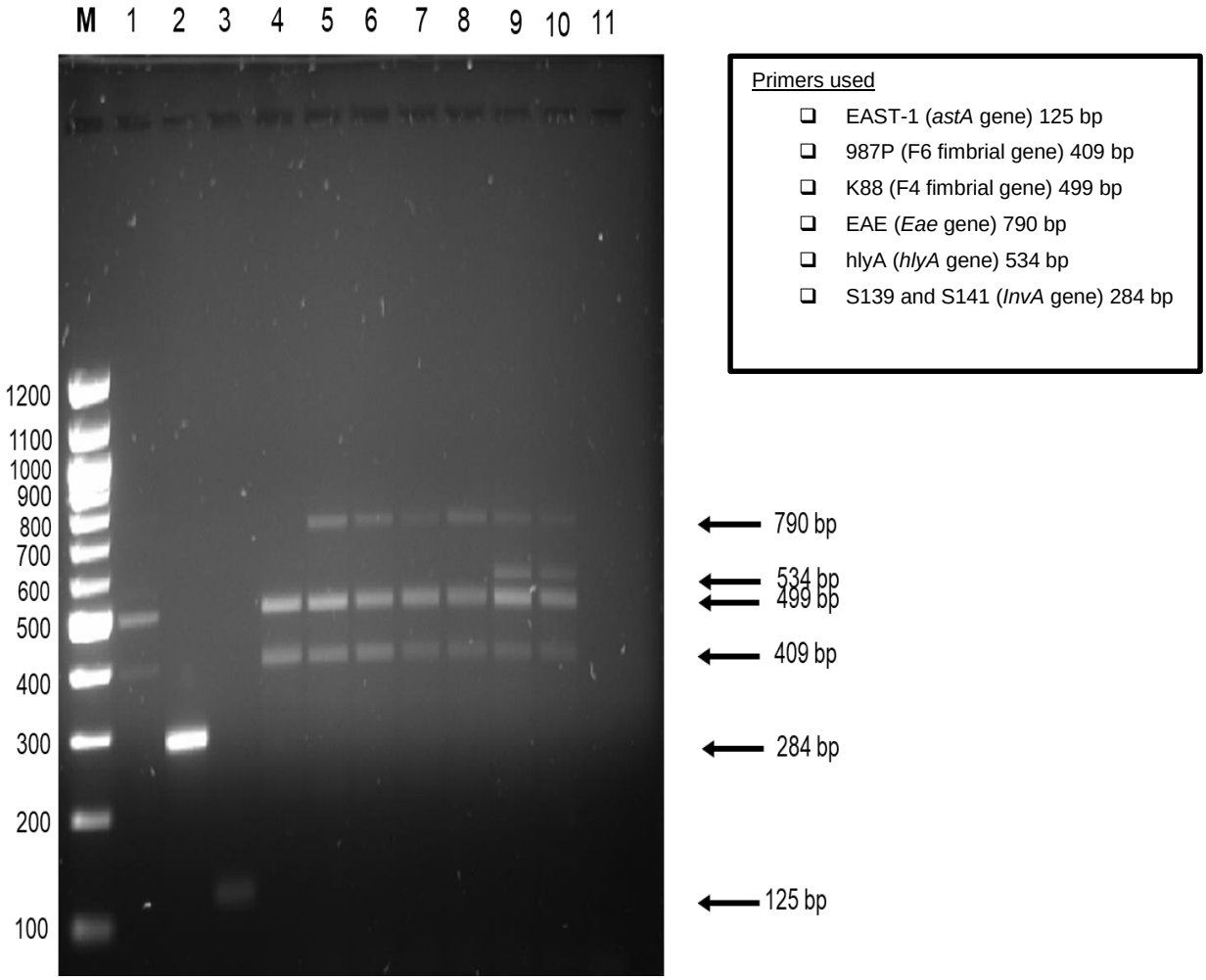


Figure 5: Agarose gel showing typical results from multiplex PCR. Lane M: 100 bp DNA ladder; lane 1: *E. coli* isolates positive for fimbrial genes of F4 and F6 with the implication sizes of 499 bp and 409 bp respectively; lane 2: *Salmonella* isolate positive for the *invA* gene with an amplicon size of 284 bp; Lane 3: *E. coli* isolate positive for the *astA* gene with an amplicon size of 125 bp; Lane 4-10: *E. coli* isolates positive for the F4 fimbrial gene with an amplicon size 499 bp and also positive for the F6 fimbrial gene with an amplicon size of 409 bp; Lane 5-10: *E. coli* isolates positive for the *eae* gene with an amplicon size of 790 bp; Lane 9 and 10: *E. coli* isolate positive for the *hlyA* gene with an amplicon size of 534 bp and Lane 11: negative control.

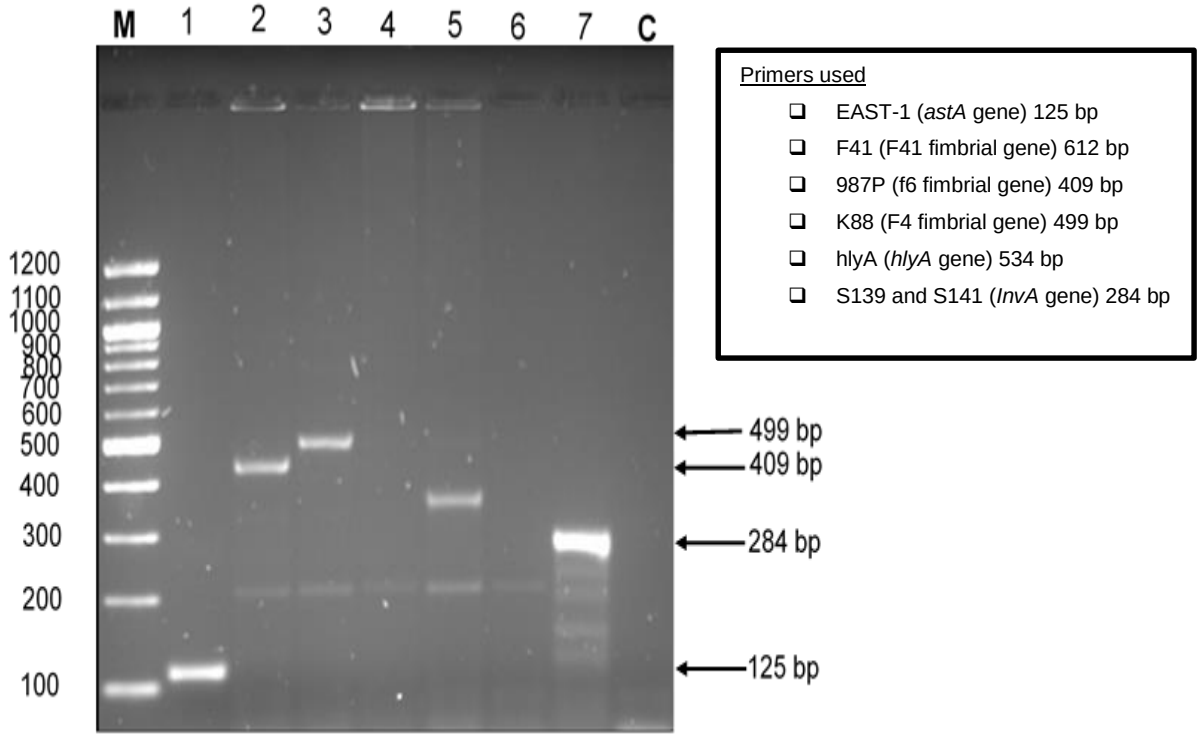


Figure 6: Agarose gel showing typical results from multiplex PCR. Lane M: 100 bp DNA ladder; lane 1: *E. coli* isolate positive for the *astA* gene with an amplicon size of 125 bp; Lane 2: *E. coli* isolates positive for the F6 fimbrial gene with an amplicon size of 409 bp; Lane 3: *E. coli* isolate positive for the F4 fimbrial gene with an amplicon size 499 bp; Lane 7: *Salmonella* isolate positive for the *InvA* gene with an amplicon size of 284 bp; and Lane 8 (labelled C) contains negative control.

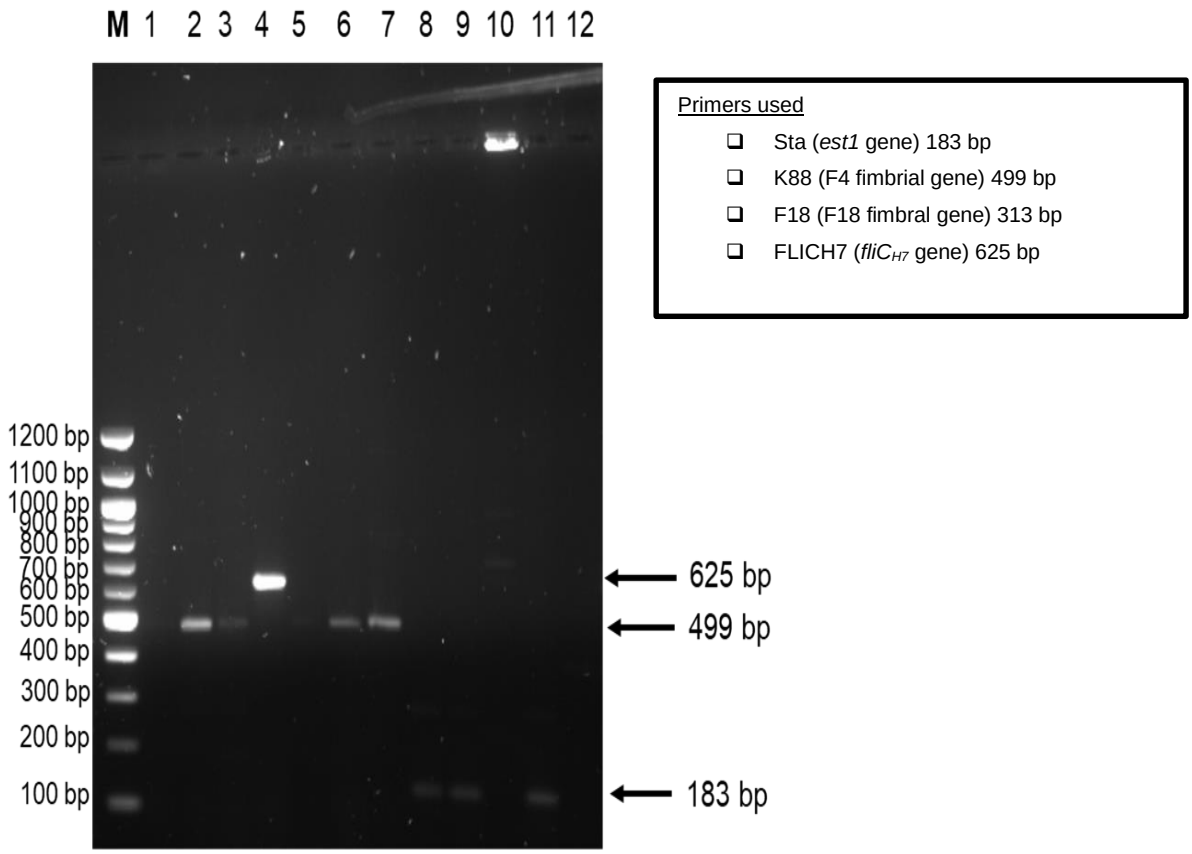


Figure 7: Agarose gel showing typical results from multiplex PCR. Lane M: 100 bp DNA ladder; lane 2, 3, 6 and lane 7: *E. coli* isolates positive for F4 fimbrial genes with an amplicon size of 499 bp; Lane 4: *E. coli* isolate positive for the $fliC_{H7}$ gene with an amplicon size of 625 bp; Lane 8, 9 and 11: *E. coli* isolates positive for the *estI* gene with an amplicon size of 183 bp and Lane 12: negative control.

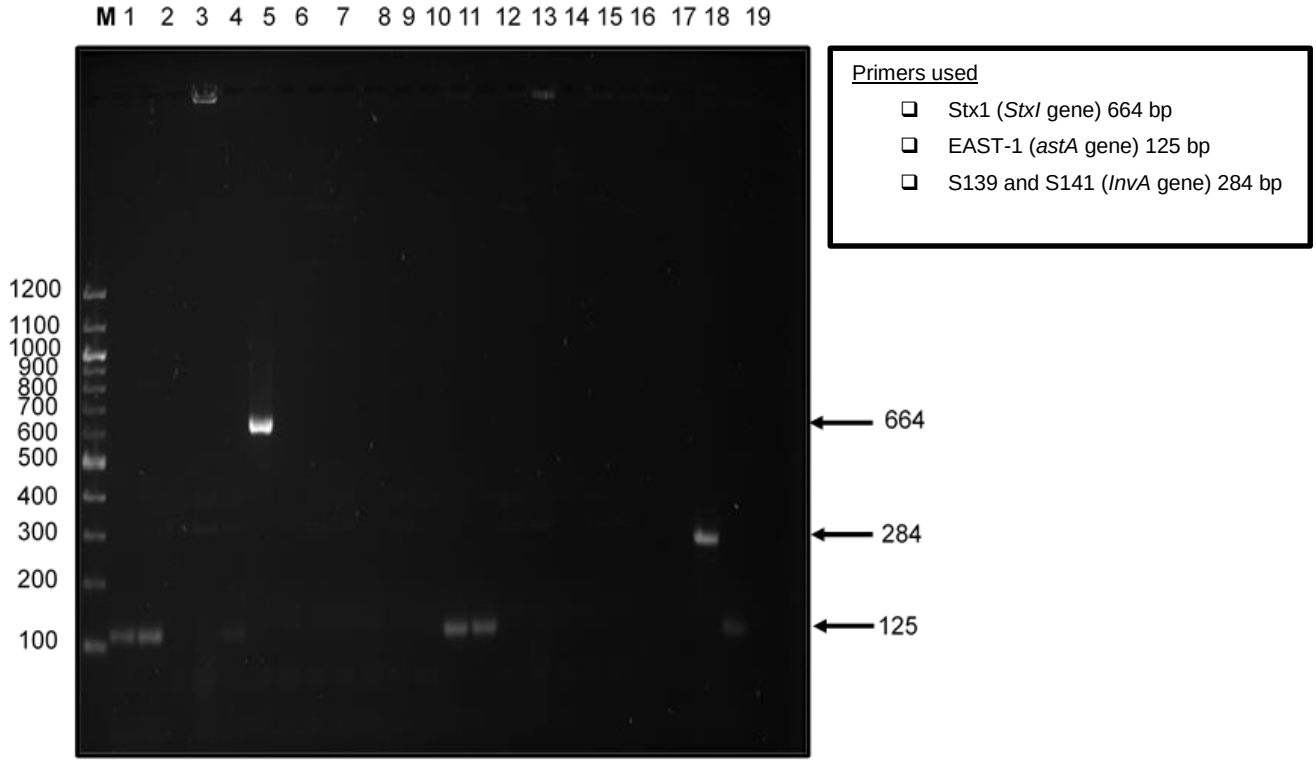


Figure 8: Agarose gel showing typical results from multiplex PCR. Lane M: 100 bp DNA ladder; lane 1; 2; 10; 11 and 19: *E. coli* isolate positive for the *astA* gene with an amplicon size of 125 bp; Lane 5: *E. coli* isolates positive for the *stxI* gene with an amplicon size of 664 bp; Lane 18: *Salmonella* isolate positive for the *InvA* gene with an amplicon size of 284 bp; and Lane 20 contains negative control.

For the confirmation of *Salmonella* using PCR, all 4 *Salmonella* isolates gave positive results with the *invA* gene, and none were positive for other tested genes (Figure 5 and 6).

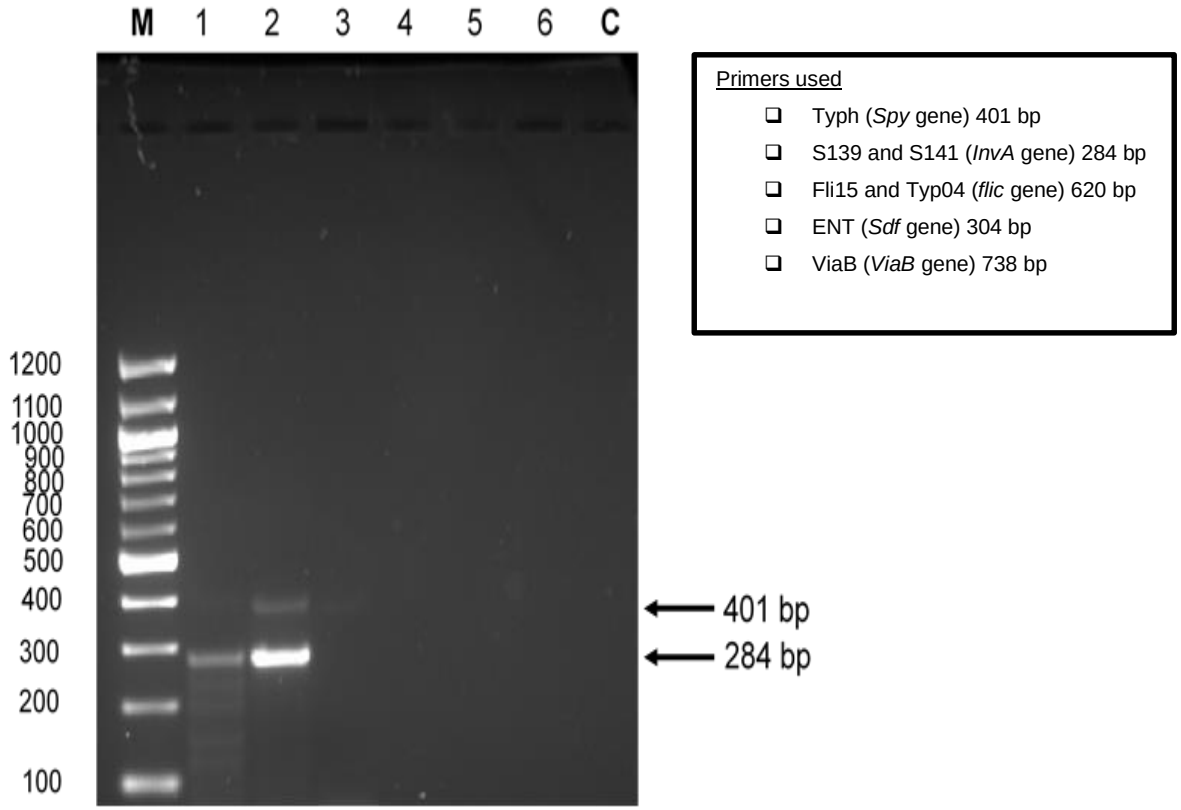


Figure 9. Agarose gel showing typical results from multiplex PCR. Lane M: DNA ladder; lane 1 and 2: *Salmonella* isolates positive for the *InvA* gene with an amplicon size of 284 bp; Lane 2: *Salmonella* isolate positive for the *Spy* gene with an amplicon size of 401 bp of the serotype *Salmonella* Typhimurium: lane 3: Negative control.

Disk diffusion method was done according to Kirby-Bauer on Muller Hinton agar to determine the antibiotic susceptibility of the isolated strains of *E. coli* and *Salmonella.*

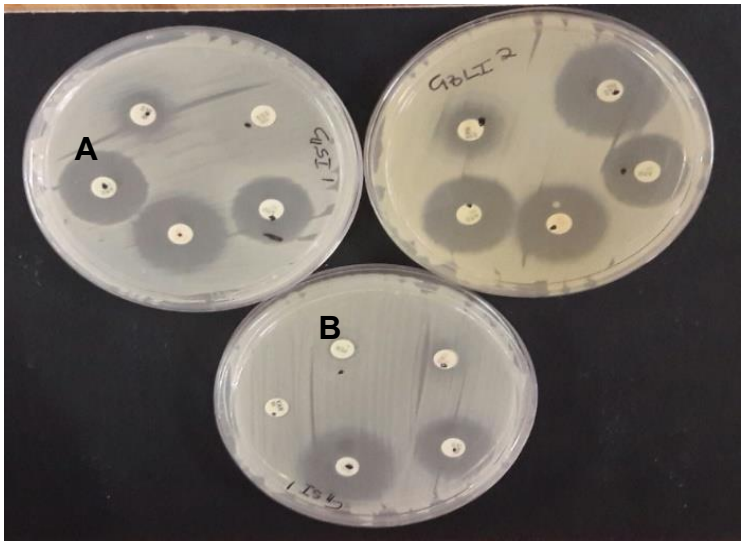


Figure 10: Mueller Hinton agar plates showing *Salmonella* and *E. coli* isolates tested for resistance to some of the ten different antibiotics used. In which clear zones around each disc are the zones of inhibition indicating the extent of the test organism's inability to survive in the presence of the test antibiotic (A) and no zone of inhibition, indicates resistance of the isolate to the test antibiotic (B).

Resistance profiles of the *E. coli* to different antibiotic groups were studied (table 5). The highest resistance by *E. coli* isolates were noted against Penicillin (100%) and Vancomycin (100%), followed by Tetracycline (56%), Amoxycillin (33%) and Ampicillin (19%) while the least resistance noted was against Gentamycin (0%), Chloramphenicol (0%) and Sulphonamide (0%) (Table 5) as shown in figure 11.

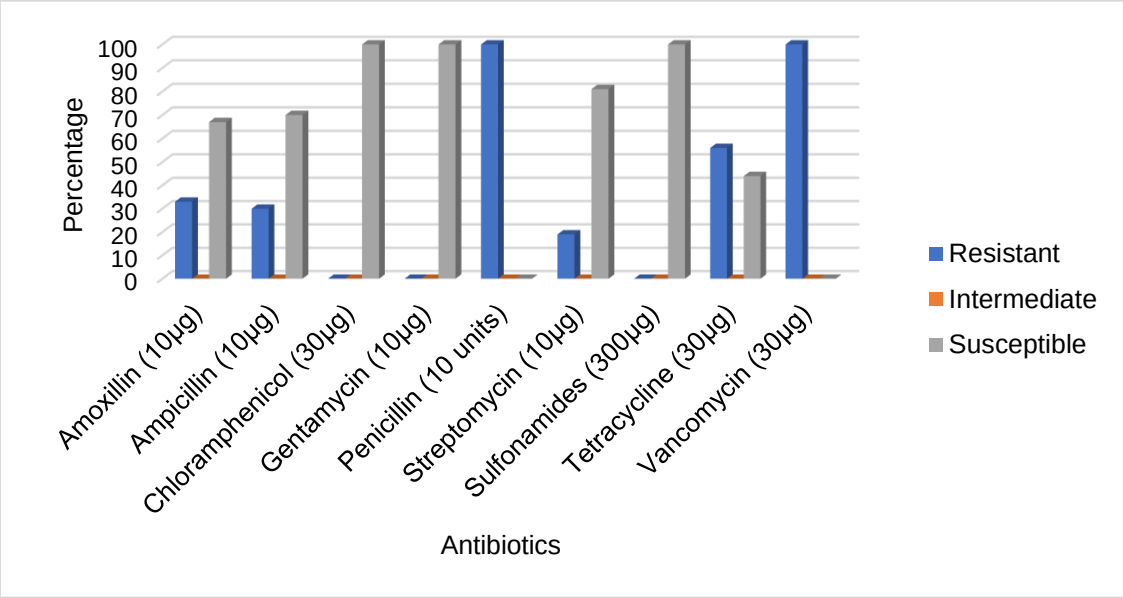


Figure 11**:** Distribution of antibiotic susceptibility profiles of *E. coli* isolated from household-raised chickens.

The highest resistance by *Salmonella* isolates, was against Penicillin (100%), Vancomycin (100%) and Gentamicin (100%), followed by Tetracycline (33%), Sulfonamides (33%), Streptomycin (16%), with the least common resistance against Chloramphenicol (0%), Amoxicillin (0%) and Ampicillin (0%) (Table 6) as shown in Figure 12.

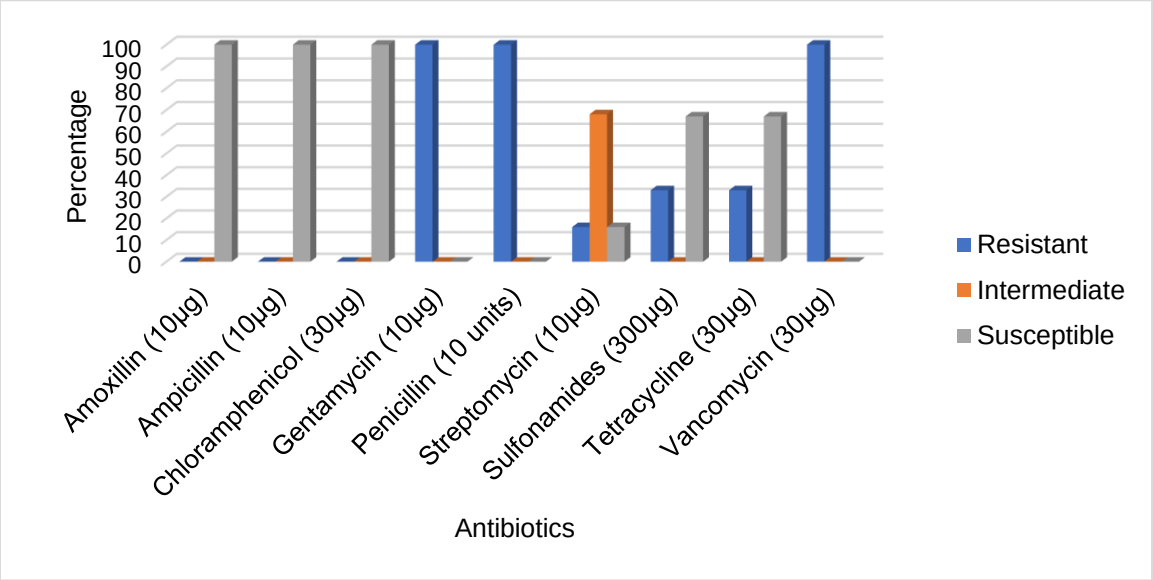


Figure 12**:** Distribution of Antibiotic resistance, susceptibility profiles of *Salmonella strains* isolated from household-raised chickens.

Table 6**:** Antimicrobial resistance profiles of pathogenic *E. coli* strains recovered from various parts of the sampled chickens.

Antimicrobial (disc concentration (µg)	Resistant	Intermediate	Susceptible
	%		
Amoxicillin (10µg)	33	0	67
Ampicillin (10µg)	30	0	70
Chloramphenicol (30 µg)	0	0	100
Gentamicin (10 µg)	0	0	100
Penicillin (10 units)	100	0	0
Streptomycin (10 µg)	19	0	81
Sulfonamides (300 µg)	0	0	100
Tetracycline (30 µg)	56	0	44
Vancomycin (30 µg)	100	0	0

Table 7**:** Antimicrobial resistance profiles of *Salmonella* strains recovered from various parts of the sampled chickens.

Antimicrobial (disc concentration (µg)	Resistant	Intermediate	Susceptible
	%		
Amoxicillin (10µg)	0	0	100
Ampicillin (10µg)	0	0	100
Chloramphenicol (30 µg)	0	0	100
Gentamicin (10 µg)	100	0	0
Penicillin (10 units)	100	0	0
Streptomycin (10 µg)	16	68	16
Sulfonamides (300 µg)	33	0	67
Tetracycline (30 µg)	33	0	67
Vancomycin (30 µg)	100	0	0

CHAPTER 5

5.0 Discussion

Salmonellosis occurs worldwide in both developed and developing countries and plays a huge role in morbidity and mortality resulting in economic loss (Obi and Ike, 2015). In the current study, *Salmonella* was isolated from household-raised chickens and was found at a rate of 5%. This low rate of *Salmonella* observed could be attributed to good hygienic conditions in the poultry environment. Obi and Ike, (2015) also reported that the low rate of *Salmonella* in their findings were probably due to improved hygienic conditions in the environment and incorporation of antimicrobials (Obi and Ike, 2015).However, in this present study, the household-raised chickens do not receive any antimicrobials for treatment of diseases or growth purposes. A lower rate of 2.5% of isolated *Salmonella* in household-raised chickens has also been reported by Salihu *et al*. (2014) in Nasawara, Northern, Nigeria. Aragaw *et al*. (2010) and Curtello, (2013) in Jamaica did not find any *Salmonella* from their chicken samples in their study.

Other studies conducted found the isolation rates of *Salmonella* to be 3.5% in Paraguay, 4% in Morocco and 15% in India (Leotta *et al*., 2010; Bouzoubaa *et al*., 1992; Samanta *et al*., 2014). The distribution of *Salmonella* in our study was low, probably since many of the sample tissues were taken from healthy chickens that showed no signs of diseases. As such, these samples had high chances of producing negative results. Furthermore, this study was based on isolation and identification of the organism as compared to other serological studies such as those conducted by Okwori *et al.* (2007).

However, a higher distribution of 51% was described in 200 chicken samples tested by amplification of the salmonella *invA* gene using PCR in South Africa and Brazil by Zishiri *et al*. (2015). In the current research, the presence of *Salmonella* was confirmed using PCR and all 5 *Salmonella* isolates gave positive results with the *invA* gene, and none were positive for other tested genes. These results are similar to the work of Oliveira *et al*. (2002), in which all the *Salmonella* isolates gave positive results in their specificity test with the *invA* primer pair.

Kabir, (2010) describe the main site of multiplication of *Salmonella* being the digestive tract (Kabir, 2010; Foley *et al.*, 2013). The high distribution of *Salmonella* in this study was in the large intestines (10%) and the lungs (2.5%). These findings revealed that large intestines were the primary target for the colonisation of *Salmonella*. Hossain *et al*. (2006) found the distribution of *Salmonella* infection in intestinal swabs of dead poultry to be 11.42%. However, they discovered that the distribution was higher in liver and ovarian samples than intestinal samples. A report published by Foley *et al*. (2014) states that there are those *Salmonella* strains that survive the low pH environment and progress to the lumen of the gastrointestinal tract (GIT) organs such as the small intestine, colon and cecum in poultry. Also, one of the reports states that survival of *Salmonella* in macrophages allows it to invade the reticulo-endothelial system (REM) and reside in the liver and spleen (Mamman *et al*., 2014), which may possibly be the reason for the presence of this organism in the liver in our study.

The distribution of *E. coli* in household chickens (83%) demonstrated in this study is related to the one reported by Sheikh *et al*. (2012) in Canada who isolated *E. coli* from retail chicken meat at a prevalence of 28%. However, Lee *et al*. (2009) reported a lower prevalence of 4.6% from poultry meat contamination in Korea. Theoretically, in poultry, *E*. *coli* resides in the lower digestive tract, where it colonizes in the first 24 hours after hatching. Many strains of this organism are harmless commensals of the gastrointestinal tract (Stromberg *et al.*, 2017). However, *E. coli* was not isolated in all the household-raised chickens in this study, as such the prevalence of 83% and not 100% was detected. However, factors such as environmental conditions, geographical regions, history of treatment with antimicrobial agents and/ or other host factors are reported to affect the distribution of the *E. coli* among animals of the same species (Amadi *et al.*, 2015; Messele *et al.*, 2017).

For the detection of virulence genes in *E. coli*, the following genes; *astA* considered to be associated with heat stable enterotoxin-1 (EAST-1) and Shigatoxin producing *E. coli* genes (*eae, eaeA, hlyA, estI* and *stxI*) were detected in some of the *E. coli* isolates recovered from the samples. No virulence genes specific for other pathotypes tested were detected. Locus of enterocyte effacement (LEE): A pathogenicity island involved in the virulence of enteropathogenic and enterohemorrhagic *Escherichia coli*, is made up of genes responsible for lesions, that involves intimate attachment of bacteria to enterocytes, as well as a signalling cascade leading to brush border and microvilli destruction, and loss of ions causing severe diarrhoea (Franzin and Sircili, 2015). These *eaeA* and *hlyA* genes detected in the study confirms the presence of the LEE pathogenicity island and the large virulence plasmid, respectively, while the detection of either stx1 or stx2 genes confirms the presence of STECs (Jamshidi *et al*., 2016).

These research findings agree with the findings of Wani *et al*. (2004) who reported none of the 426 *E. coli* isolates from faecal samples originating from chickens and pigeons in India to be positive for *stx1* and *stx2* although *eaeA* and *hlyA* genes were present at low percentages of 2.74 % and 1.74% respectively. Krause *et al*. (2005), reported 2.3% of *eaeA* positive strains from isolates which were negative for *stx* genes (Krause, Zimmermann and Beutin, 2005). Schroeder *et al*. (2003) also did not report any STEC in *E. coli* strains from retail chicken and turkey obtained from Washington DC, USA while a survey conducted by Mellata *et al*. (2001) in Algeria detected none of the intestinal avian *E. coli* investigated to harbour *eaeA* and *hlyA* genes. In contrast, Hizlisoy *et al*. (2017) found *eaeA* gene at a high percentage of 77% in their *E. coli* isolates obtained from poultry material. Also, Kobayashi *et al*. (2002) reported a higher percentage of 57% of faecal samples of contaminated chicken flocks in Finland carrying the *eaeA* gene, that were negative for the *stx* and *hlyA* genes. In South Korea, Oh *et al*. (2012) reported the presence of *eaeA* gene in *E. coli* strains obtained from the cloacal samples of chicken flocks at a percentage of 16%. This presence of genes shows clearly that intimin, hemolysin A and shiga toxins play a significant role in the virulence factors for *E. coli* infections in household-raised chickens. For *astA* gene, Mohlatlole *et al*, (2013) reports that EAST-1 is not limited to EAEC only but can be found in ETEC and EHEC.

One of the *E. coli* isolates in our study was found to contain *fliC*$_{H7}$ gene, which codes for *E. coli* O157: H7 but none were found to have *rfbE* gene when tested. Cultivation in liquid medium such as Brain Heart Infusion (BHI) broth and followed by plating on CT-SMAC may increase the number of bacteria and therefore aid in the detection of STECs which are present in low numbers or in a physiologically stressed state (Jamasidi *et al*., 2016). However, in this current study, STECS were detected by culturing on MacConkey agar followed by PCR, which could be a contributing factor to the lower results of *E. coli* O157: H7 found in one of the *E. coli* isolates. Guran *et al*. (2017) reported that the isolates that were identified as *E. coli* 0157 according to the Vitek 2 results were found to be positive for the *rfbE* gene. However, none of the isolates contained *fliC*$_{H7}$ and *hlyA* genes. Akbar *et al*. (2014) isolated *E. coli* O157 from poultry meat samples in Thailand and reported lower results of 2%. However, Jo *et al*. (2004) in their studies conducted on chicken meat samples in Korea, reported none of their isolates to be positive for *E. coli* O157. Another research conducted in Turkey on broiler chickens found liver and cecum samples to be contaminated with *E. coli* O157 at a rate of 0.1% and 0.4% respectively among 1,000 samples, while none of the *E. coli* O157 was detected in any of the broiler chicken carcass samples (Kalin, Ongor and Cetinkaya, 2012). Abdul_Raouf *et al*. (1996) from Egypt and Chinen *et al*. (2001) from Argentina reported the presence of *E. coli* O157 at a percentage of 4% in chicken samples and 10.3% in meat samples respectively.

The outcome of results obtained from multiplex PCR for fimbriae genes in this study presents evidence for the existence of F4 and F6 fimbriae genes in household-raised chickens in Limpopo Province. These fimbrial genes are mostly associated with ETEC diarrhoea in piglets, experimental animals and in the field (Mohlatlole *et al.*, 2013). The ETEC bear fimbriae F5, F6, and F41, mostly colonize the distal jejunum and ileum while that of F4 ETEC colonizes the entire jejunum and ileum (Sun and Woo, 2017).

Pasteurella multocida was not found in this current study. This means that the household-raised chickens sampled were devoid of this organism, which might be due to the low number of chickens examined in this study which did not show any signs of the disease. Although other researchers found this organism in their backyard poultry at lower

(12.41%) and higher rate (59.72%) in Gazipur Sadar and Sirajgonj district respectively (Belal, 2013; Hossain *et al*., 2017).

Looking at the distribution of these pathogens obtained in this study, most of the household-raised chickens sampled in this current study did not show any signs of diseases, which may be the reason for lower percentages or the absence of other virulent genes tested in these isolates.

The antimicrobial susceptibility data in the current study showed that household-raised chickens in Limpopo Province have *E. coli* and *Salmonella* that are resistant to various antibiotics commonly used in veterinary treatment. Resistance rates to Tetracycline (56%) and Ampicillin (19%) observed in this study is contrary to the results of other published investigations on *E. coli* strains isolated from chickens. Hamisi *et al*. (2014) revealed a higher resistance rate of 75.3% and 63.6% to Tetracycline and Ampicillin respectively, with Zinnah *et al*. (2008) reporting a resistance of 90% to Ampicillin.

Ojo *et al*. (2012), carried out a research on free range chickens in Abeokuta, Nigeria and found a higher resistance rates to Tetracycline (76.9%) and Ampicillin (92.3%). Another study carried out on commercial chickens (broilers and layers) from Grenada showed a higher resistance rate of (58.5%; broilers 66.7% and layers 37.3% (Hariharan *et al*. 2008). as compared to the resistance rate of 56% to Tetracycline observed in this study.

E. coli isolated in this study showed 0% resistance (100% susceptibility) to Chloramphenicol. These results are similar to a research work published by Amadi *et al*. (2015) that showed 0% resistance to Chloramphenicol. The findings are also related to 0.55% resistance rate reported by Hariharan *et al*. (2008). Hamisi *et al*. (2014) revealed 5.2% resistance rate to Chloramphenicol while Majalija *et al*. (2010) also reported a lower resistance to Chloramphenicol of 8% and 13%, Tetracycline 31% and 55% and Ampicillin 44% and 87.2% in *E. coli* isolated from broiler chickens in Central and Northern Uganda respectively. In contrast, a higher resistance rate of 70% to Chloramphenicol was reported by Ojo *et al*. (2012).

A complete susceptibility to Gentamicin (0% resistance) in this study agrees with the results of Manishumwe *et al*. (2017), which show a low resistance of 3.7% to Gentamicin.

Equally, a study conducted in Ethiopia revealed a lower percentage rate of resistance to Gentamicin of 0.0% (Akond *et al*. 2009).

E. coli resistance to Amoxicillin was found to be 33% in the current study, which concurs with other published by Zeryehun *et al*. (2009) and Naliaka *et al*. (2017), which revealed a higher resistance to Amoxicillin at a percentage of 56% and 66.8%, and a very high resistance of 90% to this antibiotic reported by Zinnah *et al*. (2008). *E. coli* was shown not too be susceptible to Penicillin with a 100% resistance of the organism in the present study, which is consistent to the research published by Naliaka *et al*. (2017), in which they observed resistance of *E. coli* to Penicillin at percentages of 93%. Man-made antibiotics can enter the environment in many ways, from the production of active pharmaceutical ingredients, through the excretion of residues after usage or through discarding or unused medicines. Therefore, the entire bacterial communities may become exposed to unusual antibiotic selection pressures, resulting in rapid resistance development among many pathogens (Larsson, 2014). Our results in the current study shows 33% and 100% resistance to amoxicillin and penicillin respectively, this might be due to possible exposure to antibiotic resistance strains of these organism in the environment. Apart from the dependence on household-raised chickens for food, the slaughter of chickens from other sources within the households may predispose to infection by resistance strains.
E. coli resistance to Streptomycin (19 %) was reported in this study, which is much higher than that of 10.8% reported by Ojo *et al*. (2012) in Australia. However, Akond *et al*. (2009) and Manishimwe *et al*, (2017) have reported a much higher *E. coli* resistance to Streptomycin of 70% and 78.8% in Ethiopia, Bangladesh and Rwanda respectively.

Antimicrobial susceptibility test for *Salmonella* in this study revealed 100% susceptibility to Amoxicillin, Ampicillin and Chloramphenicol, while that for Gentamicin showed a 100% resistance. Comparable results reported by Salihu *et al*. (2014) have recorded an equally high resistance of 76,1% to Gentamicin. In contrast, Obi and Ike, (2015) reported a high resistance of 100% to Amoxicillin and a high susceptibility of 100% to Gentamicin. Salihu *et al*. (2014) also reported a percentage of resistance of 65.8% Ampicillin, 66.2% Chloramphenicol and 12.9% to Amoxicillin respectively. Another study reported a complete *Salmonella* resistance (100%) to Chloramphenicol, which disagrees with our

study. The variation in resistances between findings in this study and published data can be alluded to various factors that determine the development of resistance in bacteria. The antimicrobial susceptibly data generated from the study can be a valuable reference for poultry veterinarians for treatment bacterial diseases. However, antimicrobial resistance has been identified as an emerging global issue in human and veterinary medicine both in developing and developed countries. It is also well reported that widespread use of antibiotics in agriculture and medicine is accepted as an important selective force in the high occurrence of antibiotic resistance among Gram-negative bacteria (Rasheed *et al*., 2014).

CHAPTER 6

6.0 Conclusion

Considering the presence of *Salmonella* and virulent *E. coli* in these chickens, they could pose a health hazard to other uninfected chickens around the area close by. Therefore, it is necessary to effectively prevent this contamination by educating the owners, farmers and veterinarians on the appropriate handling and storage of chickens. In other words, good hygienic practices, including good management and sanitation practices and monitoring for microbiological indicators (*Salmonella* and virulent *E. coli* as tested in this study) at their backyard (coops) should be enhanced in order minimize the risk of poultry infections and spread of resistant strains. Also, any prophylactic programs aimed at controlling *Salmonella* and *E. coli* infections must be taken into account in household-raised chickens because antibiotic administration is mostly used on commercial chickens. Advanced investigations such as serotyping to identify these strains at species level are highly recommended in the future as most were not able to be detected in this study due to limited resources. Also, more studies to distinguish virulent *E. coli* from avirulent *E. coli,* and for rapid diagnosis, ideal vaccine, development of treatment and prophylactic strategies are needed.

The presence of these major virulence genes such as *stx1, stx2, eaeA, hly* and *fliC*$_{H7}$ in *E. coli* isolates shows the significant risk of the general health of the household-raised chickens. In this current study, it was difficult to get reference strains, however, they will be used for future work.

The sampled household chickens do not receive any medication (for growth purposes or prophylaxis), the resistant bacterial contamination from the environment is the possible cause of the resistant of these bacteria. Warnings are necessary to decrease the incidence of drug resistant strains of *E. coli* and *Salmonella* in household-raised chickens. The results obtained from determining antibiotic susceptibility profiles of these pathogens, shows clearly that veterinary authorities should focus on implementing the legislation that

urges the owners to report cases of dead chickens to prevent spread of diseases to other poultry and be prepared for use of these antibiotics in the event of an outbreak.

7.0 References

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