THE INCIDENCE OF ANTIBIOTIC RESISTANT BACTERIA IN CHICKEN AND PORK

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ABSTRACT

The emergence of antibiotic resistance in important human pathogens has globally become a public health concern. Consumption of contaminated meat and meat products constitute a major route for the transmission of antibiotic resistant organisms and the dissemination of resistance genes in the human environment. The aim of this study was to determine the level of antibiotic resistance in potentially pathogenic bacteria associated with pork, chicken meat, chicken manure, chicken feed and eggs. Standard procedures were employed for the selective enrichment and isolation of *Escherichia coli, Staphylococcus, Enterobacteriaceae, Pseudomonas* and *Salmonella*, and to determine the level of their susceptibility for penicillin, oxytetracycline, tetracycline, streptomycin, as well as aminoglycoside antibiotics. It was found that 10,1% of the total number of isolates were *Pseudomonas,* 25,3% *Staphylococcus,* 21,2% *Enterobacteriaceae,* 7,0% *E. coli* and 36,4% *Salmonella.* Statistical analysis of results showed clusters of isolates exhibiting similar patterns of antibiotic resistance. Except for resistance to penicillin, *Pseudomonas* isolates were largely susceptible to the antibiotics tested. *Staphylococcus* isolates were relatively susceptible, with the highest levels of resistance, in this case to oxytetracycline and tetracycline, observed in those from pork and chicken manure. High levels of resistance to oxytetracycline (71%), tetracycline (79%), streptomycin (52%), and penicillin (100%) were detected in *Enterobacteriaceae* isolates from chicken meat samples. It was found in addition that *E. coli* from chicken meat samples 100% resistant to oxytetracycline, tetracycline, and penicillin, while *Salmonella* showed resistance to gentamycin (63%), tetracycline (46%), oxytetracycline and penicillin (99%). Indexing of multiple antibiotic resistance (MAR) confirmed the relatively high levels of resistance in *E. coli* and *Salmonella* from the chicken meat samples. Overall, results from the present study indicated that relatively high levels of resistance towards tetracycline, oxytetracycline and penicillin was observed in potentially pathogenic bacteria associated with pork, chicken meat, and the environment of the chicken industry. It was, however found that isolates from the respective bacterial groups were largely susceptible to the aminoglycoside antibiotics, as well as streptomycin and erythromycin.

OPSOMMING

Daar bestaan wêreldwyd bekommernis oor die uitwerking wat die verskyning van antibiotikumweerstandbiedendheid in belangrike menslike patogene op openbare gesondheid kan hê. Die inname van gekontamineerde vleis en vleisprodukte verteenwoordig 'n hoofroete vir die oordrag van antibiotikumweerstandbiedende organisms en die verspreiding van weerstandigheidsgene in die mens se omgewing. Die doel van hierdie studie was om die vlakke van antibiotikumweerstandbiedendheid in potensieel-patogene bakterieë wat met varkvleis, hoendervleis, hoendermis, hoendervoer en eiers geassosieer word, te bepaal. Standaard prosedures is gebruik vir die selektiewe verryking en isolering van *Escherichia coli, Staphylococcus, Enterobacteriaceae, Pseudomonas* en *Salmonella*, en om die vlakke van hul vatbaarheid vir penisillien, oksitetrasiklien, tetrasiklien, streptomisien, sowel as die aminoglikosied antibiotikums, te bepaal. Daar is in hierdie studie gevind dat 10,1% van die totale getal isolate *Pseudomonas* was, 25,3% *Staphylococcus,* 21,2% *Enterobacteriaceae,* 7,0% *E. coli* en 36,4% *Salmonella.* Statistiese ontleding van die resultate het die isolate in trosse met soortgelyke patrone van antibiotikumweerstandbiedendheid geplaas. Behalwe vir die weerstandbiedendheid teen penisillien, was *Pseudomonas* vatbaar vir die ander antibiotikums wat getoets is. *Staphylococcus* was ook vatbaar vir hierdie antibiotikums, met die hoogste vlakke van weerstandbiedendheid, in hierdie geval teen oksitetrasiklien en tetrasiklien, in isolate uit varkvleis en hoendermis. Hoë vlakke van weerstandbiedendheid teen oksitetrasklien (71%), tetrasiklien (79%), streptomisien (52%), and penisillien (100%) is egter waargeneem in *Enterobacteriaceae* isolate uit hoendervleis. Daar is verder gevind dat 100% van die *E. coli* isolate uit hoendervleis weerstandbiedendheid is teen oksitetrasiklien, tetrasiklien, and penisillien, terwyl *Salmonella* weerstandbiedendheid teen gentamisien (63%), tetrasiklien (46%), oksitetrasiklien en penisillien (99%) getoon het. Indeksering van meervoudige antibiotikum-weerstandbiedendheid (MAW) het die relatiewe hoë vlakke van weerstandbiedendheid in *E. coli* en *Salmonella* uit hoendervleismonsters bevestig. In die geheel gesien, toon die resultate van hierdie studie dat relatiewe hoë vlakke van weerstandbiedendheid teen tetrasiklien, oksitetrasiklien and penisillien in potensieel-patogene bakterieë wat met varkvleis, hoendervleis en die omgewing van die hoendervleisnywerheid geassosieer word, voorgekom het. Daar is egter gevind dat isolate verteenwoordigend van die onderskeie bakteriese groepe wel tot 'n groot mate vatbaar was vir aminoglikosiedantibiotikums, sowel as vir streptomisien en eritromisien.

CHAPTER 1

INTRODUCTION

The discovery of antibiotics with therapeutic applications in both human and veterinary medicine has been among the great achievements of our time (Vasquez-Moreno *et al.*, 1990). The modern era of antimicrobial chemotherapy began in 1929 with Fleming's discovery of the powerful bactericidal substance penicillin, and Domagk's discovery in 1935 of synthetic chemicals (sulfonamides) with broad antimicrobial activity (Todar, 1996a). In the early 1940's, spurred partially by the need for antibacterial agents in World War II, penicillin was isolated, purified and injected into experimental animals, where it was found not only to cure infections, but also to possess low toxicity towards animals (Harrison and Svec, 1998). The subsequent discovery, development, and clinical use of other antibiotics that followed this major event, resulted in the effective treatment of infection caused by major bacterial pathogens to the extent that many experts considered bacterial infectious diseases to be under complete therapeutic control (Harrison and Svec, 1998).

The emergence of antibiotic resistance in important human pathogens has globally become a continuing public health concern (Newsome *et al.*, 1987). Acquired antibiotic resistance enables pathogens to survive in the presence of antibiotics to which they were previously sensitive (Newsome *et al.*, 1987). The development of resistance in bacteria is generally related to a combination of factors including the abuse or misuse of antibiotics in human and veterinary medicine, the application of antibiotics in agriculture as growth promoters in animal feed, as well as the remarkable genetic plasticity of bacteria (Vasquez-Moreno *et al.*, 1990). Furthermore, the application of antibiotics in the treatment of viral infections, prescription by physicians of erroneous dosage, or failure of patients to complete the prescribed course are factors that probably had a major role in rendering many important antibiotics ineffective (Hardman and Limbird, 1996). Extensive use of antibiotics as the "miracle drug" to cure infection of any kind could have created a selective pressure for microorganisms to develop mechanisms to escape the inhibitory effect of the antimicrobial agents. The injudicious use of antibiotic treatment may thus lead to the selection of resistance in the target organism that causes the infection and against which antibiotic therapy was directed (Lerner, 1998).

In livestock and poultry production sub-therapeutic amounts of various antibiotics are used in animal feed mixtures for growth promotion, improved feed efficiency and for the control and prevention of diseases (Vasquez-Moreno *et al.,* 1990; Schwarz and Chaslus-Dancla, 2001). Various studies have reported that a reservoir of resistant microorganisms exists in food animals that were fed antibiotic-containing feed while being raised under intensive growth conditions (Newsome *et al,* 1987). In addition, the widespread use of antibiotics in veterinary practice is believed to be a major factor contributing to the establishment of a reservoir of antibiotic resistant enteric pathogenic and/or non-pathogenic bacteria in animals. These bacteria may transfer their resistance genes to unrelated human and animal pathogens (Newsome *et al.,* 1987; Schwarz and Chaslus-Dancla, 2001).

Meat and meat products contaminated with antibiotic resistant organisms constitute the primary means by which antibiotic resistant organisms are transmitted to humans (Mattila *et al.,* 1988). Humans may also acquire antibiotic resistant bacterial strains from environments where food animals were raised intensively on antibiotic-containing feed. Direct exposure to the manure, or contact with pets, rodents, flies and cockroaches that were exposed to the waste of these animals also constitute a possible route for the transmission of resistant bacteria to humans (Feinman, 1999).

According to Schwarz and Chaslus-Dancla (2001), the emergence and spread of antimicrobial resistance among human pathogens could be significantly reduced if published guidelines aimed at promoting the prudent and judicious use of antibiotics were followed in both medical and veterinary practice, as well as by agriculture and manufacturers of animal feed.

Because commercial feed used in the large scale production of chickens and pigs intended for human consumption probably contain various types of antibiotics in varying degrees, and fed animals raised under these conditions could be a factor contributing to the widespread problem of antibiotic resistance in modern society, the present study was designed to screen for potential human pathogenic bacteria from different settings of the chicken and pork industry. Standard procedures were employed for the isolation of *Pseudomonas*, *Staphylococcus*, *Enterobacteriaceae*, *Escherichia coli* and *Salmonella* from feed and manure samples collected from different farm settings, as well as meat products and eggs obtained from different retail outlets. Isolates were subsequently subjected to standard procedures to

determine the level of antibiotic resistance in the different bacterial groups and to compare the various samples in this regard.

In the present study, the number of isolates of the respective bacterial groups obtained from chicken meat was comparable to that from pork. The highest number of isolates picked of from the selective mediums was *Salmonella* organisms, followed by *Staphylococcus aureus*, *Enterobacteriaceae, Pseuomonas* and *Escherichia coli* organisms. *Salmonella* in particular, exhibited high resistance to three antibiotics with the highest MAR index followed by *Escherichia coli*. Findings from the present study seem to be in consensus with literature reports in suggesting that food animals may serve as reservoirs of resistant enteric bacteria from which antibiotic resistance factors could be disseminated into the environment.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

Antibiotics are chemical compounds produced as secondary metabolites of microbial metabolism. Although many antibiotics used today are produced by microorganisms, some are manufactured partly or entirely by chemical synthesis. The term antimicrobic is often used to include agents produced entirely by microorganisms, as well as commercial antibiotics that have been chemically altered to improve potency or to increase the range of species they affect (Atlas, 1997; Elliot *et al.*, 1997; Jacob, 1999).

Due to their antimicrobial activity, antibiotics are widely applied in medicine as chemotherapeutic agents to treat bacterial infections. Antibiotics act on bacteria by interfering with essential biological processes, such as cell wall synthesis, DNA replication and transcription of protein (Atlas, 1997). Bacteria vary in their sensitivity to various antibiotics. Bacteria in which the structure or function targeted by a specific antibiotic, will exhibit an intrinsic or natural resistance to the action of the specific drug. Due to their genetic plasticity, however, bacteria may also acquire antibiotic resistance after having been exposed to low levels of the drug over an extended period of time. Some antibiotics are referred to as narrow-spectrum, because they are very specific in their action and may target Gram-negative bacteria only, as opposed to wide-spectrum antibiotics that are effective in the treatment of infections caused by a wide range of bacteria, including both Gram-negative and Gram-positive bacteria (Brock *et al.*, 1994; Lancini *et al.*, 1995).

2.2 Medical, veterinary and agricultural uses of antibiotics

Antibiotics are used to treat diseases of microbial origin in both humans and animals. Their application in veterinary medicine, however, has been confined mainly to those diseases in animals that cause major economic losses, such as bovine mastitis (Atlas, 1997). Antibiotics

probably find its most important application in the treatment of infectious human diseases and these agents accounts for roughly half the antibiotics consumed every year (Jacob, 1999). Antimicrobials used in both animal or human medicine include amikacin, ampicillin, amoxicillin-clavulanic acid, apramycin, ceftiofur, cefriaxone, cephathin, chloramphenicol, ciprofloxacin, gentamycin, kanamycin, nalidixic acid, streptomycin, sulfamethoxazole, tetracycline, trimeyhoprim-sufamethoxazole and ticarcillin (Torrence, 2001).

In addition to its medical applications, a variety of antibiotics are used prophylactically and as growth promoters in commercial feed formulations used in food animal production. However, concern has repeatedly been expressed by public health authorities, infectious disease specialists and plasmid biologists that the practice of using antibiotics routinely at subtherapeutic levels in animal feed has contributed to the establishment of a reservoir of resistant zoonotic bacteria, including *Salmonella* and coliforms, to which humans may become exposed (DuPont and Steele, 1987; Teuber, 2001; Torrence, 2001).

2.3 Classes of antibiotics based on the mechanism of their antimicrobial action

Most of the therapeutically useful antimicrobial agents are effective against bacterial infections because of significant differences between the prokaryotic cells of the infecting bacteria and the eukaryotic cells of the infected human or animal. Various sites in a bacterial cell that are targeted by antibiotic action are absent in eukaryotic cells so that selective toxicity for bacteria can effectively be achieved. Antibiotics typically prevent bacterial reproduction by entering the bacterial cells and interfering with the production of components needed to form new bacterial cells (Jacob, 1999). Structures and macromolecules of prokaryotic cells that serve as potential targets for antimicrobial action include cell walls, ribosomes for protein synthesis, membranes and nucleic acids (Elliot *et al.,* 1997). Based on their mode of action and the specific structures they target in the bacterial cell, antibiotics can be arranged in five major groups (Atlas, 1997). The different classes of antibiotics include agents that affect (i) cell wall synthesis, (ii) membrane integrity, (iii) protein synthesis, (iv) DNA replication and (v) important methabolic pathways (Katzung and Trevor,1998). The different antibiotic classes, and the mode of action of

different chemotherapeutic agents belonging to each class, are discussed in the subsequent paragraphs.

2.3.1 Agents that inhibit the synthesis of the bacterial cell wall

Unlike mammalian cells, bacteria have cell walls. Some antimicrobial agents, such as the β-lactam and glycopeptide antibiotics, act selectively on the bacterial cell wall (Elliot *et al.*, 1997). For normal growth and development, the formation of peptide cross-linkages within the peptidoglycan backbone is essential to maintain the rigidity of the bacterial cell wall. This transpeptidation reaction is catalysed by certain enzymes collectively known as penicillin-binding proteins (PBPs). Beta-lactam antibiotics act by inhibiting the carboxypeptidase and transpeptidase enzymes that are required for the formation of cross-linkages to form between the peptidoglycan chains (Mandell and Petri, 1996; Atlas, 1997; Elliot *et al.,* 1997; Bush and Mobashery, 1998). Glycopeptides, such as the antibiotic vancomycin, appear to inhibit both transglycosylation and transpeptidation reactions during peptidoglycan assembly (Mims *et al.*, 1993).

Penicillins have been shown to be most effective against Gram-positive cocci, *Neisseria* and *Treponema pallidum,* but not against Gram-negative pathogens such as *Salmonella, Shigella* and *Pseudomonas* and the Gram-negative organism, *Neisseria gonorrhoeae* (Todar, 1996a; Elliot *et al.*, 1997). *Chlamydia* and *Mycoplasma* species are insensitive to the action of penicillin and cephalsporins because they lack peptidoglycan-containing cell walls. Bacteria resistant to penicillins most often produce enzymes that are collectively referred to as β-lactamases because of their ability to open the β-lactam ring of penicillins (Neu, 1976; Atlas, 1997).

In contrast to penicillins, cephalosporins are broad spectrum antibiotics relatively resistant to penicillinase, and therefore these drugs have become useful chemotherapeutic agents in the treatment of infections caused by either Gram-positive or Gram-negative bacteria (Atlas, 1997). The cephalosporin, cefamandole, is widely used in the treatment of infections caused by strains of *Klebsiella pneumoniae,* causing pneumonia and urinary tract infections (Atlas, 1997). Most Gram-positive cocci, with the exception of enterococci and methicillin-resistant *Staphylococcus*

aureus, are susceptible to cephalosporins (Mandell and Petri, 1996). Cephalosporins are used as alternatives to penicillins for patients who are allergic to penicillin and for those pathogens that are not penicillin sensitive. The cephalosporins may be used also in place of penicillins for the prophylaxis of infection by Gram-positive cocci following surgical procedures (Atlas, 1997).

2.3.2 Agents that act directly on the cell membrane of microorganisms

Polipeptide antibiotics are chemotherapeutic agents that target the microbial cell membrane. These include polymyxins, polyenes and imidazoles that damage or disorganise the structure or inhibit the function of the bacterial membrane. The integrity of the cytoplasmic membrane is vital to bacteria, and compounds that disorganise the membranes, resulting in loss of cytoplasmic content, rapidly kill bacterial cells. Antibiotics of clinical importance that act by this mechanism are the polymyxins produced by *Bacillus polymyxa* (Todar, 1996a). Polypeptide antibiotics disrupt the phospholipids that make up the structure of the membrane whereas polyene acts on the sterol components of the cell membrane. The antimicrobial activities of polymyxin b and colistin are similar and are restricted to Gram-negative bacteria such as *Enterobacter, Escherichia coli, Klebsiella, Salmonella, Pasteurella, Bordetella,* and *Shigella* (Mandell and Petri, 1996).

2.3.3 Agents that bind to ribosomal subunits causing inhibition of protein synthesis

The selectivity of this class of antibiotics is based on their ability to target either the 30S and 50S subunits of 70S bacterial ribosomes, leaving human ribosomes consisting of 80S subunits unaffected (Elliot *et al.*, 1997). Many therapeutically useful antibiotics owe their antibacterial action to the inhibition of some step in the complex process of protein synthesis (Todar, 1996a). The most important antibiotics with this mode of action are the tetracyclines, chloramphenicol, and the macrolides (Elliot *et al.*, 1997; Katzung and Trevor, 1998).

According to Elliot and coworkers (1997) chloramphenicol binds to the 50S subunit and interferes with the linkage of amino acids in the peptide chain formation, or combines with the bacterial ribosome to prevent the assembly of amino acids into a protein chain. Chloramphenicol

is active against many species of Gram-negative bacteria. Chloramphenicol is used for treating typhoid fever and various infections caused by *Salmonella.* Chloramphenicol is also effective against rickettsia and are used for the treatment of typhus fever caused by this agent, as well as conditions caused by anaerobe pathogens, e.g *Bacteriodes fragilis* (Elliot *et al.*, 1997).

Tetracyclines bind to the 30S subunit, preventing binding of aminoacyl transfer RNA to the acceptor site in the ribosome, thereby inhibiting amino acid chain elongation (Elliot *et al.*, 1997). At least two processes appear to be required for these antibiotics to gain access to the ribosomes of Gram-negative bacteria, namely (i) passive diffusion through the hydrophilic channels formed by the porin proteins of the outer membrane and (ii) active transport by an energy-depending system that pumps all tetracyclines through the inner cytoplasmic membrane (Mandell and Petri, 1996). Tetracyclines have a broad spectrum of activity against many Gram-positive and some Gram-negative bacteria (Elliot *et al.,* 1997). Tetracyclines are effective against various pathogenic bacteria, including *Rickettsia* and *Chlamydia* species. Tetracyclines are useful also against various other bacterial infections, including *Mycoplasma pneumoniae* and the causative agents of brucellosis, tularemia and cholera.

The macrolide antibiotic, erythromycin, exert it's action on the bacterial cell by binding to the 50S subunit of ribosomal RNA and inhibiting the formation of the initiation complex (Elliot *et al.*, 1997). Although not active against most aerobic Gram-negative rods, erythromycin does exhibit antibacterial activity against some Gram-negative organisms including *Legionella pneumophila, Mycoplasma pneumoniae, Corynebacterium* and *Chlamydia trachomatis* (Atlas, 1997). Erythromycin may serve as an important alternative treatment for streptococcal infections in patients who are allergic to penicillin, e.g. infection caused by *Streptococcus pyogenes* (Mandell and Petri, 1996; Todar, 1996a).

2.3.4 Agents that bind to ribosomal subunits and alter protein synthesis

Aminoglycoside antimicrobial agents include chemotherapeutic agents such as streptomycin, gentamicin, neomycin, kanamycin, tobramycin, and amikacin. After entering the bacterial cell, binding of the aminoglycoside to the 30S ribosomal subunit of the 70S bacterial ribosomes

interferes with the formation of the initiation complexes, the first step in the translation genetic code, thus affecting the fidelity of translation into a protein (Mims *et al.*, 1993; Atlas, 1997). These antimicrobics are used almost exclusively in the treatment of infections caused by Gram-negative bacteria. Aminoglycosides are relatively ineffective against anaerobic bacteria, facultative anaerobes and Gram-positive bacteria (Elliot *et al.*, 1997).

2.3.5 Agents that affect nucleic acid metabolism

The nucleic acids, DNA and RNA, of the bacterial cell are informational macromolecules that carry important biological information, and the means to process this information, in the highly specific sequence of the amino acids they are composed of (Brock *et al.*, 1994; Atlas, 1997). Some antibiotics have the ability to interfere with some processes involved in nucleic acid biosynthesis, whether at the stage of nucleotide biosynthesis, or nucleotide polimerization (Betina, 1983). The quinolone antibiotic, nalidixic acid, interferes with the DNA gyrase, preventing the formation of a replication fork, an essential step in the replication of DNA needed for cell proliferation. Although DNA synthesis is blocked, transcription and translation can still proceed (Betina, 1983; Mann and Grabbe, 1996; Atlas, 1997). Quinolones are effective against a broad range of Gram-positive and Gram-negative bacteria including the mycobacteria (Mims *et al.*, 1993).

Rifampin, a semi-synthetic derivative of rifamycin B, inhibits DNA-dependent RNA polymerase of mycobacteria and other microorganisms by forming a stable drug-enzyme complex, leading to the suppression of initiation of chain formation in RNA synthesis (Mandell and Petri, 1996). Rifampin is used in combination with other antibiotics in the treatment of *Mycobacterium tuberculosis* (Elliot *et al.*, 1997). Rifampin inhibits the growth of most Gram-positive bacteria as well as many Gram-negative microorganisms such as *Escherichia coli, Pseudomonas,* indole-positive and indole-negative *Proteus,* and *Klebsiella.* Rifampin is very active against *Staphylococcus aureus* and coagulase-negative staphylococci (Mandell and Petri, 1996).

2.3.6 Agents that act as nucleic acids analogues

Nucleic acid analogues such as zidovudine, ganciclovir, vidarabine and acyclovir inhibit viral replication by affecting enzymes that are essential for viral DNA synthesis (Mandell and Petri, 1996). Acyclovir is an acyclic guanine nucleoside analogue that lacks a 3'-hydroxyl on the side chain. The clinically useful antiviral spectrum of acyclovir is limited to Herpes viruses. By a mechanism termed suicide inactivation, the terminated DNA template containing acyclovir binds to the enzyme and leads to irreversible inactivation of the DNA polymerase (Mandell and Petri, 1996). Ganciclovir is an acyclic guanine nucleoside analogue. This agent has inhibitory activity against all herpes viruses but especially against cytomegalovirus (CMV) (Mandell and Petri, 1996). Vidarabine is an adenosine analogue with an altered sugar, and although this drug was originally developed for the treatment of leukaemia, it has proven to be more effective in treating herpes simplex virus (HSV), encephalitis and keratoconjunctivitis. Intravenous vidarabine is approved for use in HSV encephalitis, neonatal herpes, and zoster or varicella in immunocompromised patients, but can be replaced by acyclovir for these applications (Mandell and Petri, 1996; Atlas, 1997). Zidovudine is a thymidine analogue with antiviral activity against HIV-1, HIV-2, human T lymphotropic (or leukaemia) virus and other retroviruses. Following diffusion into host cells, the drug is initially phosphorylated by cellular thymidine kinase (Mandell and Petri, 1996). Being an analogue of the DNA base thymidine, azidothymidine triphosphate (AZT) inhibits viral reverse transcriptase thus terminating DNA chain elongation prematurely (Mims *et al.*, 1993).

2.3.7 Agents that interfere with important metabolic pathways

Folic acid, consisting in part of para-aminobenzoic acid (PABA) has an important role as an essential co-substrate in the biosynthesis of amino acids, purines and pirimidines. In contrast to mammalian cells, bacteria are responsible for synthesising the required amount of folic acid for themselves. The antimicrobial action of drugs such as sulphonamides and trimethoprim is due to their ability to disrupt the folic acid metabolism in bacterial cells (Atlas, 1997). Because folic acid cannot take up PABA in the presence of sulphonamides, dysfunctional molecules that are unable to perform their essential metabolic functions are formed as a result. The one-carbon

transfer required for the synthesis of thymidine and purines does not occur in the presence of thrimethoprim, an antimicrobial agent that prevents the transformation of dihydrofolic acid (DHF) into tetrahydrofolic acid (THF) (mann and Grabbe, 1996).

Many Gram-positive cocci, including *Staphylococcus aureus,* streptococci and to a variable extent the enterococci, are susceptible to trimethoprim. The enterobacteria, including *E. coli* and *Salmonella* species, are also sensitive to the action of trimethoprim. The wide-spectrum activity of trimethoprim favors the application of this drug in the treatment of gastroenteritis as well as respiratory and urinary tract infections caused by susceptible organisms. Sulphonamides and trimethoprim often are used in combination to combat bacterial infections (Mann and Grabbe, 1996; Atlas, 1997).

2.4 Non-medical uses of antibiotics

Statistics for the year 1997, released by the Federation of Animal Health (FEDESA), indicated that over 11.5 x 10^6 kg of antibiotics were used for animal growth promotion in the European Union countries and Switzerland (Ungemach, 2000). Antibiotics are also employed in the preservation of human food in order to reduce human risk of being exposed to pathogenic microorganisms (Khachatourians, 1998). Food is an excellent substrate for the growth of microorganisms, and consumption of food contaminated with pathogens could put humans at risk of food poisoning, or disease outbreaks of epidemiological proportions (Frazier and Westhoff, 1996). To ensure food safety, antimicrobials such as tetracycline and natamycin are often used as food preservatives (Jay, 1992).

2.4.1 The use of antibiotics in animal feed

In the 1940s researchers at Lederle Laboratories (USA) seeking nutritional factors that would improve the growth rate of animals raised for human consumption, discovered that small amounts of the antibiotic chlorotetracycline significantly improved the growth of food animals. An entirely new industry was generated which opened a large new market for pharmaceutical companies manufacturing antibiotics (Harrison and Svec, 1998). In agriculture, low levels of

various antibiotics are presently added to animal feeds to promote growth and improve the efficiency of feed conversion into meat (Moro *et al.*, 1998). According to Du Pont and Steele (1987), the addition of subtherapeutical amounts of certain antimicrobial agents to animal feed, not only prevents infectious diseases caused by bacteria or protozoa, but also decreases the amount of feed needed while increasing the rate of weight gain. In 1948, it was noted that chlorotetracycline containing vitamin B12 promoted the growth of chickens even when chicken feed contained more than sufficient amounts of all known vitamins (Cooke, 1974). The addition of tetracycline or penicillin to commercial swine and poultry feeds at the rate of 5 to 20 grams per ton of feed, was found to have increased the growth rate of young animals by at least 10% and sometimes more. This probably occurred because the added drugs destroyed pathogenic bacteria and intestinal parasites that could cause mild forms of disease that affect the growth and development of young animals (Pelczar *et al.*, 1993; Khachatourians, 1998). Antibiotics in animal feed could also improve the performance of animals under conditions of stress such as poor ventilation or overcrowding during transit. Chronic respiratory disease in poultry, scouring in pigs, and chronic diarrhoeal disease in pigs commonly occur under these conditions (Cooke, 1974). The use of subtherapeutic levels of antimicrobial agents is one of the tools that had facilitated confinement housing, allowing larger numbers of animals to be maintained in a production facility of a given size. The practice of adding subtherapeutical amounts of antibiotics to the feed of food animals probably contributes to lower costs of animal care and ultimately could lower costs to the consumers of meat, milk and eggs (Du Pont and Steele, 1987). However, a major concern about the use of antimicrobial agents to raise food animals is the possibility that illegal antibiotic residues of the agents may be found in meat, especially liver or kidney. Whether or not the drug to which the animals were exposed will reach the consumer, depends upon a number of factors including (i) the specific drug involved, (ii) its absorbability and pharmacokinetics, (iii) the interval from administration of the last dose of the drug until slaughtering, (iv) the tissue to be eaten and (v) the degree of cooking of the meat (Du Pont and Steele, 1987; Harrison and Svec, 1998).

2.4.2 The use of antibiotics in food preservation

Upon prolonged storage, even at refrigerator temperatures, microbial spoilage of food occurs. According to Jay (1992), internal temperatures of food are not reduced to within the refrigerator range, and spoilage that is likely to occur is caused by bacteria such as from *Clostridium perfringens* and genera of the *Enterobacteriaceae* family, originating from internal sources. Bacterial spoilage of refrigerated-stored meats may also be reflective of external conditions and sources of contamination. With respect to fungal spoilage of fresh meats, especially beef, a diverse range of molds have been recovered from various spoilage conditions of whole beef including *Thamnidium, Mucor,* and *Rhizopus,* molds that typically produce "whiskers" on beef. *Cladosporium* produces black spots, *Penicillium* green patches, and *Sporotrichum* and *Chrysosporium* white spots on the meat. Molds apparently do not grow on meat if the storage temperature is below –5 °C. Among the yeast genera associated with spoiled beef, *Candida* and *Rhodotorula* are well known (Jay, 1992).

The skin of live birds, as well as the feet, feathers and faeces contain a variety of microorganisms. Contamination of poultry usually occurs during washing, plucking and evisceration (Frazier and Westhoff, 1996). Whole poultry tends to have a lower microbial count than cut up poultry (Jay, 1992). Freshly laid eggs, although sterile inside, become contaminated on the outside by faecal matter from the hen, cage, nest, wash-water if the eggs are washed and by handling (Frazier and Westhoff, 1996). Fresh eggs may exhibit cracks and any breaks in the shell or dirt on the egg will favour spoilage on storage or transmission of pathogenic bacteria to the consumer (Frazier and Westhoff, 1996). Infected eggs can also contribute to the problem of meat contamination with potential human pathogens, since infected chicks hatched from lightly infected eggs may survive and grow into birds that continue to excrete salmonellae (Jay, 1992).

Preservation methods that have been developed to reduce the risk of food-borne outbreaks of infectious diseases include physical procedures such as irradiation, freezing, vacuum packaging or chilling (Frazier and Westhoff, 1996). Food can also be preserved using chemicals such as benzoic acid, the parabens, sorbic acid, nitrites or nitrates, sulphites or sulphur dioxide, or by increasing carbon dioxide concentrations. Nisin, a bacteriocin produced by some strains of

Lactococcus lactis, as well as antibiotics such as tetracycline, natamycin and subtilins, are often applied to preserve food (Jay, 1992).

2.5 Bacterial resistance to antibiotic action

According to Chubb (2000) infectious diseases are the third leading cause of death in the United States and the number one cause worldwide. For several decades following their discovery in the 1940's, antibiotics have been most reliable as drugs to control infectious diseases. The development of antimicrobial agents for clinical use has brought unquestionable benefits for the treatment of individual as well as community-acquired infections, because many infections that formerly were frequently fatal, have since become routinely curable. However, it soon became apparent that the frequent use of antibiotics have lead to the selection of resistance in organisms causing infection and against which antibiotic therapy was directed (Lerner, 1998). Concerns have been raised regarding the possibility of bacteria becoming increasingly antibiotic resistant due to the abuse and misuse of this miracle drug. Due to the increased in the incidence of infections caused by resistant bacteria, in particular nosocomial infections, and the importance of R-factors in clinical medicine, many studies have been focused on the biochemical mechanisms of drug resistance and the epidemiology of resistant bacteria (Mitsuhashi, 1975).

Antibiotic resistance refers to the phenomenon that bacteria causing infection are not killed or inhibited by the antibiotic to which they were previously susceptible and, therefore, they do not respond to treatment, but survive and continue to multiply and causing symptoms in the patient (Mims *et al.*, 1993; Mitcher *et al.*, 1999). Some bacteria are intrinsically resistant to a certain antibiotic. Other bacteria, although intrinsically susceptible, may develop resistance when they tolerate concentrations of an antibiotic that is significantly higher than the concentration which inhibits the growth of susceptible strains of the same species *in vitro* (WHO, 1978; Pelczar *et al.*, 1993).

Resistant strains of bacteria emerge through evolutionary selection. Whenever antibiotics are used there is selective pressure for resistance to occur, since cells that can survive exposure to the antibiotic gradually replace the more vulnerable strains. Antibiotics cause resistance, but also

create conditions for an existing strain of resistant bacteria to become dominant (Jacob, 1999; Mitcher *et al.*, 1999). Due to their ability to develop resistance, bacteria that once appeared to be under control, or at least were believed to be potentially controllable, are now causing infections that are increasingly difficult to treat. Relatively common strains of infectious agents such as *Staphylococcus, Enterococcus, Pseudomonas aeruginosa, Mycobacterium* and several pneumococci are becomming increasingly resistant to multiple antibiotics, while strains of *Enterobacter faecium* and *Pseudomonas cepacia* exist that are insensitive to any presently available antibiotics (Mitcher *et al.*, 1999).

2.5.1 Biochemical basis for resistance to antibiotics

Antibiotic resistant bacteria owe their drug insensitivity to antibiotic resistance genes that encode mechanisms that prevent the drug from reacting with its target in the bacterial cell (Jacob, 1999; Mitcher *et al.*, 1999). In some bacteria, the antibiotic is inactivated when resistant genes encode enzymes that degrade or chemically modify the drug in such a way that it no longer react with its target (Jacob, 1999). Aminoglycoside antibiotics are inactivated by bacterial enzymes that facilitate the acetylation, phosphorylation or adenylation of the drug (Mitsuhashi, 1975; Schwarz and Chaslus-Dancla, 2001). In the case of tetracyclines, enzymes encoded by resistant genes prevent the drug from reaching the target of its action inside the bacterial cell. Drug inactivation may also occur when its target inside the bacterial cell was modified (Jacob, 1999; Mitcher *et al.*, 1999; Schwarz and Chaslus-Dancla., 2001). Some bacteria develop resistance because of their ability to eliminate entry ports for the antibiotic or, more effectively may manufacture pumps that export antibiotics out of the cell before they could react with the target molecule (Jacob, 1999). Bacteria may have an alternative biochemical pathway that allows them to bypass the particular reaction that is inhibited by the antibiotic (Mandell and Petri, 1996). It has also been recognised that resistant microorganisms may carry genes encoding resistance by more than one mechanistic class. Biochemical mechanisms responsible for antibiotic resistance will be discussed in the subsequent paragraphs.

2.5.1.1 Alteration of the target to which antimicrobial agents bind

In some bacteria the structure targeted by the antibiotic is altered due to a mutation in either the genomic or plasmid DNA. By modifying targets such as the cell wall peptidoglycan, genomic DNA, ribosomal RNA or any of the functional or structural proteins, resistant bacteria prevent binding of the antimicrobic to these targets and so avoid the disruption of cell function (Black, 1996; Atlas, 1997). Bacterial resistance to antibiotics such as erythromycin, rifamycin, and the antimetabolites such as trimethoprim has developed by this mechanism (Black, 1996). Resistance to rifampin, for instance, occurs in bacterial cells that have altered the target of this drug, namely DNA-dependent RNA polymerase (Mandell and Petri, 1996). Although the degree of modification enables the bacterial cell to avoid drug action, the functionality of these structures usually remain unaffected by these changes (Black, 1996; Atlas, 1997). Plasmid-encoded dihydrofolate reductases with altered affinity for trimethoprim, allows the synthesis of tetrahydrofolic acid (THFA) to proceed unhindered in the presence of trimethoprim. The modified enzymes, although less susceptible to trimethoprim, retain their affinity for the normal substrate, namely dihydrofolate (Mims *et al.*, 1993). Some bacteria develop resistance to vancomycin by encoding an enzyme that removes the alanine residue from the peptide portion of peptidoglycan. In this case, vancomycin cannot bind to the altered peptide, but the latter remains functional in the formation of the cross-linkages during synthesis of peptidoglycan (Black, 1996; Atlas, 1997).

2.5.1.2 Role of cell wall and membrane permeability in antibiotic resistance

Resistance to bacterial beta-lactam antibiotics, such as penicillin, is caused by the inability of the antibacterial agent to penetrate to reach the site of its action. In Gram-positive bacteria the peptidoglycan polymer is very near the cell surface and beta-lactam antibiotic molecules can penetrate easily to reach the outer layer of cytoplasmic membrane and the penicillin-binding proteins (PBPs) where the final stages of cell wall synthesis occur. Gram-negative bacteria, on the other hand, are intrinsically resistant to beta-lactams, because their surface structure is more complex. An outer membrane consisting of lipopolisaccharide occurs on the outside of the peptidoglycan layer, which in some bacteria is surrounded by a capsule as well. This

composition renders Gram-negative cell walls impenetrable to the large molecules of the beta-lactam and other antibiotics (Atlas, 1997).

Small, hydrophilic antibiotics such as the aminoglycosides, however, can diffuse through aqueous channels that are formed by proteins, known as porins, in the outer membrane of the Gram-negative cell wall (Mandell and Petri, 1996; Atlas, 1997). Alteration of membrane permeability may occur when new genetic information changes the nature of proteins or pores of the outer membrane, thus preventing the antimicrobial agent from entering the bacterial cell and reach its target. Resistance to tetracyclines, quinolones, and some aminoglycosides occurs via this mechanism (Black, 1996; Mitcher *et al.*, 1999).

Bacterial resistance to tetracyclines is based on the transport of tetracyclines out of the cell fast enough to prevent the accumulation of toxic levels of tetracycline so that bacterial protein synthesis is not inhibited (Atlas, 1997). The efflux pump mechanism function in association with the inner membrane and occurs in both Gram-positive and Gram-negative bacteria.

2.5.1.3 Production of enzymes that destroy the inhibitory capacity of antibiotics

The presence of enzymes that can either destroy or inactivate the antimicrobial agent is a common cause of resistance in bacteria. Some bacteria produce enzymes that are called beta-lactamases because of their capability to hydrolyse the β-lactam ring of antimicrobial agents such as penicillins and some cephalosporins (Black, 1996; Mitcher *et al.*, 1999). Many Gram-positive microorganisms release relatively large amounts of β-lactamase into the surrounding medium. There are three classes of β-lactamase: penicillinases, oxacillinases, and carbenicillinases (Atlas, 1997). The location of the β-lactamase enzyme in the periplasmic space probably makes it more effective in destroying cephalosporins than penicillins, because the targets for cephalosporins also are located on the inner membrane of the bacterial cell (Mandell and Petri, 1996).

Certain Gram-negative bacteria possess enzymes that can destroy aminoglycosides and chloramphenicol (Black, 1996). These enzymes may act either by hydrolyzing the antimicrobial

agent or by adding a chemical group causing the enzyme to loose its inhibitory activity (Atlas, 1997). For instance, some bacterial cells produce enzymes that could either add a phosphate, an acetate, or adenyl group to an aminoglycoside antibiotic. The modified aminoglycoside cannot bind to 30S ribosomal subunits to block protein synthesis (Atlas, 1997; Schwarz and Chaslus-Dancla, 2001). Chloramphenicol resistance most often is caused by enzymatic acetylation of the antibiotic. Modified chloramphenicol is no longer effective in blocking protein synthesis since it cannot bind to the 50S ribosomal subunit of the bacterial ribosome. Most clinical isolates that are resistant to chloramphenicol possess a plasmid that carries a gene that encodes chloramphenicol acetyltransferase, leading to the inactivation of the drug soon after it has crossed the cytoplasmic membrane (Atlas, 1997; Schwarz and Chaslus-Dancla, 2001).

2.5.1.4 Alterations of enzymes targeted by antibiotic action

Certain sulfonamide resistant bacteria are capable of modifying enzymes so that the reaction that is supposed to be inhibited as a result of antibiotic action may proceed. In these organisms the modified enzyme has a higher affinity for the substrate para-amino benzoic acid (PABA), a precursor in folic acid metabolism, than for sulfonamide. Consequently, even in the presence of sulfonamide, the enzyme works well enough to allow the bacterium to function (Black, 1996).

2.5.1.5 Alterations in metabolic pathways affected by antibiotic action

Some sulphonamide resistant bacteria may alter a metabolic pathway in order to bypass the reaction inhibited by the antimicrobial agent. These organisms have acquired the ability to use ready-made folic acid from their environment and no longer need to make it from PABA (Black, 1996; Schwarz and Chaslus-Dancla., 2001).

2.5.2 Genetic aspects of antibiotic resistance in bacteria

Bacteria have evolved diverse mechanisms to transmit antibiotic resistance traits to members of their own, or other species in their environment. Genetic traits for antibiotic resistance are encoded by genes occurring either on the bacterial genome, or on extrachromosomal genetic elements called plasmids (Khachatourians, 1998). Mobile genetic elements capable of transferring resistant genes from the bacterial genome to plasmids, and from one plasmid to another, contribute to the rapid rise of R-plasmids that contain multiple genes for antibiotic resistance (Atlas, 1997). According to Harrison and Svec (1998), the frequent exchange of these R-plasmids is a major factor in the rapid distribution of resistance genes among bacteria in the environment.

2.5.2.1 Antibiotic resistance associated with the bacterial genome

In bacteria genetic information is passed on to the progeny during the process of binary division (Harrison and Svec, 1998). However, during replication of DNA, copying errors, called mutations, may occur leading to changes in the sequence of nucleic acids. Mutations may either arise spontaneously, or could be induced by external stress factors in the environment, including chemical agents such as antibiotics, heat or irradiation (Todar, 1996b; Elliot *et al.*, 1997; Harrison and Svec, 1998). Mutations causing a change in only a single nucleotide with no detectable alteration in the end product, namely the transcribed protein, are referred to as point mutations. Point mutations probably are of less consequence to the problem of antibiotic resistance compared to the major genetic changes that cause significant alterations in bacterial cells. Such alterations often are detrimental and the mutant organism may not survive. However, alterations in the bacterial genome may also result in mutant cells having new properties of significant advantage under particular environmental conditions allowing them to outcompete other daughter cells. For example, antibiotic resistance mutants may become dominant in an environment where the presence of an antibiotic exerts the selective pressure (Todar, 1996b; Elliot *et al.*, 1997). Mutations that cause chromosomal genes to encode resistance in stead of sensitivity occur at a rate of one per million to one per billion cells (Khachatourians, 1998).

2.5.2.2 Antibiotic resistance associated with extrachromosomal genetic elements

In bacteria extrachromosomal genetic material occur in plasmids and transposons. Plasmids carry those genes that encode properties or functions that are not essential for growth and multiplication, but rather give the organism an advantage for survival in environments where they are exposed to a particular stress factor, such as antibiotics (Harrison and Svec, 1998). Transposons, often referred to as jumping genes, are mobile genetic elements that move from one site to another, inevitably causing the amino acid sequence in these sites to change. (Harrison and Svec, 1998). At each end of the transposon is specific base sequences known as insertion sequences which allow the transposon DNA to be inserted into existing DNA strands. Transposons allow genetic information to be transferred rapidly between plasmids and chromosomal DNA, and also facilitate the dissemination of genetic information among bacteria in the environment (Elliot *et al.*, 1997; Harrison and Svec, 1998).

Plasmids are relatively large, independent, self-replicating genetic units carrying several genes that control the activities of the plasmid itself as well as those of the parent cell, such as plasmid replication, production of sex pili, conjugation, DNA transfer, antibiotic resistance and toxin production (Mims *et al*, 1993). R-factors are extrachromosomal genetic elements which are conjugationally transmissible and capable of conferring resistance to various chemotherapeutic agents and heavy metal ions on their host bacteria. All genetic characters of the R-factor are conjugationally transmissible as a whole or are jointly transduced with bacteriophage when the phage has the same (or larger) size of DNA as that of the R-factor (Mitsuhashi, 1976). R-factor mediated multivalent resistance was noted in the 1960s, as was the emergence of resistance during the course of chemotherapy (Mitcher *et al.*, 1999).

2.6 The transmission of genetic information

Genetic information, including resistance genes, are exchanged at relatively high frequency between different bacterial species sharing the same environment (Feinman, 1999). Genetic transfer between bacteria is facilitated by three mechanisms, namely conjugation, transformation

and transduction (Ho *et al.*, 1998). These mechanisms will be discussed in subsequent paragraphs.

2.6.1 Conjugation

According to Sirotnek (1976), genetic material is transferred from one cell to another through a process known to involve the transfer of a large portion, if not all, of the chromosome from the donor (F^+) to a recipient cell. Close contact between donor and recipient is one of the major requirements for efficient conjugation (Schwarz and Chaslus-Dancla, 2001). This process of genetic transfer, termed conjugation, is recognised as an extremely important mechanism for the spread of antibiotic resistance. Genes coding for resistance to multiple drugs may be transferred in this manner (Mandell and Petri, 1996). This process also allows for genetic transfer between different species of organisms and the transfer of resistance to several antibiotics at the same time (Cooke, 1974; Schwarz and Chaslus-Dancla, 2001).

It is known that genes for antibiotic resistance, located on conjugative R-plasmids in bacteria such as *E. coli*, can be readily transferred to various other bacterial cells (Pelczar *et al.*,1993). Conjugative plasmids contain genes which control the formation of pili, which allow the bacterial cell to attach to a second cell via a cytoplasmic bridge. Following attachment, the conjugative plasmid divides and a copy is transferred across the cytoplasmic bridge into the recipient cell (Elliot *et al.*, 1997). R-plasmids contain a resistance transfer factor (RTF) that controls conjugative transfer of resistant genes (Atlas, 1997). Organisms that serve as recipients of R-plasmids from *E. coli* include species of *Enterobacter, Klebsiella, Salmonella,* and *Shigella* (Pelczar *et al,* 1993). Genetic transfer by conjugation occurs predominantly among Gram-negative bacilli, and resistance is conferred on a susceptible cell as a single event. Enterococci also contain broad host range conjugative plasmids which are involved in the transfer and spread of resistance genes among Gram-positive organisms. Conjugation can take place in the intestinal tract between non-pathogenic and pathogenic microorganisms. While the efficiency of transfer is low *in vitro* and still lower *in vivo,* antibiotics can exert a powerful selective pressure to allow emergence of the resistant strain (Mandell and Petri, 1996).

R-plasmids have contributed largely to the problems of multiple antimicrobic resistance since they can be transferred from one species to another, giving rise to antimicrobic resistant strains during an infection which then may become difficult to treat.

2.6.2 Transduction

Transduction is the transfer of DNA from a donor cell to a recipient cell by bacteriophages. In most cases only a small segment of the host (the donor) DNA is transferred. Two kinds of transduction can be distinguished, namely (i) non-specific or generalised transduction during which any part of the host DNA is transferred, and (ii) specialised transduction which is restricted to the transfer of specific DNA segments. In non-specific transduction the host DNA segment is integrated into the virus particle, either in addition to, or in place of the phage genome. In specific transduction some of the phage genes are replaced by the host genes (Schlegel, 1992; Mitcher *et al.,* 1999). If these altered particles successfully infect another cell then the donated DNA can be integrated into the chromosome of the recipient cell. Since bacteriophages infect only a narrow range of bacteria, this form of DNA recombination can occur only between closely related bacterial strains. A bacteriophage can also incorporate its own viral DNA into the bacterial chromosome; occasionally, this can result in the bacteria synthesizing new proteins (Mandell and Petri, 1996; Elliot *et al.*, 1997).

2.6.3 Transformation

Gene transfer by soluble DNA, which has been extracted, or otherwise liberated from donor bacterium, to a recipient bacterium is called transformation (Schlegel, 1992; Mitcher *et al.,* 1999). In the case of chromosomal DNA transfer, the process is initiated by direct transfusion of the protoplasts. This brings together genome sections from different parent cells, resulting in mutant or recombinant cells under certain experimental conditions. The recombinant cells obtained from such fusion exhibit properties of both parents, by virtue of homologous recombination (Schlegel, 1992). Some bacteria are able to take up soluble DNA fragments derived from other, normally closely related, species directly across their cell wall (Elliot *et al.*, 1997). According to Mims *et al.* (1993), lysing of bacteria could release DNA fragments of

competent cells into the environment. After the released DNA have been broken up in smaller lengths, the double-stranded donor DNA is reduced to single strand, which can then be incorporated into the recipient's chromosome (Mims *et al.* 1993; Schwarz and Chaslus-Dancla, 2001).

2.7 Prevalence of antibiotic resistance among pathogenic bacteria

The following important organisms have been shown to be resistant to one or more of the presently available antibiotics, to the extent that treatment of infections caused by them has become extremely difficult: *Staphylococcus aureus* (toxic shock syndrome, postoperative infections), *Streptococcus pneumoniae* (pneumonia), *Streptococcus pyogenes* (rheumatic fever), *Haemophilus influenzae* (meningitis), *Mycobacterium leprae* (leprosy), *Neisseria gonorrhoeae* (gonorrhea), *Shigella dysenteriae* (dysentry) and several other species of bacteria that infect the human gut including *E. coli*, *Klebsiella*, *Proteus*, *Salmonella*, *Serrratia marcescens*, *Pseudomonas*, *Enterococcus faecium*, *Enterobacteriaceae* and *Vibrio cholerae* (Khan *et al.*, 2000).

Pseudomonas aeruginosa, a human opportunistic pathogen, is a common cause of nosocomial infections and can be found growing in a large variety of environmental locations (Kaiser, 1999). This organism characteristically causes infections in hospitalised patients, particularly those who are immunocompromised. In addition to it being a normal commensal organism in the human gastrointestinal tract, *Pseudomonas aeruginosa* may also colonise other sites when host defences are compromised (Elliot *et al.*, 1997). Opportunistic infections caused by *P. aeruginosa* skin infections associated with burns and venous ulcers, urinary tract infections particularly with associated long-term urethral catheterisation and destructive infections of the eye. Contact lens wearers are one group at increased risk for such infections (Baron *et al.*, 1994; Elliot *et al.*, 1997). Clinically important antibiotics used for control of *Pseudomonas* infection include aminoglycosides such as gentamycin, cephalosporins such as ceftazidime and the quinolones (Elliot *et al.*, 1997). *Pseudomanas* species are genetically complex, often possessing one or more plasmids in addition to their chromosomal genes. These plasmids often contain antibiotic resistance genes that frequently are transferred to neighbouring bacteria sharing the same

environment transfer (Elliot *et al.*, 1997). Resistance in *Pseudomonas* has been shown to involve antibiotics such as gentamicin, kanamycin, streptomycin, tetracycline and sulfonamide. An antibiotic that can be used with caution against *Pseudomonas* is polymyxin, which is not ordinarily used in human therapy because of its toxicity. Although effective, polymyxin may cause damage to the kidneys and other organs, and should therefore only be given under close supervision in the hospital (Todar, 1996a). Some newer antibiotics have been designed specifically to combat *P. aeruginosa* (Elliot *et al.,* 1997).

Staphylococci cause diseases because of their ability to spread in tissues and form abscesses, produce extracellular enzymes or exotoxins and combat host defences (Elliot *et al.*, 1997). *Staphylococcus aureus* produces the enzyme, hyaluronidase that may contribute to the spread of infections through tissues (Baron *et al.*, 1994). Infections caused by staphylococci include deep and superficial abscesses, endocarditis, mastitis, pneumonia, meningitis, wound infections and sepsis. Several toxins are produced by *Staphylococcus* and are the cause of diseases such as staphylococcal food poisoning and toxic shock syndrome (Atlas, 1997). Among the toxins produced by *S. aureus* are alpha, beta, gamma and delta toxins and leukocidan, which act on the red and white blood cell membranes of some species. The frequencies of infection caused by penicillin-resistant staphylococci is reported to increase (Atlas, 1997) and in most cases this resistance is attributed to β-lactamase production due to genes located on extrachromosomal plasmids (Baron *et al.,* 1994). Strains of *S. aureus* were found to be resistant to macrolide antibiotics, aminoglycosides and tetracyclines (Thomson and Holding, 1986; Schwarz *et al.*, 1989). Antimicrobial agents such as flucloxacillin or erythromycin with fusidic acid remain the first line treatment for *S. aureus* infections (Elliot *et al.*, 1997).

Many genera of the family *Enterobacteriaceae* are normal biota of the human intestinal tract and are considered opportunistic pathogens when they are introduced into body locations where they are not normally found, especially if the host is debilitated or immunocompromised. The most common genera of *Enterobacteriaceae* causing opportunistic infections in humans are *Escherichia coli*, *Proteus, Enterobacter, Klebsiella, Citrobacter*, and *Serratia*. Infections, associated with *Enterobacteriaceae* include urinary tract infections, wound infections, pneumonia, and septicemia (Kaiser, 1999). Multiple antibiotic resistance occur in hospital strains

of *Enterobacteriaceae* most commonly involved in serious nosocomial infections. Treatment of these infections usually consists of a β-lactam or quinolone, either alone, or in combination with another agent such as one of the aminoglycosides (Baron *et al.*, 1994). Environmental strains of *Enterobacteriaceae* usually are susceptible to aminoglycosides, imipenem, quinolones and third generation cephalosporins (Baron *et al.*, 1994).

Most strains of *E. coli* are harmless commensals that help protect us against infection by competing with enteric pathogens such as *Shigella* and *Salmonella* (Ingraham and Ingraham, 2000). Although *E. coli* is a commensal of the human intestine, it can cause a variety of important infections, including infections of the gastrointestinal tract, urinary tract, bilary tract, lower respiratory tract and septicaemia (Elliot *et al.*, 1997). *E. coli* strains are also involved in foodborne illnesses. Enterohemorrhagic *E. coli* strains (EHEC) associated with consumption of undercooked hamburgers can cause disease outbreaks, including infections that cause bloody diarrhoea which may eventually may develop into hemolytic uremic syndrome leading to anaemia and renal failure (Atlas, 1997). Infection by enteropathogenic *E. coli* (EPEC) is typically associated with epidemic diarrhoea in areas of the developing world, particularly in infants younger than 6 months (Kaiser, 1999). *E. coli* "O" serotypes (EPEC) cause direct damage to the intestinal villi (Elliot *et al.*, 1997). Enterotoxigenic *E. coli* (ETEC) produces enterotoxins that cause the loss of sodium ions in water from the intestines resulting in watery diarrhoea (Kaiser, 1999). ETEC is an important cause of travellers' diarrhoea and diarrhoea disease in children in developing countries (Elliot *et al.*, 1997). Enteroinvasive *E. coli* (EIEC) produces a shigella-like toxin, with invasion of the intestinal mucosa, resulting in diarrhoea containing blood, pus and mucus (Elliot *et al.*, 1997). The toxin inhibits protein synthesis and eventually kills cells in the intestinal lining, which leads to dysentery and colitis (Ingraham and Ingraham, 2000). *E. coli* is commonly resistant to penicillin and ampicillin due to production of β-lactamases by those organisms. Interestingly, a higher degree of resistance to these antibiotics was reported for strains isolated from hospital patients. Therefore, infections caused by *E. coli* commonly is treated using antibiotics not affected by β-lactamases such as cephalosporins, trimethoprin and aminoglycosides (Petrosino *et al.*, 1998).

Several *Salmonella* species are pathogenic to humans. Typhoid fever, enterocolitis and various other gastrointestinal diseases are caused by *Salmonella* species (Atlas, 1997). Other infections associated with salmonellae are osteomylitis, septic arthritis or abscesses and enteric fever caused by *S. typhi* or *S. paratyphi.* (Elliot *et al.*, 1997).

2.8 The misuse and abuse of antibiotics

Antibiotics are vital to treat human infections, but their effectiveness is threatened by their overuse and often misuse which have been shown to contribute to bacteria developing resistance to the action of these type of drugs (Feinman, 1999). Antibiotics are often prescribed for infections caused by agents, such as viruses, that have been shown to be insensitive for their action. The majority of diseases caused by viruses are self-limited and generally do not respond to most of the currently available anti-infective compounds (Mandell and Petri, 1996). Erroneous dosage of antimicrobial agents by physicians prescribing them, whether in excessive amounts or suboptimal quantities, is a problem in modern medicine, since overdosage may harm the patient. Applying anti-infective agents in sub-optimal concentrations can also lead to overdosage (Mandell and Petri, 1996). Another misuse is failure to finish an antibiotic prescription, because if the infectious agent is not eliminated completely, exposure to the antibiotic probably would induce resistance in surviving cells. All the afore-mentioned problems are prevalent in countries where antibiotics are available without prescription by a doctor. Many scientists believe that poorly regulated use of antibiotics in medicine as well as agriculture, whether for growth promotion, prophylaxis or therapy, has contributed to a build-up of reservoirs of antibiotic resistant bacteria of animal origin (Mandell and Petri, 1996; Harrison and Svec, 1998; Khachatourians, 1998; Jacob, 1999). The danger of this to humans is that (i) antibiotic resistant pathogens common to both animals and humans may reach the latter by cross-infection, and (ii) antibiotic resistant non-pathogenic zoonotic organisms occurring in the gastrointestinal tract of both humans and animals, may carry R-plasmids into the human environment. These R-plasmids may subsequently be transferred to human pathogens or to indigenous biota in the human body (Moro *et al.*, 1998). According to a World Health Organisation report in 1978 (WHO, 1978) the rapid emergence and spread of drug-resistant *Salmonella* resulted from the antibiotic use in animals during the 1960's. Transmission of these resistant *Salmonella* strains to

man resulted in many human infections and the resistance was acquired in the animal host. In the CDC Annual *Salmonella* Surveillance (1974), it was reported that 6 of the most common human *Salmonella* serotypes were among the 10 most common animal serotypes. In fact, according to Novick (1978), it is probable that virtually all salmonellae of farm animal origin are capable of infecting humans as well.

In 1998 the American National Research Council's report stated that the use of antibiotics to control and treat diseases in animals improves the safety of our food supply by providing healthier sources of meat, cheese, milk and eggs, but cases have also been reported of resistant bacteria that were passed on to humans through the consumption of these foods (Jacob, 1999). Thus, although the evidence is unclear, many scientists believe that the use of antibiotics in animal feed should be discontinued (Jacob, 1999).

2.9 Addressing the problem of antibiotic resistance

The often unrestricted and indiscriminate use of antimicrobial agents in humans, animals and agriculture in past decades probably has contributed largely to present conditions that apparently promote antibiotic resistance in bacterial species that often are of clinical significance. The widespread occurrence of multiple antibiotic resistance has rendered many useful antimicrobials ineffective for the treatment of infections thought to have been under control. Since relatively few new classes of antimicrobial agents are being developed, the need for prudent use of the available agents is becoming increasingly apparent.

When novel antibacterial compounds are developed, early studies should be directed to the investigation of mechanisms of resistance that are already present in microorganisms and of resistance that can be selected *in vitro,* since such findings might detect novel resistance mechanisms that might arise in targeted or other bacteria once the drug has come into clinical use (Lerner, 1998).

Levy and co-workers have warned in 1975 that new antibiotic development lagged several years behind the need, and that "no magic bullet around the corner" could be expected to solve the

problem. According to the authors, doctors, who often are pressurised by patients, should refrain from prescribing antimicrobial drugs for minor infections. They also suggested that fortified cleansers could contribute to the selection of resistance in bacteria and should, therfore, be avoided.

Nobel laureate and the National Institute of General Medical Sciences (NIGMS) grantee Sidney Altman and his colleagues at Yale University are taking an entirely different approach (Machalek, 1997). Instead of developing new antibiotics, they are forcing bacteria to submit once again to existing antibiotics. Using one of the same genetic methods that spread resistance, plus some biochemical wizardry of their own, the scientists destroyed bacterial genes that confer resistance, rendering previously resistant microbes susceptible to certain antibiotics.

There can be no argument that the ultimate responsibility for the excessive human therapeutic use of antibiotics in developed countries lies with the world's health care providers, primarily physicians and dentists. Clinicians must be educated to understand the resistance problem, accept the importance of their role in both the problem and the solution and resolve to use these drugs in a far more appropriate manner (Harrison and Svec, 1998).

The indiscriminate use of antibiotics in agriculture also have far-reaching medical consquences, because the selective pressure induced by these practices promotes the establishment of resistance reservoirs in food animals. In view of the risks for human health, the uncontrolled of antibiotics as growth promoters in animal feed, particular those that are valuable in human medicine, has become unacceptable and should be regulated (Teuber, 2001). Treatment of bacterial infections in herds or flocks consisting of food animals should also be limited. This practice is aimed at preventing the spread of infection among animals of the herd of flock, and more often than not, all animals are dosed with antibiotcs regardless of whether or not they are infected. To minimise the risk that resistant strains will be selected, this mode of treatment should be carefully considered and performed in a controlled manner in order to prevent the transfer of low levels of antibiotics to uninfected animals (Gustafson and Bowen, 1997; Johnston, 2001). Care must also be taken to limit or avoid routes of transmission that could facilitate the dissemination of resistance genes into environments shared by both food animals

and humans, such as human contact with animal feces, or contaminated water and utensils (Teuber, 2001).

The American Veterinary and Medical Association has established a steering committee for antimicrobial use. The purpose of this committee was the development of guidelines for judicious therapeutic use of antimicrobials by veterinarians, and the development of educational materials and programs to increase awareness the problem of antibiotic resistance among veterinarians (Torrence, 2001). These Guidelines have been published to assist all those people involved in the effort towards prudent and judicious use of antimicrobials. By complying to these guidelines, medical and veterinary practitioners, manufacturers of animal food and meat producers could help to contain the problem of antibiotic resistance. At this stage this seems to be the only broad scale approach available to retain the efficiency of antimicrobial agents for medical use (Schwarz and Chaslus-Danla, 2001).

2.10 The aim of the present study

There seems to be general consensus in scientific literature concerning the important role of food animals in the development of antibiotic resistance in human pathogenic bacteria worldwide. In view of the problem antibiotic resistance is causing in human medicine, the present study was aimed at determining the level of antibiotic resistance in bacteria derived from chicken meat and pork, as well as from the environment of the respective meat-producing industries.

The first objective was to collect representative samples of the the different products, namely various parts of chicken, eggs and pork, as well as feed and manure samples.

The second objective was to isolate *Pseudomonas, Staphylococcus, Enterobacteriaceae, E. coli* and *Salmonella* from the respective samples.

Following isolation, the third objective was to test isolates for potential resistance to one or more of eight commonly used antibiotics, using the Calibrated Dichotomous Sensitivity Method.

Based on the outcome of the Calibrated Dichotomous Sensitivity Test, the fourth objective was to subject potential antibiotic resistant strains to antibiotic susceptibility testing, employing the standard Kirby Bauer Susceptibility Test.

The fifth objective was to statistically process data derived from the Kirby Bauer Susceptibility Test and present the processed data in dendograms that can be used to infer trends in the antibiotic resistance levels of isolates.

CHAPTER 3

MATERIALS AND METHODS

3.1 Sampling and processing of meat

Meat samples were collected and handled according to prescribed procedures (SABS Method 758, 1975a). Chicken and pork samples were obtained from two different chain stores, as well as a poultry and pig farm respectively. The chicken and pork samples were not derived from the farms. Eggs were collected from a chain store as well as two different farms from where the chicken feed and manure samples also derived. All samples were transported and stored under refrigerated conditions (4 °C) and processed within 24 hours to prevent changes in the composition of bacterial populations.

Refrigerated samples were aseptically processed and prepared for use in subsequent isolation procedures. Chicken and pork were aseptically cut into smaller pieces before a 10 g mass of each sample was grounded aseptically for 60 seconds using a sterile Drehzahiregler CH 6000 Kriens-Lu homogeniser. The contents of one egg per sample locality was homogenised for 60 seconds. A 10 g mass of chicken feed and chicken manure sample respectively was aseptically crushed in a crucible until fine before it was grounded aseptically for 60 seconds using a sterile homogeniser. Subsequently, a one to ten dispersion of each sample was aseptically prepared for use in the respective standard isolation procedures.

3.2 Enrichment and isolation of the different groups of bacteria on selective media

Standard procedures of the British Pharmacopoeia (1993) and South African Bureau of Standards (SABS, 1975a, 1975b and 1975c) respectively were used for the enrichment and subsequent isolation of the experimental organisms, namely *Pseudomonas, Staphylococcus, Enterobacteriaceae, Salmonella* and *Escherichia coli.*

3.2.1 Isolation of *Pseudomonas*

Enrichment and isolation of *Pseudomonas* from respective samples were carried out according to the procedure described in the British Pharmacopoeia (1993:A197). A 1:10 (m/v) dispersion was prepared for each sample by aseptically transferring a 5 g mass of the prepared sample to a sterile flask and making that up to 50 ml with sterile NaCl peptone. Subsequently a 5 ml volume of the prepared dispersion was transferred aseptically to a sterile tube containing 50 ml of Triptone Soy Broth. After 24/48 hours of incubation at 37 °C tubes showing growth were used for the isolation of *Pseudomonas*.

To obtain single colonies, a serial dilution of 10^{-2} to 10^{-7} were prepared for each enriched Triptone Soy Broth cultures that was used for the plating of the 10^{-4} to 10^{-8} Cetrimide Agar spread plates. After incubation at 37 °C for 24/48 hours, *Pseudomonas* colonies on Cetrimide Agar were well develop and typically showed a blue-green fluorescence colour. A statistically representative number of typical colonies (i.e. all the colonies on plates from the highest dilution that showed growth (10^{-5}) plus half of those on plates from the second highest dilution that showed growth) were selected and subjected to Gram-staining to confirm pureness of colonies and a Gram-negative reaction. Pure cultures of Gram-negative rods were transferred to Nutrient Agar slants for subsequent use in antibiotic susceptibility testing.

3.2.2 Isolation of *Staphylococcus aureus*

Enrichment and isolation of *Staphylococcus aureus* from the respective samples were carried out according to procedures described in the SABS method 760 (1975c). To prepare the required 1:10 (m/v) dispersion for each sample, a total mass of 5 g of the prepared sample were aseptically transferred to a sterile flask, and made up to 50 ml using Physiological Salt Solution. Subsequently a 5 ml volume of dispersion was transferred aseptically to sterile tubes containing 10 ml volumes of *Staphylococcus* broth. After 48 hours of incubation at 37 °C tubes were examined for the typical colour change to yellow or orange.

To obtain single colonies a serial dilution of 10^{-2} to 10^{-7} was prepared from the enriched *Staphylococcus* broth cultures that were used for the plating of 10^{-4} to 10^{-8} Mannitol Salt Agar spread plates. After incubation at 37 °C for 48 hours, *Staphylococcus aureus* colonies were well developed and turned the Mannitol Salt Agar yellow. To confirm pureness of colonies and a Gram-positive reaction, a statistically representative number of typical colonies (i.e. all the colonies on plates from the highest dilution that showed growth (10^{-5}) plus half of those on plates from the second highest dilution that showed growth) were selected and subjected to Gram-staining. Pure cultures of Gram-positive cocci were transferred to Nutrient Agar slants for subsequent use in antibiotic susceptibility testing.

3.2.3 Isolation of *Enterobacteriaceae*

Enrichment and isolation of *Enterobacteriaceae* from respective samples were carried out according to procedures described in the British Pharmacopoeia (1993:A196). To prepare the required 1:10 (m/v) dispersion of each sample, a total mass of 5 g of the prepared sample were aseptically transferred to a sterile flask, and made up to 50 ml using Lactose Broth. The dispersion was mixed well using a vortex, and incubated at 37 °C for 5 hours that would have allowed sufficient time for reviving bacteria, but was insufficient to allow multiplication. After incubation 5 ml of the homogenate were transferred to sterile tubes containing 50 ml volumes of *Enterobacteriaceae* Enrichment Broth-Mossel and incubated at 37 °C for 18/24 hours.

After incubation tubes containing 10 ml of Violet-Red Bile Broth as a selective medium for *Enterobacteriaceae* were inoculated from the enriched cultures. Following incubation at 37 °C for 18/48 hours, flasks showing growth were used to prepare serial dilutions of 10^{-1} to 10^{-7} that were used for the plating of 10^{-4} to 10^{-8} spread plates on Violet-Red Bile Agar containing glucose and lactose. After incubation at 37 °C for 18/48 hours plates were examined for the presence of colonies with red or reddish colour that were typical of potential *Enterobacteriaceae*. To confirm pureness of colonies and a Gram-negative reaction, a statistically representative number of typical colonies (i.e. all the colonies on plates from the

highest dilution that showed growth (10^{-5}) plus half of those on plates from the second highest dilution that showed growth) were selected and subjected to Gram-staining. Pure cultures of Gram-negative bacilli were transferred to Nutrient Agar slants for subsequent use in antibiotic susceptibility testing.

3.2.4 Isolation of *Escherichia coli*

Enrichment and isolation of *Escherichia coli* from respective samples were carried out according to the SABS method 758 (1975a). To prepare the required 1:10 (m/v) dispersion of each sample, a total mass of 5 g of the prepared sample were aseptically transferred to a sterile flask, and made up to 50 ml using Physiological Salt Solution. Subsequently 5 ml volumes the dispersion was transferred to a sterile tube with a 50 ml volume of MacConkey Broth containing a Durham tube. Following 18/24 hours of incubation at 37 °C, tubes were examined for production of acid and gas, typical of *Escherichia coli* due to fermentation of lactose in MacConkey Broth.

To obtain single colonies from the MacConkey Broth cultures, a serial dilution of 10^{-2} to 10^{-7} was prepared from the MacConkey Broth cultures and used for the plating of the 10^{-4} to 10^{-8} MacConkey Agar spread plates. After incubation at 37 °C for 24/48 hours, *Escherichia coli* colonies on MacConkey Agar spread plates were well develop and typically showed a red colour. A statistically representative number of typical colonies (i.e. all the colonies on plates from the highest dilution that showed growth (10^{-5}) plus half of those on plates from the second highest dilution that showed growth) were selected and subjected to Gram-staining to confirm pureness of colonies and a Gram-negative reaction. Pure cultures of Gram-negative rods were transferred to Nutrient Agar slants for subsequent use in antibiotic susceptibility testing.

3.2.5 Isolation of *Salmonella*

To facilitate the isolation of *Salmonella* from the respective samples, enrichment prior to isolation was carried out according to the procedure described by the British Pharmacopoeia, (1993:A197). The required 1:10 (m/v) dispersion of each sample was prepared by transferring aseptically 5 g of the prepared sample to a sterile flask,

which was then made up to 50ml using Buffered Peptone Water. Following incubation at 37 °C for 16/20 hours, a 10ml volume of the dispersion was aseptically transferred to a tube containing a 100ml volume of Tetrathionate broth, and subsequently incubated at 43 °C for 48 hours. A 10 ml volume of the dispersion was also used to inoculate a tube containing 100 ml of Selinite broth that was subsequently incubated at 37 °C for 48 hours. After 18/24 hours of incubation, Selinite broth cultures had been shaken at 160 revolutions per minute for the rest of the incubation period. To obtain single colonies, a serial dilution of 10^{-1} to 10^{-7} was prepared from each of the Tetrathionate broth and Selinite broth cultures that were subsequently used to plate Brilliant Green agar and Desoxycholate Citrate agar spread plates respectively (SABS method 759, 1975b). Following incubation of the Brilliant Green agar and Desoxycholate Citrate agar plates at 37 °C for 18/24 hours, *Salmonella* colonies were well developed and typically small, transparent and colourless. A statistically representative number of typical colonies (i.e. all the colonies on plates from the highest dilution that showed growth (10^{-5}) plus half of those on plates from the second highest dilution that showed growth) were selected and subjected to Gram-staining to confirm pureness of colonies and a Gram-negative reaction. Pure cultures of Gram-negative rods were transferred to Nutrient agar slants for subsequent use in antibiotic susceptibility testing.

3.3 Antibiotic susceptibility testing with the Calibrated Dichotomous Sensitivity (CDS) method

Susceptibility of isolates for eight antibiotics of different classes was assayed using the CDS method (Bell *et al.*, 1999). All antibiotics were used in 30 $\mu g.l^{-1}$ concentrations as recommended in the Standard Antimicrobial Disc Susceptibility Tests (NCCLS, 1997; Bell *et al.*, 1999). The antibiotics used in the present study, and the classes they represent are listed in Table 3.1.

Table 3.1 - Antibiotics used in the present study

Antibiotic	Antibiotic Class
Erythromycin USP (Ery)	Macrolides
Gentamicin Sulphate (Gen)	Aminoglycosides
Kanamycin Acid Sulphate (Kan)	Aminoglycosides
Neomycin Sulphate (Neo)	Aminoglycosides
Oxyteracycline (Oxy)	Tetracyclines
Potassium Penicillin (Pen)	Beta –lactam
Streptomycin (Strep)	Aminoglycosides
Tetracycline (Tet)	Tetracyclines

3.3.1 Preparation of isolates for use in the Calibrated Dichotomous Sensitivity (CDS) method

To prepare isolates for susceptibility testing employing the Calibrated Dichotomous Sensitivity (CDS) procedure, Nutrient agar slant cultures were inoculated into volumes of 5 ml Triptone Soy broth and incubated at 37 °C until the absorbency measured 0,1 at 640 nm on the spectrophotometer (Bell *et al.*, 1999).

3.3.2 Inoculation of plates

A dried (half an hour in a laminar flow cabinet) Mueller-Hinton agar plate containing 20 ml agar in a 90 mm petridish was flooded with 1 ml of the inoculum described in par. 3.3.1 (Bell *et al.*, 1999). The plate was swivelled carefully to distribute the suspension evenly over the surface of the petridish. The excess bacterial suspension was removed from the plate by pouring it off. The inoculated plate was dried in a laminar flow cabinet for 10 minutes. With the aid of a sterile cork bore, four holes of 7 mm in diameter were made in the agar plate. Holes were made one and a half centimetre from the edge of the plate. The bottom of each hole was sealed with 0,01

µl molten agar. Wells were filled with 20 µl of antibiotic. After antibiotics were allowed to diffuse into the medium surrounding the wells, plates were incubated at 35 °C for 18 hours. Following incubation, plates were examined for clear zones in the lawn of bacterial growth where the antibiotic diffused from the well into the surrounding medium.

3.3.3 Measurement and interpretation of growth inhibition zones

Zones of growth inhibition were measured using a vernier to determine the annular radius, i.e. the shortest distance from the edge of the well to the edge of the confluent growth. The diameter of the growth inhibition zone around the well containing the antibiotic correlates inversely with minimum inhibitory concentration of the antibiotic indicated by the Standard Antimicrobial Disc Susceptibility Tests (NCCLS, 1997). According to Bell *et al.* (1999), an annular radius equal or larger than 6 mm indicated susceptibility for the antibiotic tested, and a radius smaller than 6 mm, resistance.

3.3.4 Processing of data

Results from the isolation of *Pseudomonas, Staphylococcus, Enterobacteriaceae, Salmonella* and *Escherichia coli* from the respective meat, egg, feed and manure samples, as well as the antibiotic susceptibility testing are presented in tables. To determine the level of multiple antibiotic resistance in isolates, antibiotic susceptibility data were statistically processed using Ward's method*. Results from isolation, susceptibility testing and statistical processing are presented in Chapter 4.

* An analysis of variance approach that attempts to minimise the Sum of Squares (SS) of any two (hypothetical) clusters that can be formed at each step. This method is regarded as very efficient, although it tends to create clusters of small size (Ward, 1993).

CHAPTER 4

RESULTS

4.1 Isolation of bacteria from samples collected from retail outlets and farms

A total of 198 organisms were isolated from chicken meat, pork, chicken manure, chicken feed and eggs (par. 3.2). Of these organisms, 20 was *Pseudomonas* isolates, 50 *Staphylococcus* isolates, 42 *Enterobacteriaceae* isolates , 14 *Escherichia coli* isolates and 72 *Salmonella* isolates (see Table 4.1).

Table 4.1 - The total number of isolates from representative of the various bacterial groups

Pseudomonas	*Staphylococcus*	*Enterobacteriaceae*	*E. coli*	*Salmonella*
20	50	42	14	72

The relative numbers of isolates representative of each of the respective bacterial groups obtained from each sample are illustrated in Table 4.2.

Table 4.2 - The relative numbers of isolates of the different bacterial groups obtained from the various samples

Sample	*Pseudomonas*	*Staphylococcus*	*Enterobacteriaceae*	*E. coli*	*Salmonella*
CH	0	5	2	1	5
CV	3	4	2	0	5
PH	1	7	5	1	2
PV	4	3	1	1	0
P	0	0	2	0	0
H1	0	0	0	0	0
H2	0	0	3	0	0
H3	1	3	2	0	12
S1	0	0	1	0	0

Table 4.2 (continued)

S2	2	15	6	4	17
S3	1	5	2	3	3
DA	2	0	4	2	8
DB	3	1	7	1	6
WL	3	4	4	0	5
WV	0	3	1	1	9
Total	20	50	42	14	72

CH = chicken meat from Chain store C
CV = pork meat from Chain store C
PH = chicken meat from Chain store P
PV = chicken meat from Chain store P
P = eggs from Chain store P
H1 = eggs from Farm H
H2 = chicken manure from Farm H
H3 = chicken feed from Farm H
S1 = eggs from Farm S
S2 = chicken manure from Farm S
S3 = chicken feed from Farm S
DA = chicken liver from Farm D
DB = chicken stomach from Farm D
WL = pork liver from Farm W
WV = pork meat from Farm W

Table 4.3 depicts the relative numbers of isolates representative of the respective bacterial groups obtained from chicken meat from different retail outlets, namely chain stores C and P, as well as farm D respectively.

Table 4.3 - The relative numbers of the different bacterial groups isolated from chicken meat obtained from two chain stores and a farm respectively

Sampling point	***Pseudomonas***	***Staphylococcus***	***Enterobacteriaceae***	***E. coli***	***Salmonella***
Chain store C	0	5	2	1	5
Chain store P	1	7	5	1	2
Farm D	5	1	11	3	14
Total	6	13	18	5	21

According to results depicted in Table 4.3, a total of 63 isolates representative of all five bacterial groups were obtained from chicken meat collected at the respective sampling points. Percentages of different bacterial groups relative to total number of isolates were as follows:

Pseudomonas: 9.5 %

Staphylococcus: 20.6 %

Enterobacteriaceae: 28.6 %

Escherichia coli: 7.9 %

Salmonella: 33. 4 %.

Table 4.4 depicts the relative numbers of isolates representative of the respective bacterial groups obtained from pork collected at the different retail outlets, namely chain stores C and P, as well as farm W, respectively.

Table 4.4 - The relative numbers of the different bacterial groups isolated from pork obtained from chain stores and a farm respectively

Sampling point	*Pseudomonas*	*Staphylococcus*	*Enterobacteriaceae*	*E. coli*	*Salmonella*
Chain store C	3	4	2	0	5
Chain store P	4	3	1	1	0
Farm W	3	7	5	1	14
Total	10	14	8	2	19

According to results depicted in Table 4.4, a total of 53 isolates representative of all five bacterial groups were obtained from pork collected at the respective sampling points. Percentages of different bacterial groups relative to total number of isolates were as follows:

Pseudomonas: 18.9 %

Staphylococcus: 26.4 %

Enterobacteriaceae: 15.1 %

Escherichia coli: 3.8 %

Salmonella: 35.8 %.

Table 4.5 shows the relative numbers of isolates representative of the respective bacterial groups obtained from chicken feed and chicken manure collected at the different chicken farm settings, farm H and farm S respectively.

Table 4.5 - The relative numbers of the different bacterial groups isolated from feed and manure obtained from the farms respectively

Sampling point	*Pseudomonas*	*Staphylococcus*	*Enterobacteriaceae*	*E. coli*	*Salmonella*
Farm H-feed	1	3	2	0	12
Farm S-feed	1	5	2	3	3
Farm H-manure	0	0	3	0	0
Farm S-manure	2	15	6	4	17
Total	4	23	13	7	32

According to results depicted in Table 4.5, a total of 79 isolates representative of all five bacterial groups were obtained from pork collected at the respective sampling points. Percentages of different bacterial groups relative to total number of isolates were as follows:

Pseudomonas: 5.1 %

Staphylococcus: 29.1 %

Enterobacteriaceae: 16.5 %

Escherichia coli: 8.8 % and

Salmonella: 40.5 %.

From eggs the only isolates obtained were 3 representative of the family *Enterobacteriaceae*

4.2 Antibiotic susceptibility testing using the Calibrated Dichotomous Sensitivity (CDS) method

Antibiotic susceptibility testing of isolates from various samples were performed according to the Calibrated Dichotomous Sensitivity (CDS) method (Bell *et al.*, 1999). Results were interpreted based on the diameter of the zones of growth inhibition caused by the presence of the specific antibiotic in the growth medium.

4.2.1 Reading the inhibition zones

The diameter of the growth inhibition zone around the well correlate inversely with minimum inhibitory concentration from standard dilution tests (NCCLS, 1997). According to the standard interpretation suggested by Bell *et al.* (1999) an annular radius equal or larger than 6 mm, indicates that the isolate is susceptible to the action of the antibiotic and smaller than 6 mm, that the isolate is resistant.

4.2.2 Level of antibiotic resistance in *Pseudomonas* isolates

According to the results depicted in Table 4.6, 10 % of the total of 20 *Pseudomonas* isolated from the respective samples were found resistant to three antibiotics, namely kanamycin (Kana), erythromycin (Ery) and neomycin (Neo), 5 % to both tetracycline (Tetra) and streptomycin (Strep), 20 % to gentamicin (Genta), 25 % to oxytetracycline (Oxy) and 90 % to penicillin (Pen).

Table 4.6 - The relative numbers of *Pseudomonas* isolates resistant or susceptible to the different antibiotics

	Kana	Ery	Neo	Genta	Oxy	Tetra	Strep	Pen
Resistant	2	2	2	4	5	1	1	18
Susceptible	18	18	18	16	15	19	19	2

The number of the resistant *Pseudomonas* isolated from each of the respective samples is shown in Table 4.7.

Table 4.7 - The relative numbers of *Pseudomonas* isolates from chicken meat and pork showing resistance to the different antibiotics

Samples	Kana	Ery	Neo	Genta	Oxy	Tetra	Strep	Pen
Chicken	0	0	0	1	2	0	1	5
Pork	1	1	0	2	2	0	0	9
Manure	0	0	0	0	0	0	0	2
Feed	1	1	2	1	1	1	0	2

4.2.3 Level of antibiotic resistance in *Staphylococcus* isolates

Results depicted in Table 4.8 showed that 12 % of the total of 50 *Staphylococcus* isolated from the respective samples were resistant to both kanamycin (Kana) and gentamicin (Genta), 44 % to erythromycin (Ery), 20 % to both neomycin (Neo) and streptomycin (Strep), 76 % to oxytetracycline (Oxy), 52 % to tetracycline (Tet) and 8 % to penicillin (Pen).

Table 4.8 - The relative numbers of *Staphylococcus* isolates resistant or susceptible to the different antibiotics

	Kana	Ery	Neo	Genta	Oxy	Tetra	Strep	Pen
Resistant	6	22	10	6	38	26	10	4
Susceptible	44	28	40	44	12	24	40	46

The number of the resistant *Staphylococus* isolated from each of the respective samples is shown in Table 4.9.

Table 4.9 - The relative numbers of *Staphylococcus* isolated from chicken meat and pork showing resistance to the different antibiotics

Samples	Kana	Ery	Neo	Genta	Oxy	Tetra	Strep	Pen
Chicken	1	8	1	1	9	7	1	3
Pork	1	8	1	1	11	8	3	1
Manure	3	3	7	3	12	7	6	0
Feed	1	3	1	1	6	4	0	0

4.2.4 Level of antibiotic resistance in *Enterobacteriaceae* isolates

According to results depicted in Table 4.10, 14 % of the total of 42 *Enterobacteriaceae* isolated from the respective samples were resistant to kanamycin (Kana), 39 % to erythromycin (Ery), 21 % to neomycin (Neo), 7 % to gentamicin (Genta), 71 % to oxytetracycline (Oxy), 79 % to tetracycline (Tetra), 52 % to streptomycin (Strep), while 100 % of the isolates were resistant to penicillin (Pen).

Table 4.10 - The relative numbers of *Enterobacteriaceae* isolates resistant or susceptible to the different antibiotics

	Kana	Ery	Neo	Genta	Oxy	Tetra	Strep	Pen
Resistant	6	16	9	3	30	33	22	42
Susceptible	36	26	33	39	12	9	20	0

The number of the resistant *Enterobacteriaceae* isolated from each of the respective samples is shown in Table 4.11.

Table 4.11 - The relative numbers of *Enterobacteriaceae* isolates from chicken meat and pork showing resistance to the different antibiotics

Samples	Kana	Ery	Neo	Genta	Oxy	Tetra	Strep	Pen
Chicken	2	3	3	2	16	17	8	17
Pork	2	7	2	0	3	5	6	9
Manure	0	3	4	1	7	8	4	9
Feed	2	3	0	0	2	3	2	4
Eggs	0	0	0	0	2	0	2	2

4.2.5 Level of antibiotic resistance in *Escherichia coli* isolates

Results depicted in Table 4.12 showed that 43 % of the total of the 14 *Escherichia coli* isolated from the respective samples were resistant to erythromycin (Ery), 21 % to neomycin (Neo), 36 % to both gentamicin (Gen) and streptomycin (Strep) and 100 % of isolates the to three antibiotics, namely oxytetracycline (Oxy), tetracycline (Tet) and penicillin (Pen).

Table 4.12 - The relative numbers of *Escherichia coli* isolates resistant or susceptible to the different antibiotics

	Kana	Ery	Neo	Genta	Oxy	Tetra	Strep	Pen
Resistant	0	6	3	5	14	14	5	14
Susceptible	14	8	11	9	0	0	9	0

The number of the resistant *E. coli* isolated from each of the respective samples are shown in Table 4.13

Table 4.13 - The relative numbers of *Escherichia coli* isolates from chicken meat and pork meat showing resistance to the different antibiotics

Samples	Kana	Ery	Neo	Genta	Oxy	Tetra	Strep	Pen
Chicken	0	2	2	1	5	5	1	12
Pork	0	1	1	0	2	2	0	2
Manure	0	1	0	1	4	4	2	0
Feed	0	2	0	3	3	3	2	0

4.2.6 Level of antibiotic resistance in *Salmonella* isolates

According to results shown in Table 4.14, 33 % of the total of 72 *Salmonella* isolated from the various samples were resistant to kanamycin (Kana), 38 % to erythromycin (Ery), 11 % to neomycin (Neo), 63 % to gentamicin (Gen), 99 % to oxytetracycline (Oxy), 88 % to tetracycline (Tetra), 99 % to penicillin (Pen) and 46 % to streptomycin (Strep).

Table 4.14 - The relative numbers of *Salmonella* isolates resistant or susceptible to the different antibiotics

	Kana	Ery	Neo	Genta	Oxy	Tetra	Strep	Pen
Resistant	24	27	8	45	71	63	33	71
Susceptible	48	45	64	27	1	9	39	1

The number of the resistant *Salmonella* isolated from each of the respective samples are shown in Table 4.15.

Table 4.15 - The relative numbers of *Salmonella* isolates from chicken meat and pork showing resistance to the different antibiotics

Samples	Kana	Ery	Neo	Genta	Oxy	Tetra	Strep	Pen
Chicken	5	8	1	15	24	23	13	24
Pork	5	4	2	11	16	12	5	16
Manure	8	5	4	11	17	15	11	16
Feed	6	10	1	8	14	13	4	15

4.3 Cluster analysis

Cluster analysis was done by the Statistical Consulting Services (PU for CHE). The Wards Method was applied using STATISTICA for Windows Release 5.1 (1998) StaSoft, Inc, Tulsa, USA. Isolates were grouped together in clusters on the basis of similar pattern of antibiotic resistance.

4.3.1 Antibiotic resistance in *Pseudomonas* isolates

Clusters of *Pseudomonas* isolates exhibiting similar patterns of antibiotic resistance are illustrated in Figure 4.1. A large cluster consisting of 10 isolates has been identified.

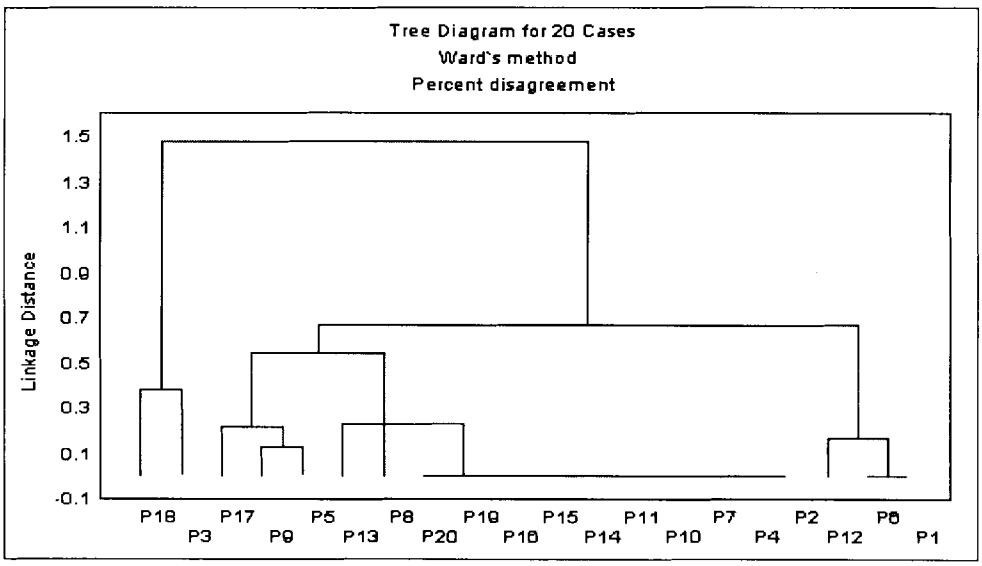

Figure 4.1 - Dendogram illustrating clusters of antibiotic resistant *Pseudomonas* isolates

The susceptibility/resistance pattern to the various antibiotics together with the Multiple Antibiotic Resistance (MAR) Index for each individual isolate is showed in Table 4.16.

Table 4.16 - Antibiotic susceptibility/resistance patterns of the individual *Pseudomonas* isolates

Isolate	Kana	Ery	Neo	Genta	Oxy	Tetra	Strep	Pen	MAR Index*	Cluster nr.
P1	s	s	s	R	s	s	s	R	0.25	Pse 2
P2	s	s	s	s	s	s	s	R	0.125	Pse 1
P3	R	R	s	s	R	s	s	R	0.5	
P4	s	s	s	s	s	s	s	R	0.125	Pse 1
P5	s	s	s	s	R	s	s	R	0.25	
P6	s	s	s	R	s	s	s	R	0.25	Pse 2
P7	s	s	s	s	s	s	s	R	0.125	Pse 1
P8	s	s	s	s	s	s	R	R	0.25	
P9	s	s	s	s	R	s	s	s	0.125	
P10	s	s	s	s	s	s	s	R	0.125	Pse 1
P11	s	s	s	s	s	s	s	R	0.125	Pse 1
P12	s	s	s	R	R	s	s	R	0.375	
P13	s	s	R	s	s	s	s	R	0.25	
P14	s	s	s	s	s	s	s	R	0.125	Pse 1
P15	s	s	s	s	s	s	s	R	0.125	Pse 1
P16	s	s	s	s	s	s	s	R	0.125	Pse 1
P17	s	s	s	s	s	s	s	s	0.0	
P18	R	R	R	R	R	R	s	R	0.875	
P19	s	s	s	s	s	s	s	R	0.125	Pse 1
P20	s	s	s	s	s	s	s	R	0.125	Pse 1
Res/Sus	2/18	2/18	2/18	4/16	5/15	1/19	1/19	18/2		

* The MAR Index for each individual isolate was calculated. For an individual isolate the MAR Index = the number of antibiotics to which the isolate was resistant, divided by the total number of antibiotics tested.

According to Figure 4.1 and Table 4.16, two clusters of antibiotic resistant isolates were identified for *Pseudomonas:* one large cluster (Pse 1) contained 10 isolates namely P2, P4, P7, P10, P11, P14, P15, P16, P19, and P20. The other cluster (Pse 2) contained 2 isolates, namely P1 and P6. Eight single isolates exhibited each a different resistant pattern where isolate P18 (from chicken feed) 87.5 % resistance showed. Cluster Pse 1 contain 40% isolates from pork meat, 10 % isolates from pork liver, 20 % chicken stomach and 10 % from chicken liver and 20 % from chicken manure while cluster Pse 2 consisted of 100 %

pork liver isolates (See Appendix A). The individual isolates were obtained from chicken, pork, chicken feed and manure (See Appendix A).

The antibiotic resistant patterns for the two major clusters for the *Pseudomonas* isolates are showed in Table 4.17.

Table 4.17 - Antibiotic resistant patterns of the two clusters identified for *Pseudomonas*

Cluster	Kana	Ery	Neo	Genta	Oxy	Tetra	Strep	Pen	MAR Index
Pse 1	s	s	s	s	s	s	s	R	0.125
Pse 2	s	s	s	R	s	s	s	R	0.25

4.3.2 Antibiotic resistance in *Staphylococcus* isolates

Clusters of *Staphylococcus* isolates exhibiting similar patterns of antibiotic resistance are illustrated in Figure 4.2. Two relatively large clusters consisting of 9 isolates each have been identified, as well as one cluster consisting of three isolates and three clusters of two isolates each.

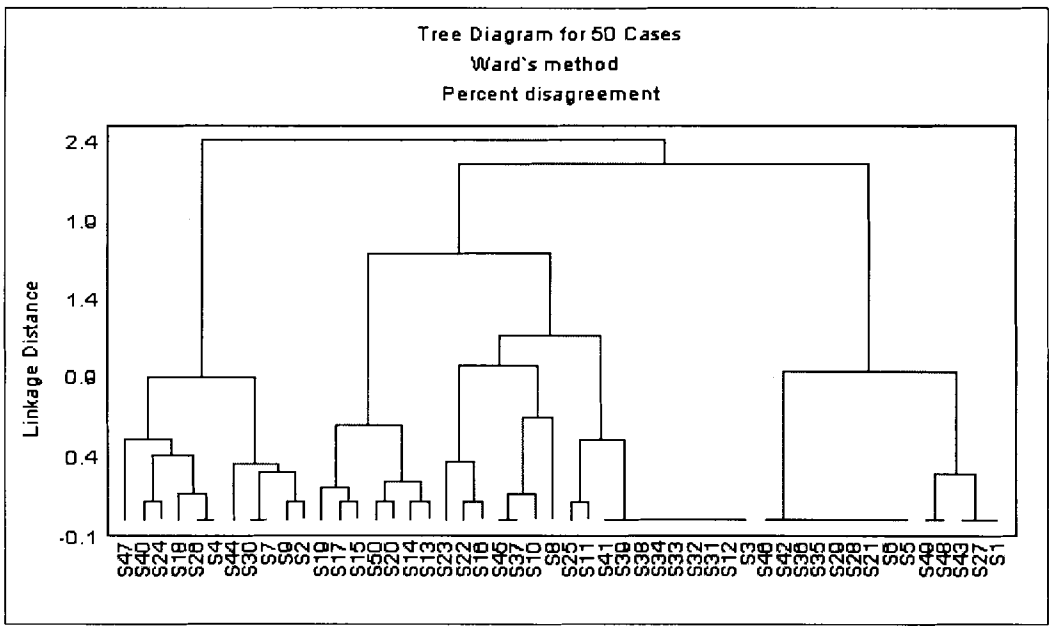

Figure 4.2 - Dendogram illustrating clusters of antibiotic resistant *Staphylococcus* isolates

The susceptibility/resistance pattern to the various antibiotics together with the MAR Index for each individual isolate is showed in Table 4.18.

Table 4.18 - Antibiotic susceptibility/resistance patterns of *Staphylococcus* isolates

Isolate	Kana	Ery	Neo	Genta	Oxy	Tetra	Strep	Pen	MAR Index	Cluster nr.
S1	s	R	s	s	R	s	s	s	0.25	Sta 7
S2	s	s	s	s	R	s	s	s	0.125	
S3	s	s	s	s	R	R	s	s	0.25	Sta 4
S4	s	R	s	s	S	s	s	s	0.125	Sta 1
S5	s	R	s	s	R	R	s	s	0.375	Sta 5
S6	s	R	s	s	R	R	s	s	0.375	Sta 5
S7	s	s	s	s	s	s	s	s	0.0	Sta 2
S8	R	R	R	s	R	s	s	R	0.625	
S9	R	s	s	s	R	s	s	s	0.125	
S10	s	s	s	s	R	s	s	R	0.25	
S11	s	s	s	s	s	R	s	s	0.125	
S12	s	s	s	s	R	R	s	s	0.25	Sta 4
S13	s	s	s	s	R	s	R	s	0.25	
S14	s	s	R	s	R	s	R	s	0.375	
S15	s	s	s	s	R	R	R	s	0.375	
S16	s	s	s	R	R	s	s	s	0.25	
S17	s	s	R	s	R	R	R	s	0.5	
S18	R	R	s	s	s	s	s	s	0.25	
S19	R	s	s	s	R	R	R	s	0.5	
S20	s	s	R	s	s	s	R	s	0.25	
S21	s	R	s	s	R	R	s	s	0.375	Sta 5
S22	s	s	R	R	R	s	s	s	0.375	
S23	s	R	R	R	R	R	s	s	0.625	
S24	s	R	R	R	s	s	s	s	0.375	
S25	s	s	s	R	s	R	s	s	0.25	
S26	s	R	s	s	s	s	s	s	0.125	Sta 1
S27	s	R	s	s	R	s	s	s	0.25	Sta 7
S28	s	R	s	s	R	R	s	s	0.375	Sta 5
S29	s	R	s	s	R	R	s	s	0.375	Sta 5
S30	s	s	s	s	s	s	s	s	0.0	Sta 2
S31	s	s	s	s	R	R	s	s	0.25	Sta 4
S32	s	s	s	s	R	R	s	s	0.25	Sta 4
S33	s	s	s	s	R	R	s	s	0.25	Sta 4
S34	s	s	s	s	R	R	s	s	0.25	Sta 4
S35	s	R	s	s	R	R	s	s	0.375	Sta 5

Table 4.18 (continued)

S36	s	R	s	s	R	R	s	s	0.375	Sta 5
S37	s	s	s	s	R	R	s	R	0.375	Sta 3
S38	s	s	s	s	R	R	s	s	0.25	Sta 4
S39	s	s	s	s	R	R	s	s	0.25	Sta 4
S40	s	R	R	s	s	s	s	s	0.25	
S41	s	s	s	s	R	R	s	s	0.25	Sta 4
S42	s	R	s	s	R	R	s	s	0.375	Sta 5
S43	s	R	s	s	R	s	s	s	0.25	Sta 7
S44	R	s	R	s	s	s	s	s	0.25	
S45	s	s	s	s	R	R	s	R	0.375	Sta 3
S46	s	R	s	s	R	R	s	s	0.375	Sta 5
S47	R	R	s	R	s	s	R	s	0.5	
S48	s	R	s	s	R	s	R	s	0.375	Sta 6
S49	s	R	s	s	R	s	R	s	0.375	Sta 6
S50	s	s	s	s	s	s	R	s	0.125	
Res/Sus	6/44	22/28	10/40	6/44	38/12	26/24	10/40	4/46		

According to Figure 4.2 and Table 4.18, seven clusters of antibiotic resistant isolates were identified for *Staphylococcus*. One large cluster, (Sta 4) contained the following isolates: S3, S12, S31, S32, S33, S34, S38, S39 and S41. The other large cluster (Sta 5) contained 9 isolates namely S5, S6, S21, S28, S29, S35, S36, S42 and S46. Cluster Sta 7 contained the following 3 isolates: S1, S27, S43 while clusters Sta 1 (S4, S26), Sta 2 (S7, S30), Sta 3 (S37, S45) and Sta 6 (S48, S49) each 2 isolates contained. Twenty-one single isolates exhibited each a different resistant pattern. The single isolate S23 (isolated from chicken manure) showed a very high resistance of 62.5 % to the antibiotics. Twenty-two percent of cluster Sta 4 consisted of isolates from chicken meat and chicken feed each, 44.4 % isolates from pork meat and 11.1 % isolates from chicken manure. Cluster Sta 5 contained 22.2 % isolates from pork liver, 44.4 % isolates from chicken meat and pork meat each. Chicken feed and chicken manure contained 11.1 % isolates. The smaller cluster, Sta 7 contained 33.3 % isolates from pork meat, chicken meat and chicken manure respectively (See Appendix B).

The antibiotic resistant patterns of the 7 clusters for the *Staphylococcus* isolates are showed in Table 4.19.

Table 4.19 - Antibiotic resistant patterns of the seven clusters identified for *Staphylococcus*

Cluster	Kana	Ery	Neo	Genta	Oxy	Tetra	Strep	Pen	MAR Index
Sta 1	s	R	s	s	s	s	s	s	0.125
Sta 2	s	s	s	s	s	s	s	s	0.0
Sta 3	s	s	s	s	R	R	s	R	0.375
Sta 4	s	s	s	s	R	R	s	s	0.25
Sta 5	s	R	s	s	R	R	s	s	0.375
Sta 6	s	R	s	s	R	s	R	s	0.375
Sta 7	s	R	s	s	R	s	s	s	0.25

4.3.3 Antibiotic resistance in *Enterobacteriaceae* isolates

Clusters of *Enterobacteriaceae* isolates exhibiting similar patterns of antibiotic resistance are illustrated in Figure 4.3. Clusters consisting of 7 and 5 isolates respectively have been identified, as well as two clusters of 4 isolates, one of three isolates and four small clusters of two isolates each.

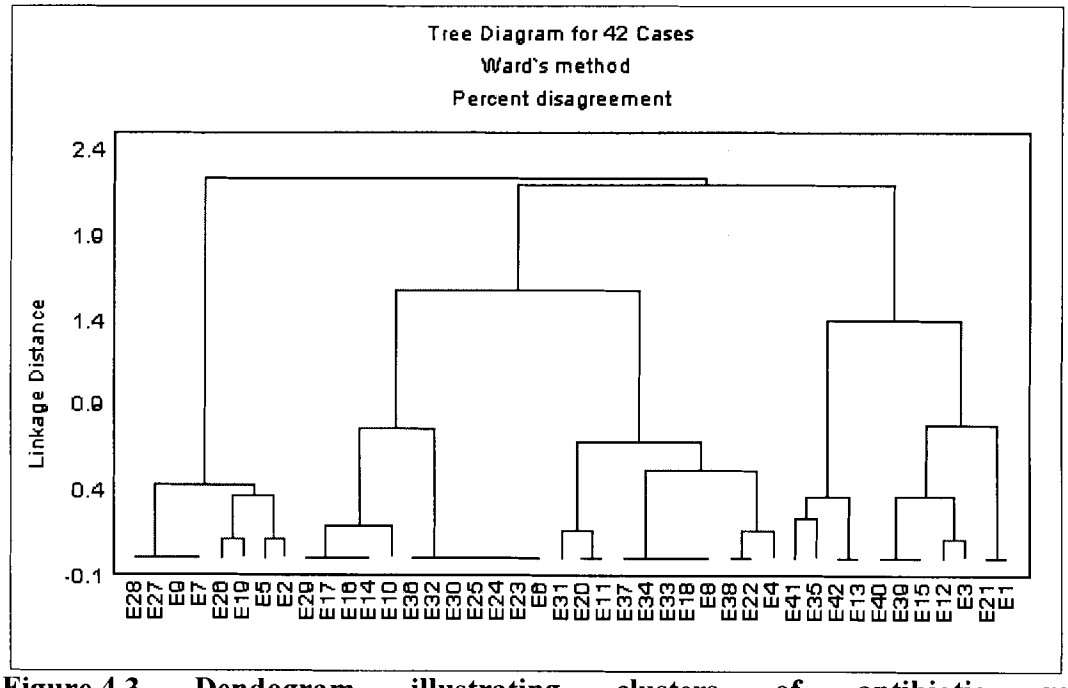

Figure 4.3 - Dendogram illustrating clusters of antibiotic resistant *Enterobacteriaceae* isolates

The susceptibility/resistance pattern to the various antibiotics together with the MAR Index for each individual isolate is showed in Table 4.20.

Table 4.20 - Susceptible/resistance pattern of the individual *Enterobacteriaceae* isolates and MAR Index values

Isolate	Kana	Ery	Neo	Genta	Oxy	Tetra	Strep	Pen	MAR Index	Cluster nr.
E1	s	R	R	s	s	s	R	R	0.125	Ent 9
E2	s	s	s	s	R	s	R	R	0.375	
E3	s	s	R	s	R	R	R	R	0.625	
E4	s	R	R	s	R	R	s	R	0.625	
E5	s	s	s	s	R	s	s	R	0.25	
E6	s	s	s	s	R	R	R	R	0.5	Ent 3
E7	s	s	s	s	s	s	s	R	0.125	Ent 1
E8	s	s	s	s	R	R	s	R	0.375	Ent 5
E9	s	s	s	s	s	s	s	R	0.125	Ent 1
E10	R	R	s	s	R	R	R	R	0.75	
E11	s	s	s	R	R	R	s	R	0.5	Ent 4
E12	s	s	R	s	R	R	s	R	0.5	
E13	R	R	s	s	s	R	s	R	0.5	Ent 7
E14	s	R	s	s	R	R	R	R	0.625	Ent 2
E15	s	R	R	s	R	R	R	R	0.75	Ent 8
E16	s	R	s	s	R	R	R	R	0.625	Ent 2
E17	s	R	s	s	R	R	R	R	0.625	Ent 2
E18	s	s	s	s	R	R	s	R	0.375	Ent 5
E19	s	s	s	s	s	s	R	R	0.25	
E20	s	s	s	R	R	R	s	R	0.5	Ent 4
E21	s	R	R	s	s	s	R	R	0.5	Ent 9
E22	s	R	s	s	R	R	s	R	0.5	Ent 6
E23	s	s	s	s	R	R	R	R	0.5	Ent 3
E24	s	s	s	s	R	R	R	R	0.5	Ent 3
E25	s	s	s	s	R	R	R	R	0.5	Ent 3
E26	s	s	s	s	s	R	R	R	0.375	
E27	s	s	s	s	s	s	s	R	0.125	Ent 1
E28	s	s	s	s	s	s	s	R	0.125	Ent 1
E29	s	R	s	s	R	R	R	R	0.625	Ent 2
E30	s	s	s	s	R	R	R	R	0.5	Ent 3
E31	R	s	s	R	R	R	s	R	0.625	
E32	s	s	s	s	R	R	R	R	0.5	Ent 3
E33	s	s	s	s	R	R	s	R	0.375	Ent 5
E34	s	s	s	s	R	R	s	R	0.375	Ent 5
E35	R	s	R	s	s	R	s	R	0.5	
E36	s	s	s	s	R	R	R	R	0.5	Ent 3

E37	s	s	s	s	R	R	s	R	0.375	Ent 5

Table 4.20 (continued)

E38	s	R	s	s	R	R	s	R	0.5	Ent 6
E39	s	R	R	s	R	R	R	R	0.75	Ent 8
E40	s	R	R	s	R	R	R	R	0.75	Ent 8
E41	R	R	R	s	s	R	R	R	0.75	
E42	R	R	s	s	s	R	s	R	0.5	Ent 7
Res/ Susc	6/36	16/26	9/33	3/39	30/12	33/9	22/20	42/0		

According to Figure 4.3 and Table 4.20 nine clusters of antibiotic resistant isolates were identified for *Enterobacteriaceae*. Cluster (Ent 3) contained 7 isolates namely E6, E23, E24, E25, E30, E32 and E36. The other large cluster (Ent 5) contained five isolates namely E8, E18, E33, E34 and E37. Clusters Ent 1 (E7, E9, E27 and E28) and Ent 2 (E14, E16, E17 and E29) contained 4 isolates, clusters Ent 8 (E18, E39 and E40) contained 3 isolates and clusters Ent 4 (E11 and E20), Ent 6 (E22 and E38), Ent 7 (E13 and E42) and Ent 9 (E1 and E21) contained 2 isolates each. Eleven single isolates exhibited each a different resistant pattern. Isolate E10 (from chicken feed) and E41(from pork liver) each showed an 87.5% resistance to the different antibiotics. Cluster Ent 3 consisted of 14.24 % organisms isolated from chicken manure, chicken stomach, chicken meat, chicken liver and eggsrepestively. Cluster Ent 5 contained 20 % isolates from chicken manure, 60 % isolates from chicken stomach and 20 % isolates from chicken liver (See Appendix C).

The antibiotic resistant patterns of the 9 different clusters for the *Enterobacteriaceae* isolates are showed in Table 4.21.

Table 4.21 - Antibiotic resistant patterns of the seven clusters identified for *Enterobacteriaceae* and MAR Index values

Cluster	Kana	Ery	Neo	Genta	Oxy	Tetra	Strep	Pen	MAR Index
Ent 1	s	s	s	s	s	s	s	R	0.125
Ent 2	s	R	s	s	R	R	R	R	0.625
Ent 3	s	s	s	s	R	R	R	R	0.5
Ent 4	s	s	s	R	R	R	s	R	0.5
Ent 5	s	s	s	s	R	R	s	R	0.375
Ent 6	s	R	s	s	R	R	s	R	0.5
Ent 7	R	R	s	s	s	R	s	R	0.5
Ent 8	s	R	R	s	R	R	R	R	0.75

Ent 9	s	R	R	s	s	s	R	R	0.125

4.3.4 Antibiotic resistance in *Escherichia coli* isolates

Clusters of *Escherichia coli* isolates exhibiting similar patterns of antibiotic resistance are illustrated in Figure 4.3. One clusters consisting of 3 isolates, and two of two isolates each have been identified for *Escherichia coli* isolates.

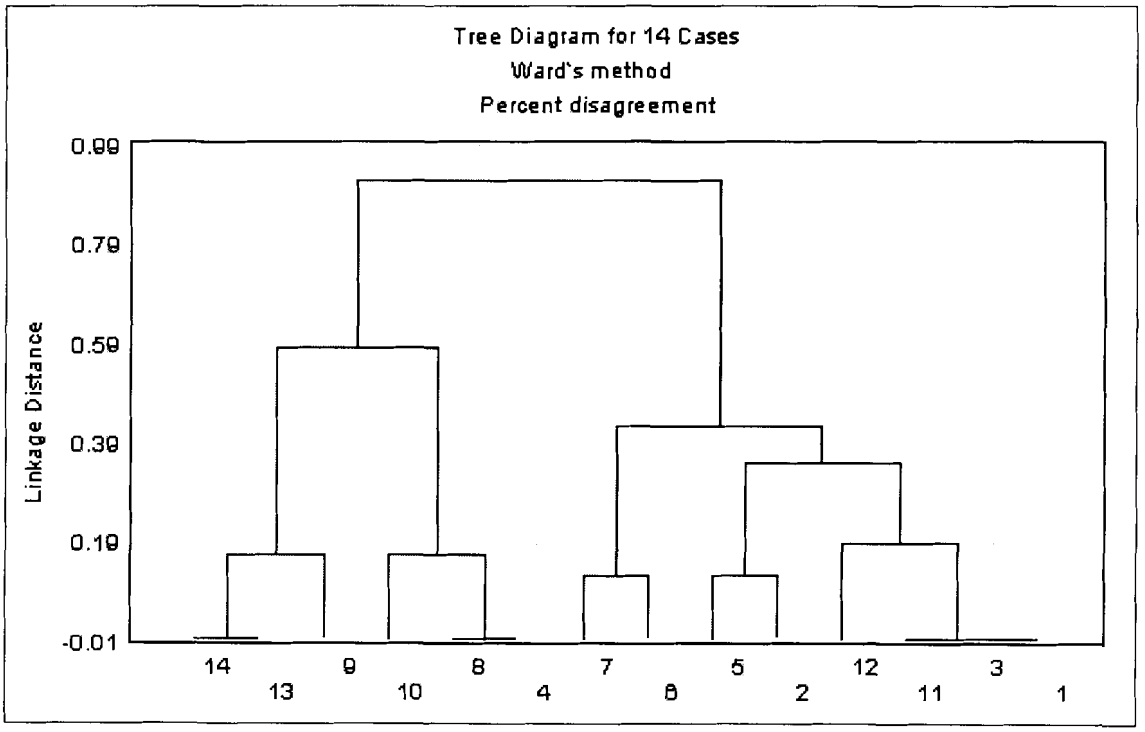

Figure 4.4 - Dendogram illustrating clusters of antibiotic resistant *Escherichia coli* isolates

The pattern of susceptibility for, or resistance to the different antibiotics together with the MAR Index for each individual isolate are shown in Table 4.22.

Table 4.22 - Antibiotic susceptibility/resistance patterns of the individual *Escherichia coli* isolates

Isolate	Kana	Ery	Neo	Genta	Oxy	Tetra	Strep	Pen	MAR Index	Cluster nr.
C1	s	s	s	s	R	R	s	R	0.375	Esc 3
C2	s	s	R	R	R	R	s	R	0.625	
C3	s	s	s	s	R	R	s	R	0.375	Esc 3
C4	s	s	s	s	R	R	R	R	0.5	Esc 2
C5	s	s	s	R	R	R	s	R	0.5	
C6	s	R	R	s	R	R	s	R	0.625	
C7	s	R	s	s	R	R	s	R	0.5	
C8	s	s	s	s	R	R	R	R	0.5	Esc 2
C9	s	R	s	R	R	R	s	R	0.625	
C10	s	R	s	s	R	R	R	R	0.625	
C11	s	s	s	s	R	R	s	R	0.375	Esc 3
C12	s	s	R	s	R	R	s	R	0.5	
C13	s	R	s	R	R	R	R	R	0.75	Esc 1
C14	s	R	s	R	R	R	R	R	0.75	Esc 1
Res/Sus	0/14	6/8	3/11	5/9	14/0	14/0	5/9	14/0		

According to Figure 4.4 and Table 4.22 three clusters of antibiotic resistant isolates were identified for *Escherichia coli.* Cluster (Esc 3) contained the following isolates: C1, C3 and C11. The other clusters (Esc 2) contained 2 isolates namely C4 and C8 and Esc 1 contained C13 and C14. Seven single isolates exhibited each a different resistant pattern. Cluster Esc 1 contained 100 % isolates from chicken feed while Esc 2 100 % isolates from chicken manure contained. Thirty-three percent of cluster Esc 3 was isolates from chicken manure, pork meat and chicken liver each (See Appendix D).

The antibiotic resistant patterns of the 3 different clusters for the *Escherichia coli* isolates are showed in Table 4.23.

Table 4.23 - Antibiotic resistant patterns of the seven clusters identified for *Escherichia coli*

luster	Kana	Ery	Neo	Genta	Oxy	Tetra	Strep	Pen	MAR Index
Esc 1	s	R	s	R	R	R	R	R	0.75
Esc 2	s	S	s	s	R	R	R	R	0.5
Esc 3	s	S	s	s	R	R	s	R	0.375

4.3.5 Antibiotic resistance in *Salmonella* isolates

Clusters of *Salmonella* isolates exhibiting similar patterns of antibiotic resistance are illustrated in Figure 4.5. One cluster consisting of 10 isolates, two of seven isolates each, two clusters of six isolates each, three of four isolates each and four clusters of two isolates each have been identified for *Salmonella* isolates.

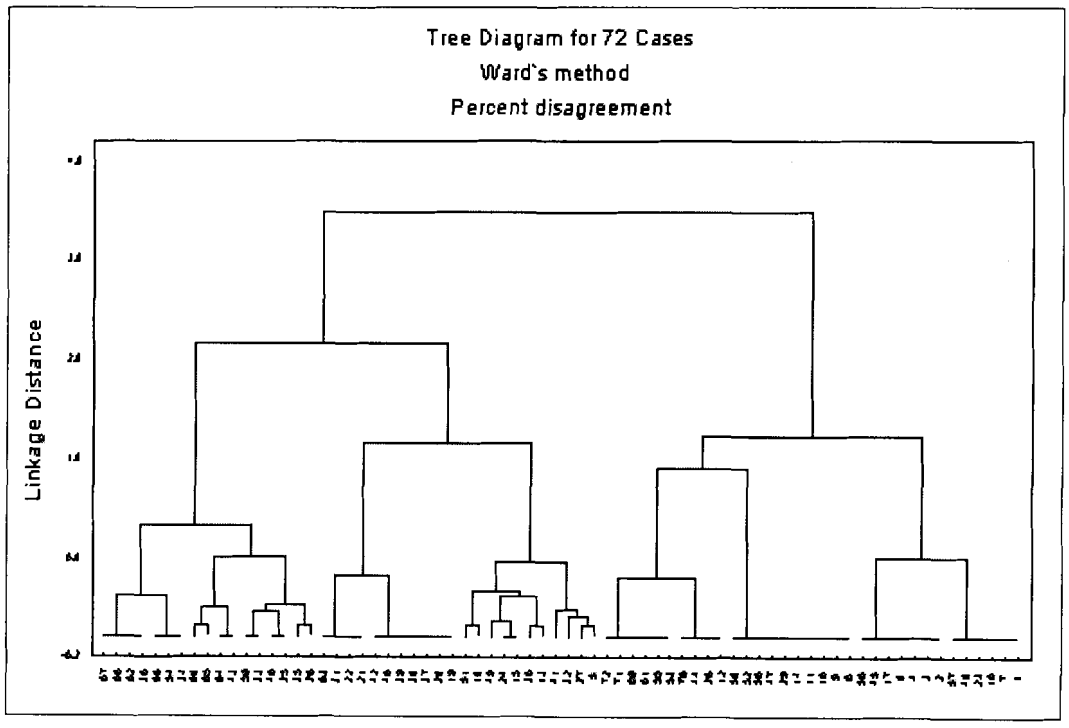

Figure 4.5 - Dendogram illustrating clusters of antibiotic resistant *Salmonella* isolates

The susceptibility/resistance pattern to the various antibiotics together with the MAR Index for each individual isolate is showed in Table 4.24.

Table 4.24 - Antibiotic susceptibility/resistance patterns of the individual *Salmonella* isolates

Salmo-nella isolate	Kana	Ery	Neo	Genta	Oxy	Tetra	Strep	Pen	MAR Index	Cluster nr.
1	s	s	s	R	R	R	R	R	0.625	Sal 13
2	s	s	s	R	R	R	s	R	0.5	Sal 12
3	s	s	s	R	R	R	s	R	0.5	Sal 12
4	s	s	s	R	R	R	s	R	0.5	Sal 12
5	s	s	s	s	R	S	s	R	0.25	
6	s	R	s	R	R	R	R	R	0.75	Sal 11
7	s	s	s	R	R	R	R	R	0.625	Sal 13
8	s	s	s	R	R	R	s	R	0.5	Sal 12
9	s	R	s	R	R	R	R	R	0.75	Sal 11
10	s	R	s	R	R	R	R	R	0.75	Sal 11
11	s	R	s	R	R	R	R	R	0.75	Sal 11
12	R	R	s	R	R	R	R	R	0.875	Sal 10
13	s	s	s	R	R	s	R	R	0.5	
14	s	R	s	R	R	R	R	R	0.75	Sal 11
15	s	s	s	s	R	s	R	R	0.375	Sal 8
16	s	s	s	R	R	R	R	R	0.625	Sal 13
17	s	s	s	R	R	R	s	R	0.5	Sal 12
18	s	s	s	s	R	R	R	R	0.5	
19	s	s	s	s	R	R	s	R	0.25	Sal 7
20	s	R	s	s	R	R	s	R	0.5	
21	s	s	R	s	R	R	s	R	0.5	Sal 6
22	s	s	R	s	R	R	s	R	0.5	Sal 6
23	s	s	s	R	R	R	R	R	0.625	Sal 13
24	s	s	s	s	R	s	R	R	0.375	Sal 8
25	s	R	s	R	R	R	s	R	0.625	Sal 5
26	R	R	s	R	R	R	R	R	0.875	Sal 10
27	s	R	s	s	R	s	s	R	0.375	
28	s	s	s	s	R	R	s	R	0.25	Sal 7
29	s	R	s	R	R	R	R	R	0.75	Sal 11
30	s	R	s	R	R	s	R	R	0.625	
31	s	s	R	s	R	R	s	R	0.5	Sal 6
32	s	s	s	R	R	s	s	R	0.375	
33	R	R	s	R	R	R	S	R	0.75	Sal 4
34	R	s	s	R	R	R	s	R	0.625	Sal 2
35	R	R	s	s	R	R	s	R	0.625	
36	R	s	s	s	R	R	s	R	0.5	Sal 1
37	s	s	s	s	R	R	s	R	0.25	Sal 7

Table 4.24 (continued)

38	s	s	s	s	R	R	s	R	0.25	Sal 7
39	s	s	s	s	R	R	s	R	0.25	Sal 7
40	s	s	s	s	R	R	s	R	0.25	Sal 7
41	s	R	s	s	s	s	s	R	0.25	
42	s	s	s	s	R	R	s	R	0.25	Sal 7
43	R	R	R	R	R	R	s	R	0.875	Sal 3
44	R	R	s	R	R	R	R	R	0.875	Sal 10
45	s	s	s	R	R	R	s	R	0.5	Sal 12
46	s	R	s	R	R	R	s	R	0.625	Sal 5
47	s	R	s	R	R	R	R	R	0.75	Sal 11
48	s	s	s	R	R	R	R	R	0.625	Sal 13
49	s	s	s	s	R	s	R	s	0.25	
50	s	R	s	R	R	R	R	R	0.75	Sal 11
51	s	R	s	s	R	R	R	R	0.625	
52	s	R	s	R	R	R	R	R	0.75	Sal 11
53	R	s	s	R	R	R	R	R	0.75	Sal 9
54	R	s	s	R	R	R	S	R	0.625	Sal 2
55	R	s	s	R	R	R	R	R	0.75	Sal 9
56	s	s	s	R	R	R	s	R	0.5	Sal 12
57	s	s	s	R	R	R	R	R	0.625	Sal 13
58	s	R	s	R	R	R	R	R	0.75	Sal 11
59	R	R	s	R	R	R	s	R	0.75	Sal 4
60	R	s	s	R	R	R	s	R	0.625	Sal 2
61	R	s	s	R	R	R	R	R	0.75	Sal 9
62	R	s	s	s	R	R	s	R	0.5	Sal 1
63	s	s	R	s	R	R	s	R	0.5	Sal 6
64	R	R	R	R	R	R	s	R	0.875	Sal 3
65	R	R	R	s	R	R	s	R	0.75	
66	R	s	s	s	R	R	s	R	0.5	Sal 1
67	R	s	s	s	R	R	s	R	0.5	Sal 1
68	R	s	R	s	R	R	s	R	0.625	
69	R	s	s	R	R	R	R	R	0.75	Sal 9
70	R	R	s	R	R	R	R	R	0.875	Sal 10
71	R	s	s	R	R	R	R	R	0.75	Sal 9
72	R	s	s	R	R	R	R	R	0.75	Sal 9
Res/Sus	24/48	27/45	8/64	45/27	71/1	63/9	33/39	71/1		

According to Figure 4.5 and Table 4.24 thirteen clusters of antibiotic resistant isolates were identified for *Salmonella.* Cluster Sal 11 (isolates 6, 9, 10, 11, 14, 29, 47, 50, 52 and 58) contained 10 isolates while cluster Sal 7 (isolates 19, 28, 37, 38, 39, 40 and 42) and

Sal 12 (isolates 2, 3, 4, 8, 17 45 and 56) each 7 isolates and, Sal 9 (isolates 53, 55, 61, 69, 71 and 72) and Sal 13 (isolates 1, 7, 16, 23, 48 and 57) each 6 isolates contained. Three clusters contained each four isolates, namely Sal 1 (isolates 36, 62, 66 and 67), Sal 6 (isolates 21, 22, 31, 63) and Sal 10 (isolate 12, 26, 44, 70). Cluster Sal 2 contained 3 isolates (isolates 34, 54, 60) and clusters Sal 3 (isolates 43, 64), Sal 4 (isolates 33,59), Sal 5 (isolates 25, 46) and Sal 8 (isolates 15, 24) contained 2 isolates each. Thirteen single isolates exhibited each a different resistant pattern. Sal 11 contained 30 % chicken manure isolates, 20 % chicken stomach isolates and 10 % isolates from chicken meat, pork meat, chicken feed, pork liver and chicken liver each. Fifty-seven percent of cluster Sal 7 was pork meat isolates, 28.57 % chicken feed isolates and 14.28 % isolates from chicken stomach. Sal 12 contained 42.85 % chicken liver isolates, 28.57 % pork liver isolates and 14.28 % isolates from chicken feed and chicken meat each. Fifty percent of cluster Sal 9 contained chicken manure isolates, 33.3 % isolates was from chicken liver and 16.6 % was from pork meat. Cluster Sal 13 contained 33.3 % chicken manure isolates and 16.6 % isolates from chicken liver, chicken meat, pork meat and chicken stomach (See Appendix E).

The antibiotic resistant patterns of the 13 different clusters for *Salmonella* isolates are showed in Table 4.25.

Table 4.25 - Antibiotic resistant patterns of the thirteen clusters identified for *Salmonella*

Cluster	Kana	Ery	Neo	Genta	Oxy	Tetra	Strep	Pen	MAR Index
Sal 1	R	s	s	s	R	R	s	R	0.5
Sal 2	R	s	s	R	R	R	s	R	0.625
Sal 3	R	R	R	R	R	R	s	R	0.875
Sal 4	R	R	s	R	R	R	s	R	0.75
Sal 5	s	R	s	R	R	R	s	R	0.625
Sal 6	s	s	R	s	R	R	s	R	0.5
Sal 7	s	s	s	s	R	R	s	s	0.25
Sal 8	s	s	s	s	R	s	R	R	0.375
Sal 9	R	s	s	R	R	R	R	R	0.75
Sal 10	R	R	s	R	R	R	R	R	0.875
Sal 11	s	R	s	R	R	R	R	R	0.75
Sal 12	s	s	s	R	R	R	s	R	0.5
Sal 13	s	s	s	R	R	R	R	R	0.625

4.4 Major antibiotic resistant clusters

Table 4.26 shows the different major clusters for each isolated bacterial group with the represented number of isolates in each cluster.

Table 4.26 - Major clusters with the number of isolates in each cluster

Pseudomonas		***Staphylococcus***		***Enterobacteriaceae***		***Escherichia coli***		***Salmonella***	
Clusters	No. of isolates	Clusters	No. of isolates	Clusters	No. of isolates	Clusters	No. of isolates	Clusters	No. of isolates
Pse 1	10	Sta 1	2	Ent 1	4	Esc 1	2	Sal 1	4
Pse 2	2	Sta 2	2	Ent 2	4	Esc 2	2	Sal 2	3
		Sta 3	2	Ent 3	7	Esc 3	3	Sal 3	2
		Sta 4	9	Ent 4	2			Sal 4	2
		Sta 5	9	Ent 5	5			Sal 5	2
		Sta 6	2	Ent 6	2			Sal 6	4
		Sta 7	3	Ent 7	2			Sal 7	7
				Ent 8	3			Sal 8	2
				Ent 9	2			Sal 9	6
								Sal 10	4
								Sal 11	10
								Sal 12	7
								Sal 13	6
Single isolates (not contained in a cluster)									
8		21		11		7		13	

Thirteen different clusters are present in the *Salmonella* group, with 9 clusters in the *Enterobacteriaceae*, 7 in the *Staphylococcus* isolates, 3 in the *Escherichia coli* group and 2 in the *Pseudomonas* isolates. Cluster Pse 1 and Sal 11 exhibit each 10 isolates, while 2 clusters (Sta 5 and Sta 6) each 9 isolates separately contain. Three clusters (Ent 3, Sal 7 and Sal 12) each contain 7 isolates. Clusters Sal 9 and Sal 13 each contain 6 isolates. The rest of the major clusters contain 5 or less isolates. The total number of isolates present in the major clusters is 138 with 60 individual isolates. The antibiotic resistant patterns of the

thirty-three clusters (see Table 4.26) are showed in Table 4.27. They were named, M1 to M33.

Table 4.27 - Antibiotic resistant patterns for clusters M1 to M33

Cluster	Kana	Ery	Neo	Genta	Oxy	Tetra	Strep	Pen	Representive clusters for each group of organisms
M1	R	R	s	R	R	R	R	R	Sal 10
M2	s	R	s	R	R	R	R	R	Esc 1, Sal 11
M3	s	R	R	s	S	s	R	R	Ent 9
M4	s	R	R	s	R	R	R	R	Ent 8
M5	s	R	s	s	R	R	R	R	Ent 2
M6	R	R	R	R	R	R	s	R	Sal 3
M7	R	R	s	R	R	R	s	R	Sal 4
M8	s	R	s	R	R	R	s	R	Sal 5
M9	s	R	s	s	R	R	s	R	Ent 6
M10	s	R	R	s	R	R	s	R	*
M11	R	R	s	s	s	R	s	R	Ent 7
M12	s	s	s	s	R	s	R	R	Sal 8
M13	s	s	s	s	s	s	R	R	**
M14	s	s	s	R	R	R	R	R	Sal 13
M15	s	s	s	s	R	R	R	R	Ent 3, Esc 2
M16	s	R	s	s	R	s	R	s	Sta 6
M17	s	s	s	s	R	s	R	s	***
M18	R	s	s	R	R	R	R	R	Sal 9
M19	R	s	s	R	R	R	s	R	Sal 2
M20	s	s	s	R	R	R	s	R	Ent 4, Sal 12
M21	R	s	s	s	R	R	s	R	Sal 1
M22	s	s	R	s	R	R	s	R	Sal 6
M23	s	s	s	s	R	R	s	R	Sta 3, Ent 5, Esc 3, Sal 7
M24	s	R	s	s	R	R	s	s	Sta 5
M25	s	s	s	s	R	R	s	s	Sta 4
M26	s	s	s	s	s	s	s	R	Pse 1, Ent 1
M27	s	s	s	R	R	s	s	R	****
M28	s	s	s	R	s	s	s	R	Pse 2
M29	s	s	s	s	R	s	s	R	*****
M30	s	s	s	s	R	s	s	s	******
M31	s	s	s	s	s	s	s	s	Sta 2
M32	s	R	s	s	s	s	s	s	Sta 1
M33	s	R	s	s	R	s	s	s	Sta 7

* Individual isolate Ent 4 (see Table 4.20) and Esc 6 (see Table 4.22).

** Individual isolate Ent 19 (see Table 4.20) and Pse 8 (see Table 4.16).

*** Individual isolate Sal 49 (see Table 4.24) and Sta 13 (see Table 4.18).

**** Individual isolate Pse 12 (see Table 4.16) and Sal 32 (see Table 4.24).

***** Individual isolate Pse 5 (see Table 4.16), Ent 5 (see Table 4.20), Sal 5 (see Table 4.24) and Sta 10 (see Table 4.18).

****** Individual isolate Pse 9 (see Table 4.16) and Sta 2 (see Table 4.18).

According to the number of isolates, 11 large clusters with five or more organisms were present (see Table 4.28). The rest of the 22 major clusters contain less than 5 isolates.

Table 4.28 - Number of isolates in each of the major clusters

Clusters	Number of Isolates	Clusters	Number of Isolates	Clusters	Number of Isolates	Clusters	Number of Isolates
M 1	4	**M 11**	2	**M 21**	4	**M 31**	3
M 2	12	**M 12**	3	**M 22**	6	**M 32**	2
M 3	2	**M 13**	2	**M 23**	17	**M 33**	3
M 4	3	**M 14**	6	**M 24**	9		
M 5	6	**M 15**	10	**M 25**	9		
M 6	3	**M 16**	2	**M 26**	14		
M 7	2	**M 17**	2	**M 27**	2		
M 8	3	**M 18**	6	**M 28**	2		
M 9	4	**M 19**	4	**M 29**	4		
M 10	2	**M 20**	10	**M 30**	2		
Single isolates (not contained in a cluster)							
33							

4.5 Ranking of the antibiotics

Using the data depicted in Tables 4.6 to 4.14, the respective antibiotics were ranked according to the number of isolates resistant to each of them. Results shown in Table 4.29 give an indication of the overall level of resistance towards each of the respective antibiotics that was observed amongst isolates from the various groups of bacteria.

Table 4.29 - Ranking of antibiotics according to number of resistant isolates

Antibiotic	No. of resistant isolates
Oxy	158
Pen	149
Tetra	137
Ery	73
Strep	71
Genta	63
Kana	38
Neo	32

According to results in Table 4.29, the highest number of resistant isolates was obtained for oxytetracycline (Oxy), followed by penicillin (Pen), tetracycline (Tetra), erythromycin (Ery), streptomycin (Srep), gentamicin (Gen) and kanamycin (Kan). The smallest number of resistant isolates was towards neomycin.

CHAPTER 5

DISCUSSION AND CONCLUSION

5.1 Isolation

Single and multiple antibiotic resistance (MAR) in microorganisms raise serious concerns in researchers all over the world. In consequence of studies about MAR bacteria in other countries (Newsome *et al.,* 1987; Mattila *et al.,* 1988; Epling and Carpenter, 1990; Vazquez-Moreno *et al.,* 1990; Krumperman, 1993; Salmon *et al.,* 1995; Klein *et al.,* 1998; Moro *et al.,* 1998) this investigation of MAR bacteria in pork and chicken meat as well as chicken manure, feed and eggs was conducted.

A total of 198 organisms were isolated from various samples of chicken and pork meat, chicken manure, chicken feed and eggs (see par. 3.2). Seventy-two of the total isolates picked off were *Salmonella* species (see Table 4.1). Studies conducted by Khachatourians (1998), showed that poultry that are given antibiotics often carry antibiotic-resistant strains of *Salmonella* or antibiotic-resistant transposons, which eventually reach humans through the meat and eggs. It has been found repeatedly that chickens, pigs, and cattle are frequent asymptomatic chronic *Salmonella* carriers and that these animals, especially pigs, infect others en route to the abattoir as they travel in crowded and unsanitary conditions and are stressed by such crowding and also by food and water deprivation. As a consequence, they excrete large number of *Salmonella* organisms that contaminate the carcasses and meat products: The *Salmonella* organisms isolated from animals and their products show a high frequency of multiple resistance (Novick, 1978).

The rest of the total percentage of the isolates picked off during this study was *Staphylococcus* species (50), followed by the *Enterobacteriaceae* (42), *Pseudomonas* species (20) and *E. coli* isolates (14) (see Table 4.1).

The frequency of microorganisms isolated from chicken tissues according to Vazquez-Moreno *et al.* (1990) were as followed: *E. coli* (80%), *Staphylococcus epidermis* (70%) and *Hafnia alvei* (50%), while *Salmonella* and *Enterobacter agglomerans* were present in

20-30% of the samples. Other microorganisms, for example *Pseudomonas* and *Staphylococcus aureus*, were present in less than 10% of the samples. According to Novick (1978) during the slaughtering process, contamination of carcasses with intestinal microorganisms cannot be prevented. Meat and meat products are often contaminated with antibiotic-resistant *E. coli* and these often reach the human consumer. It was demonstrated that 52 percent of the bovine (beef) and 83 percent of porcine (pork) carcasses slaughtered at commercial abattoirs are contaminated with *E. coli.*

The highest number of isolates during this investigation was obtained from chicken feed and manure (79 isolates, see Table 4.5). This was followed by isolates from chicken meat (63 isolates, see Table 4.3), pork meat (53 isolates, see Table 4.4) and eggs (3 isolates).

In previous studies antibiotic resistance was investigated in 474 *E. coli* isolates recovered from animal faeces (broilers, pigs, pets, bulls and horses), human faeces (patients and healthy volunteers) and food products of animal origin. *E. coli* isolates (3260) recovered from human significant infectious samples were also included. There was a high frequency of nalidixic acid, ciprofloxacin and gentamicin resistance in *E. coli* isolates from broilers (88%, 38% and 40%, respectively), and from foods (53, 13 and 17%). High levels of resistance to trimethoprim-sulphamethoxazole and tetracycline have been found in *E. coli* isolates from broilers, pigs and foods. These data raise important questions about the potential impact of antibiotic use in animals and the possible entry of resistant pathogens into the food chain (Saénz *et al.,* 2001).

5.2 Antibiotic susceptibility testing using the CDS method

5.2.1 *Pseudomonas*

Pseudomonas species occur as free-living bacteria or in association with plants and animals (Atlas, 1997). *Pseudomonas* often possess one or more plasmids in addition to their chromosomal genes (Atlas, 1997). In this study little resistance was observed to kanamycin (10%), erythromycin (10%), neomycin (10%), tetracycline (5%) and streptomycin (5%) while moderate resistance was indicated to gentamicin (20%) and oxytetracycline (25%) (see Table 4.6). From the 20 isolates of *Pseudomonas,* 90% showed resistance to penicillin (see Table 4.6). Isolates from chicken and pork meat were

observed to be most resistant to penicillin while little resistance was observed to the other antibiotics (see Table 4.7). It is clear from Table 4.7 that the highest number of resistant *Pseudomonas* isolates was obtained from pork samples (meat and liver) (15 isolates), followed by chicken samples (meat, stomach and liver) (9 isolates) and feed (9 isolates) and 2 isolates from chicken manure. Research done by Vazquez-Moreno *et al.* (1990) showed penicillin, streptomycin and tetracycline resistant *Pseudomonas* species were also isolated from chicken tissue. They, however, found none gentamicin resistant *Pseudomonas* species during their investigation.

5.2.2 *Staphylococcus*

Staphylococcus aureus has been recognized to encode for a variety of antibiotic resistance determinants (Thomson and Holding, 1986). In this study all the *Staphylococcus* isolates show little resistance to kanamycin (12%), gentamicin (12%) and penicillin (8%), whereas 76% showed resistance to oxytetracycline (see Table 4.8). The chicken manure isolates (41 isolates) show multiple antibiotic resistance to all the antibiotics except penicillin while the isolates from chicken samples (meat, stomach and liver) (31 isolates), chicken feed (16 isolates) and pork samples (meat and liver) (34 isolates) contribute less to resistance (see Table 4.9).

Studies done by Newsome *et al.* (1987) showed that 67% of 160 staphylococci isolates from beef steaks were sensitive to 13 antimicrobial agents tested. The study also revealed that antibiotic resistance was greater for staphylococci from restructured steaks (6%) than from conventional steaks (16%). Thirty eight percent of the restructured and 15% of the conventional steak isolates were resistant to tetracycline.

5.2.3 *Enterobacteriaceae*

In the Netherlands, the emergence of resistance to the fluoroquinolone antibiotics coincide with their increased use in human and veterinary medicine to treat infections caused by *Enterobacter* and *Campylobacter* species (Khachatourians, 1998).

In this study little resistance was observed to kanamycin (14%), neomycin (21%) and gentamicin (7%) while moderate resistance was indicated to erythromycin (39%) (see

Table 4.10). Isolates were observed to be most resistant to oxytetracycline (71%), tetracycline (79%) and streptomycin (52%) and 100 % resistant to penicillin (see Table 4.10). Isolates of chicken samples (meat, stomach and liver) (68 isolates) show high resistance to oxytetracycline, tetracycline and penicillin while the isolates from chicken manure (36 isolates), chicken feed (16 isolates) pork samples (meat and liver) (34 isolates) and eggs (6 isolates) contribute less to resistance (see Table 4.11).

A study done by Newsome *et al.* (1987) showed that 63% of 97 *Enterobacteriaceae* steak isolates were resistant to cephalothin. 10% was resistant to streptomycin and 11% was resistant to tetracycline. Less than 10% had multiple resistance.

According to Bonfiglio and coworkers (2002), the prevalence of extended-spectrum β-lactamase (ESBL) production by consecutive non-repeated isolates of *Enterobacteriaceae* was determined over a 6-month period. A total of 8015 strains were isolated from ten Italian laboratories and 509 (6.3%) of these were designated ESBL producers from the results of a double-disc synergy test. *Escherichia coli* was the most isolated microorganism, followed by *Klebsiella pneumoniae* and *Proteus mirabilis. Providencia stuartii* (28.1%) was the most frequently isolated ESBL producer. *K. pneumoniae* (38.2%) was the most represented followed by *P. mirabilis* (25.7%).

5.2.4 *Escherichia coli*

Escherichia coli is ubiquitous and is also found in the gastrointestinal tracts of humans and warmblooded animals (Atlas, 1997). Antibiotic resistance is very common among gut coliforms, and it is likely that everyone, well or ill, carries some resistant coliforms at all times (Richmond, 1972). *E. coli* can harbour antibiotic resistant plasmids which can spread naturally among species and can colonise the intestinal tracts of humans and other species who live in the same environment (Feinman, 1999).

In this study the *E. coli* isolates shows 100% resistance to oxytetracycline, tetracycline and penicillin and no resistance to kanamycin (see Table 4.12). Isolates of all the samples also show resistance to erythromycin (43%) (see Table 4.13). It is clear from Table 4.13 that the highest number of resistant *E. coli* isolates was obtained from chicken samples

(meat, stomach and liver) (28 isolates) followed by chicken feed (13 isolates), chicken manure (12 isolates) and pork samples (meat and liver) (8 isolates).

Moro *et al.* (1998) showed antimicrobial resistance patterns for *E. coli* isolated from pigs, ranged from 5 to 10% for ampicillin and from 25 to 55% for tetracycline. According to studies done by Vazquez-Moreno *et al.* (1990) *E. coli* isolated from chicken carcasses had similar multiple resistance except for lower frequency to ampicillin and gentamicin. Levy *et al.* (1975) found that 78% of *E. coli* isolates from faecal matter of chickens was resistant to chloramphenicol. The isolates also showed resistance to streptomycin and sulphonamides, but not to tetracycline.

5.2.5 *Salmonella*

During the past decade, numerous investigations have been initiated to examine the effects of sub-therapeutic doses of antibiotics in animal feeds on *Salmonella* (Epling and Carpenter, 1990). An earlier study (Novick, 1978) shows it is very probable that virtually all *Salmonella* of farm origin are capable of infecting humans. A single specific serotype, namely *S. typhimurium,* is by far the most common cause of salmonellosis both in animals and in man, moreover, certain other serotypes are very commonly isolated from both man and animals. For example, in the CDC 1974 Annual *Salmonella* Surveillance (CDC, 1974), it is reported that 6 of the most common human serotypes were among the 10 most common animal serotypes. Similar results have been reported for European isolates. It has even been granted that there are some biotypes that are more commonly isolated from chickens, others from pigs, etc and most or all strains and serotypes can cause illness both in man and in farm animals (Novick, 1978).

In this study little resistance was observed to neomycin (11%), while moderate resistance was indicated to kanamycin (33%), erythromycin (38%) and streptomycin (46%) (see Table 4.14). Lack of resistance to neomycin, kanamycin, erythromycin and streptomycin does not mean that the bacteria were not exposed to antibiotics at all, but may suggest that bacterial strains have been exposed to these agents for a short while (Vazquez-Moreno *et al.*, 1990). Isolates were observed to be most resistant to gentamicin (63%), tetracycline (88%) and 99% resistance to oxytetracycline and penicillin (see Table 4.14). Isolates of chicken samples (meat, stomach and liver) (113 isolates) and chicken manure

(87 isolates) show high resistance to all antibiotics except to kanamycin (33%), erythromycin (38%) and neomycin (11%), while isolates from pork samples (meat and liver) (71 isolates) and chicken feed (71 isolates) show less resistance (see Table 4.15).

Epling and Carpenter (1990) found that *Salmonella* isolates from pork carcasses showed resistance to penicillin (95%), trimethoprim (80%), ampicillin (80%) and tetracycline (65%). No resistance to gentamicin and neomycin was detected. Chloramphenicol showed a 12% resistance in all strains. Research done by Vazquez-Moreno *et al.* (1990) showed that *Salmonella* species isolated from chicken tissues presented resistance mainly to penicillin, tetracycline and streptomycin.

An epidemiological survey of antibiotic resistance in *Salmonella* recovered from market-age swine at five different Texas farms showed during preliminary analysis of the first 183 samples out of approximately 400 *Salmonella* samples that 183 (100%) of the *Salmonella* samples were resistant to penicillin G, and 122 (66.7%) were resistant chlortetracycline. Six (3.3%) were resistant to four antibiotics (chlortetracycline, penicillin G, streptomycin, and sulfisoxazole), and 25 (13.7%) were resistant to three antibiotics (chlortetracycline, penicillin G, and either streptomycin, sulfisoxazole, or ampicillin) (Farrington *et al.*, 1999).

5.3 Cluster analysis

MAR is the ability to resist the effects of two or more unrelated antibiotics by bacterial strains generally containing R-plasmids. The higher the MAR index, the greater the resistance and the lower the MAR index, the greater the susceptibility (Krumperman, 1983). This study showed that the average MAR for the different groups of organisms isolated during the study are as follow: *Pseudomonas* (0.219), *Staphylococcus* (0.288), *Enterobacteriaceae* (0.473), *Escherichia coli* (0.545) and *Salmonella* (0,582) (see Tables 4.16 - 4.24). Two isolates (P3 and P18) of *Pseudomonas* showed high resistance to the antibiotics with a MAR of 0.5 and 0.875 respectively (see Table 4.16). Five isolates of *Staphylococcus aureus* showed a MAR value equal or higher than 0.5: (S8 and S23 = (0.625); S17, S19 and S47 = (0.5) (See Table 4.18). Twenty-eight isolates of *Enterobacteriaceae* showed high resistance to the antibiotics with a MAR equal or higher than 0.5. Sixteen isolates showed a value of 0.5, seven isolates showed a value of 0.624

and five isolates showed high resistance of 0.725 (see Table 4.20). Eleven isolates of *Escherichia coli* showed high resistance to the antibiotics. Five isolates (C4, C5, C7, C8 and C12) showed a MAR of 0.5. Four isolates (C2, C6, C9 and C10) showed a MAR of 0.625, while two isolates (C13 and C14) showed a MAR of 0.75 (see Table 4.22). Sixteen isolates of *Salmonella* showed a MAR of 0.5 and 14 isolates showed a MAR of 0.625. Nineteen isolates showed a MAR of 0.75 and 6 isolates showed a MAR of 0.875 (see Table 4.24).

Krumperman (1983) demonstrated that the sub-therapeutic use of antibiotics in the mass production of poultry, eggs and pork had promoted the emergence of resistance and has maintained the prevalence of MAR *E. coli* in the faecal environment of these animals. The wide use and abuse of antibiotics in human therapy has also produced MAR *E. coli* in the faeces of humans as well. These practices have resulted in the coexistence of MAR *E. coli* within these major reservoirs of enteric disease for humans. The consequence of these practices may provide a fortuitous opportunity to identify *E. coli* contamination of food originating from these high-risk environments by MAR indexing of *E. coli* isolates obtained from food (Krumperman, 1983).

Research done by Vazquez-Moreno *et al.* (1990) showed that MAR was more common than single resistance among bacteria isolated from chicken and beef tissues. Epling and Carpenter (1990) found that multiple resistance was most often observed among *Salmonella* isolates from chickens, cattle and swine with 91% resistance to 2 or more antibiotics. Krumperman (1983) suggested that an unexpected increase in the MAR index of isolates from any sample should prompt an immediate investigation even though the number of isolates present was below the established guideline or standard. He also suggested MAR indexing was likely to provide a useful tool for better risk assessment by identifying contamination from high-risk environments.

Multiple drug resistance to antibacterial agents, antifungals, antivirals, antiprotozoals, and antitumor agents has risen spectacularly in the last decade and presently threatens to put an end to successful chemotherapy in all of the above fields. Multiple drug resistance against chemotherapy of infections and other diseases characterized by rapid growth of genetically versatile cells has developed in recent years. Development of carefully chosen

new drugs devising more intelligent ways of administering them, will reduce the negative impact of multiple drug resistance, but will not abolish it (Mitcher *et al.*, 1999).

5.4 Major clusters

From the 198 isolates, 33 clusters containing 165 isolates and 33 individuals were obtained (see Table 4.26). Of the 165 isolates present in the major clusters, 67 of the isolates were from the *Salmonella* species. Thirty-two isolates were from *Staphylococcus* species, 36 isolates from the *Enterobacteriaceae* species, 18 from *Pseudomonas* species and 12 from the *Escherichia coli* species (see Table 4.26).

According to Tables 4.27 and 4.28 the 33 major clusters can be divided as follows:

- 2 clusters (M1 and M6, represent 7 isolates) were resistant to 7 antibiotics (87.5% resistance).
- 4 clusters (M2, M4, M7 and M18, represent 23 isolates) were resistant to 6 antibiotics (75% resistance).
- 3 clusters (M5, M8 and M14, represent 15 isolates) were resistant to 5 antibiotics (62.5% resistance).
- 8 clusters (M3, M9, M11, M15, M19, M20, M21 and M22, represent 42 isolates) were resistant to 4 antibiotics (50% resistance).
- 6 clusters (M10, M12, M16, M23, M24 and M27, represent 35 isolates) were resistant to 3 antibiotics (37.5% resistance).
- 6 clusters (M13, M17, M25, M28, M29 and M33, represent 22 isolates) were resistant to 2 antibiotics (25% resistance).
- 3 clusters (M26, M30 and M32, represent 18 isolates) were resistant to 1 antibiotics (12.5% resistance).
- 1 cluster (M31, represent 3 isolates) was resistant to 0 antibiotics (0% resistance).

Of the individual isolates that did not cluster (See Table 4.27), 2 was resistant to 5 antibiotics (62.5%), 2 resistant to 3 antibiotics (37.5%), 8 resistant to 2 antibiotics (25%) and 2 was resistant to 1 antibiotic (12.5%).

5.5 Ranking of antibiotics

This study showed a high percentage of resistance of the isolates to oxytetracycline, tetracycline and penicillin. Little resistance was shown to kanamycin and neomycin. Of the 198 isolates, 158 (80%) of the isolates were resistant to oxytetracycline, 149 (75%) of the isolates resistant to penicillin and 137 (70%) resistant to tetracycline (see Table 4.29). Isolates showed resistance between 37% and 32% for erythromycin, streptomycin and gentamicin (see Table 4.29). Of the 198 isolates, 38 (19%) showed resistance to kanamycin and 32 (16%) showed resistance to neomycin (see Table 4.29).

Epling and Carpenter (1990) examined isolates of *Salmonella* from pork carcasses, for resistance to antimicrobial agents and found that the most commonly resistance was to penicillin (95%), trimethoprim (80.2%), ampicillin (80.1%), chloramphenicol (11.6%) and tetracycline (64.5%). No resistance to gentamicin and neomycin was detected. Khachatourians (1998) showed antibiotic resistant *Salmonella* isolates associated with human infections had a 93% resistance to chloramphenicol, streptomycin, sulfonamides and tetracyclines for each antibiotic respectively.

Newsome *et al.* (1987) determined antibiotic resistance of *Staphylococcus* and *Enterobacteriaceae* isolated from restructured and conventional steaks. Thirty eight percent of the restructured and 15% of the conventional steak isolates were resistant to tetracycline. One percent of the restructured steak isolates was resistant to erythromycin, kanamycin and penicillin. No resistance to chloramphenicol and neomycin was detected. Ten percent of the *Enterobacteriaceae* isolates was resistant to streptomycin and 11% was resistant to tetracycline.

Vazquez-Moreno *et al.* (1990) showed antibiotic resistance in various bacteria isolated from chicken tissues. *E. coli* was resistant to penicillin (71%), tetracycline (71%), streptomycin (78%) and gentamicin (21%) and no resistance to chloramphenicol was detected. *Staphylococcus* was resistant to penicillin (38%), tetracycline (38%) and streptomycin (19%) and not resistant to chloramphenicol and gentamicin. *Enterobacter* was resistant to penicillin (80%), tetracycline (70%) and streptomycin (60%) and no resistance to chloramphenicol and gentamicin was detected. *Salmonella* was resistant to

penicillin (75%), tetracycline (80%), streptomycin (38%) and chloramphenicol (50%) and not resistant to gentamicin.

According to Geornares *et al.* (2001), *E. coli* strains from poultry carcasses were analysed to determine their susceptibility to antimicrobial agents (chlortetracycline, oxytetracycline and neomycin) used in the South African poultry industry. The majority (76%) of the strains were resistant to chlortetracycline and oxytetracycline only, while 14% resistant to both tetracyclines as well as neomycin. Six percent of the isolates were resistant to neomycin only, while the remaining 2% of the isolates were susceptible to neomycin.

Animal-adapted bacterial strains harbouring antibiotic resistant plasmids may spread naturally among animals and, at least transiently, can colonise the intestinal tracts of humans and other species (Feinman, 1999). Humans may also acquire antibiotic resistant strains from the environment through direct exposure to manure or contact with pets or mice that are in contact with waste of farm animals. It can also spread to humans from pets fed contaminated poultry products; by human contact with farm animals, freshwater fish and wild animals or from animals to humans by vectors, such as flies, cockroaches and rats (Feinman, 1999).

The genetic material coding for antibiotic resistance is termed the R-factor and is usually carried on plasmids, which can be transferred between strains of the same species of bacteria, and also between different species (Mattila *et al.*, 1988). According to Mattila *et al.* (1988) there are three mechanisms by which R-factors of animal origin could be transferred to man. The first is the direct transmission of *E. coli* harbouring the R-factor to the human gut, where it may colonise. The second is the transmission of the organism to the human gut where it does not colonise but may transfer R-factors to resident strains of *E. coli* in which they are maintained. This may also occur, especially if antibiotics are present to provide selective pressure, a phenomenon in which the more susceptible bacteria are destroyed and the more resistant bacteria survive (Harrison and Svec, 1998). Epidemiological evidence indicates it must occur occasionally, and this may be all that is required to allow the slow but steady spread of R-factors through a range of bacterial species in the environment where the use of antibiotics is widely practised. The third mechanism is indirect, where the organism, after leaving the host animal, may transfer R-

factors to other organisms, such as another strain of *E. coli* or *Salmonella* species which may subsequently colonise the human gut (Mattila *et al.*, 1988).

Epidemiological studies (Mitsuhashi, 1976) of resistant bacteria have produced the following findings:

- many resistant bacteria carry transmissible plasmids (R-factor) or nontransmissible plasmids (r-plasmid).
- there are two groups of drug resistant bacterial species, one group which easily acquires multiple resistance and is isolated frequently from clinical specimens, and another which is still sensitive to various antibiotics.
- biochemical mechanisms of resistance in clinical isolates are mostly different from those in resistant mutants developed *in vitro* and are common in many clinical isolates which are isolated all over the world.

5.6 Conclusion

This study has revealed that bacteria are common in chicken and pork meat as well as chicken feed and manure and the surrounding area. Isolates from these samples, tested against 8 different antibiotics, exhibit resistance towards most of the antibiotics tested. These antibiotics are mainly used in human therapy, but some are also used in subtherapeutic levels in animal feed. From this survey it is evident that most of the isolates from the samples exhibit multiple antibiotic resistance. A variety of resistance patterns were observed when all the isolates were compared. When comparing the major resistant patterns (33 patterns) only 1 pattern consist of 17 isolates. The rest of the patterns contain either 14 isolates to 1 isolate. This study showed that antibiotic resistance to the 8 antibiotics can vary from 87.5% to 0% resistance. From the total of 198 isolates, seven isolates (3.5%) showed a MAR of 0.875. Twenty-six isolates (13.13%) with a MAR of 0.75, twenty-seven isolates (13.6%) with a MAR of 0.625 and 40 isolates (20.2%) have a MAR of 0.5. Thus 50.5% of the total isolates showed multiple antibiotic resistance to 4 or more antibiotics.

The first antimicrobial agents were introduced in the 1940s and a large number of new compounds were discovered in the following decades. Shortly after their introduction, resistance began to emerge and over the years bacteria have acquired resistance to all

known antimicrobial agents (Aarestrup *et al.*, 1998). Emergence of antimicrobial resistance in bacteria from food animals may compromise the treatment of infections in animals, but may also be of consequence for human health (Aarestrup *et al.*, 1998). The wide use and abuse of this wonderful drug in human therapy and subtherapeutic use of antibiotics, has produced MAR *E. coli* in human faeces (Krumperman, 1983). The bacteria became resistant and the antibiotic inadequate against the bacteria. Extensive and sometimes careless usage of antibiotics in medicine, dentistry, and agriculture is rendering these wonder drugs less reliable for treating important infections (Feinman, 1999). Clinicians must be educated to understand the resistance problem, accept the importance of their role in both the problem and the solution, and resolve to use these drugs in a far more appropriate manner. Humans have ignored clear warnings that excessive or improper use of antibiotics would lead to a world filled with human carriers of bacterial resistant to most known antibiotics (Harrison and Svec, 1998).

The consequence of the abuse and subtherapeutic use of antibiotics has resulted in the coexistence of MAR *E. coli* within major reservoirs of enteric disease for humans. The consequence of these practices may provide opportunity to identify *E. coli* contamination of food originating from these high-risk environments by MAR indexing of *E. coli* isolates obtained from food (Krumperman, 1983).

The role of the veterinary profession in the control and prevention of disease in animals is critical. Ideally all farms should have a veterinary health plan that is based on the results from the good surveillance of the disease trends on the farm including antibiotic resistance. This surveillance should also enable emerging antibiotic resistance to be recognized. To reduce the need for antibiotics, the method of production may have to change. Methods for rapid diagnosis must be developed to enable improved empirical therapy of sick animals. The use of antibiotics as part of the farm veterinary health plan is the way forward and must be an essential component of any Farm Quality Assurance Scheme (Johnston, 2001).

According to Novick (1978) the problem stem from the fact that the meat producers tend to give antibiotics throughout the entire life of the animal with the consequence that the normal (and abnormal) bacterial flora becomes highly resistant to many antibiotics simultaneously, so that the benefits of the drugs at times of stress are drastically reduced.

The conclusion is that it is unquestionably true that many clinically effective antibiotics have distinct growth promoting properties for a variety of animals, when fed at low levels as part of their food, and undoubtedly the use of antibiotics for this purpose can make a substantial difference, to the efficiency of conversion of food to edible protein. But at whose expense? Where do we draw the line? So, in summary, all our efforts should be directed towards reducing the selection pressure as much as possible. Only in this way can we hope to reduce the reservoir of resistant bacteria (Richmond, 1972).

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Appendix A

Table A: The source of the *Pseudomonas* isolates obtain from the different samples

Isolate	Source of isolation
P1	Pork meat
P2	Pork meat
P3	Pork meat
P4	Pork meat
P5	Pork liver
P6	Pork liver
P7	Pork liver
P8	Chicken meat
P9	Chicken stomach
P10	Chicken stomach
P11	Chicken liver
P12	Chicken liver
P13	Chicken feed
P14	Chicken manure
P15	Chicken stomach
P16	Pork meat
P17	Pork meat
P18	Chicken feed
P19	Pork meat
P20	Chicken manure

Appendix B

Table B: The source of the *Staphylococcus aureus* isolates obtain from the different samples

Isolate	Source of isolation
S1	Chicken meat
S2	Chicken meat
S3	Chicken meat
S4	Chicken meat
S5	Pork liver
S6	Pork liver
S7	Pork liver
S8	Pork liver
S9	Chicken feed
S10	Chicken feed
S11	Chicken feed
S12	Chicken feed
S13	Chicken manure
S14	Chicken manure
S15	Chicken manure
S16	Chicken manure
S17	Chicken manure
S18	Chicken manure
S19	Chicken manure
S20	Chicken manure
S21	Chicken manure
S22	Chicken manure
S23	Chicken manure
S24	Chicken feed
S25	Pork meat
S26	Chicken feed
S27	Pork meat
S28	Pork meat
S29	Chicken meat
S30	Chicken meat
S31	Pork meat
S32	Pork meat
S33	Pork meat
S34	Pork meat
S35	Chicken meat
S36	Chicken meat
S37	Chicken meat
S38	Chicken meat

Appendix B (continued)

S39	Chicken manure
S40	Chicken stomach
S41	Chicken feed
S42	Chicken feed
S43	Chicken manure
S44	Chicken manure
S45	Chicken manure
S46	Chicken meat
S47	Chicken meat
S48	Pork meat
S49	Pork meat
S50	Pork meat

Appendix C

Table C: The source of the *Enterobacteriaceae* isolates obtain from the different samples

Isolate	Source of isolation
E1	Pork meat
E2	Chicken meat
E3	Chicken meat
E4	Chicken manure
E5	Eggs
E6	Chicken manure
E7	Chicken manure
E8	Chicken manure
E9	Chicken feed
E10	Chicken feed
E11	Chicken manure
E12	Chicken manure
E13	Chicken feed
E14	Chicken stomach
E15	Chicken stomach
E16	Pork liver
E17	Chicken feed
E18	Chicken stomach
E19	Pork liver
E20	Chicken stomach
E21	Pork meat
E22	Pork meat
E23	Chicken stomach
E24	Chicken liver
E25	Eggs
E26	Chicken manure
E27	Chicken liver
E28	Pork meat
E29	Eggs
E30	Chicken meat
E31	Chicken meat
E32	Chicken meat
E33	Chicken stomach
E34	Chicken stomach
E35	Chicken meat
E36	Chicken meat
E37	Chicken liver
E38	Chicken liver

Appendix C (continued)

E39	Chicken manure
E40	Chicken manure
E41	Pork liver
E42	Pork liver

Appendix D

Table D: The source of the *Escherichia coli* isolates obtain from the different samples

Isolate	Source of isolation
C1	Chicken manure
C2	Chicken meat
C3	Pork meat
C4	Chicken manure
C5	Chicken feed
C6	Pork meat
C7	Chicken liver
C8	Chicken manure
C9	Chicken manure
C10	Chicken meat
C11	Chicken liver
C12	Chicken stomach
C13	Chicken feed
C14	Chicken feed

Appendix E

Table E: The source of the *Salmonella* isolates obtain from the different samples

Isolate	Source of isolation
1	Chicken liver
2	Chicken liver
3	Chicken liver
4	Chicken liver
5	Pork liver
6	Chicken meat
7	Chicken meat
8	Pork liver
9	Chicken manure
10	Chicken manure
11	Chicken manure
12	Chicken manure
13	Pork meat
14	Pork meat
15	Pork meat
16	Pork meat
17	Chicken feed
18	Chicken feed
19	Chicken feed
20	Chicken feed
21	Chicken manure
22	Chicken manure
23	Chicken manure
24	Chicken manure
25	Chicken feed
26	Chicken feed
27	Chicken feed
28	Chicken stomach
29	Chicken stomach
30	Chicken meat
31	Pork meat
32	Pork meat
33	Chicken feed
34	Pork liver
35	Chicken meat
36	Chicken meat
37	Pork meat
38	Pork meat
39	Pork meat

Appendix E (continued)

40	Pork meat
41	Chicken feed
42	Chicken feed
43	Chicken feed
44	Chicken meat
45	Chicken meat
46	Chicken stomach
47	Chicken stomach
48	Chicken stomach
49	Chicken stomach
50	Chicken feed
51	Chicken feed
52	Pork liver
53	Chicken liver
54	Chicken liver
55	Chicken liver
56	Pork liver
57	Chicken manure
58	Chicken liver
59	Chicken feed
60	Chicken feed
61	Pork meat
62	Chicken manure
63	Chicken manure
64	Pork meat
65	Pork meat
66	Pork meat
67	Chicken manure
68	Chicken manure
69	Chicken manure
70	Chicken manure
71	Chicken manure
72	Chicken manure