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The Establishment and Standardization of a Veterinary Antimicrobial Resistance Surveillance Programme in South Africa

By

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Submitted in partial fulfilment of the requirements for the degree of Master of Science

Department of Veterinary Tropical Diseases

Faculty of Veterinary Science

University of Pretoria

2001

Supervisor: Prof. M van Vuuren

Co-supervisor: Prof. G.E Swan

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SUMMARY

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The rapid development and spread of bacterial resistance to antimicrobial drugs is an increasing threat to human and animal health. Information on the prevalence of bacterial resistance to specific antimicrobial agents in both humans and animals together with changes occurring over time, is required to understand the magnitude of the problem, to make decisions and to take actions that are based on risk assessment (3). The ultimate goal is to preserve the effectiveness of available antimicrobial drugs for the benefit of future generations of animals and humans. The emergence of resistance and the spread of resistant bacteria can be limited by implementing a

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veterinary antimicrobial drug policy, in which *inter alia* systematic monitoring and prudent use play essential parts (1).

Testing the susceptibility of bacteria to antimicrobial drugs is fundamental to the study of resistance. Sensitivity testing serves two purposes: to provide reliable results to the prescriber of antimicrobial drugs and to monitor changes in susceptibility of microbial populations. Standardized methods are needed for surveillance in the national and international context and harmonization of methods to provide meaningful comparisons between individual centres and countries (1, 3).

The objective of this study was to establish a repeatable, standardized laboratory procedure for monitoring the development of antimicrobial resistance in bacteria isolated from animals and food of animal origin in South Africa.

The bacteria included in this study represented three different groups, namely zoonotic bacteria (*Salmonella),* indicator bacteria *(Escherichia coli*, *Enterococcus faecalis*, *E. faecium)* and veterinary pathogens (*Mannheimia haemolytica*). Thirty isolates of each organism were collected with the aim of standardizing the laboratory methodology for a future national veterinary surveillance programme. Susceptibility to 10 antimicrobial agents was determined by means of minimum inhibitory concentrations (MIC's) using the microdilution method. The method according to the National Committee for Clinical Laboratory Standards was used as the standard. Susceptibility tests were repeated once for each individual organism. Quality control measures were included to ensure that accurate results were obtained.

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Repeatability was satisfactory as results from duplicate tests differed by only one two-fold dilution. Multi-well plates prepared in-house for MIC determinations also yielded repeatable results after two months of storage at -70°C. Within this limited sample of bacteria, MIC results did not indicate meaningful resistance against any of the 10 selected antimicrobials.

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OPSOMMING

Die Vestiging en Standardisering van 'n Program vir die Waarneming van Veterinêre Antimikrobiese Weerstand in Suid-Afrika

Deur

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Voorgelê ter vervulling van 'n deel van die vereistes vir die graad MSc

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Die vinnige ontwikkeling en verspreiding van bakteriese weerstand teenoor antimikrobiese middels is 'n bedreiging vir mens en dier se gesondheid. Inligting oor die voorkoms van bakteriese weerstand teen spesifieke antimikrobiese middels in beide mens en dier, tesame met die veranderinge oor tyd, is nodig om die probleem as 'n geheel te beskou, besluite hieroor te neem, en om verdere stappe te neem wat op risikobepaling gebaseer is (3). Die hoofdoel is om die effektiwiteit van beskikbare antimikrobiese middels te bewaar tot die voordeel van toekomstige generasies. Die ontstaan van weerstand en die verspreiding van weerstandbiedende bakterieë kan deur die implementering van 'n veterinêre antimikrobiese middels beleid, waarin onder andere sistematiese monitering en gekontroleerde gebruik belangrike rolle speel, beperk word (1).

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Die toetsing van die vatbaarheid van bakterieë vir antimikrobiese middels is ‘n fundamentele aspek van die studie van weerstandbiedenheid. Die twee hoof doele van sensitiwiteitstoetsing is om betroubare resultate aan die voorskrywer van antimikrobiese middels te bied, en om die veranderinge van sensitiwiteit in mikrobiese populasies te moniteer. Gestandardiseerde metodes word vereis vir die waarneming in beide die nasionale en internasionale konteks, sowel as die harmonisering van metodes om sinvolle vergelykings te tref tussen individuele sentra en lande (1,3).

Die doel van hierdie studie was om ‘n herhaalbare, gestandardiseerde laboratorium prosedure te vestig vir die monitering van die ontwikkeling van antimikrobiese weerstand in bakterieë geïsoleer uit diere en dierlike produkte in Suid-Afrika.

Die bakterieë wat in die studie ingesluit was het drie groepe verteenwoordig naamlik zoönotiese bakterieë (*Salmonella)*, indikator bakterieë (*Escherichia coli, Enterococcus faecalis, E. faecium)* en veterinêre patogene (*Mannheimia haemolytica*). Dertig isolate van elke organisme is versamel met die doel om die laboratorium metodes te standardiseer vir ‘n toekomstige nasionale veterinêre waarnemingsprogram. Vatbaarheid teenoor 10 antimikrobiese middels was deur middel van minimum inhibitoriese konsentrasies (MIK) bepaal deur gebruik te maak van die mikro-verdunnings metode. Die metode volgens die ‘National Committee for Clinical Laboratory Standards’ (NCCLS) was as standaard gebruik. Sensitiwiteitstoetse is eenkeer herhaal vir elke individuele organisme. Maatreëls vir kwaliteitskontrole is toegepas om die akkuraatheid van die resultate te verseker.

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Die herhaalbaarheid van die resultate was aanvaarbaar. Duplikaat toetse het slegs met een tweevoudige verdunning verskil. Die mikrotiter plate wat self voorberei is vir die MIK bepalings het ook herhaalbare resultate getoon na 2 maande se bevriesing by –70°C. Binne die beperkte steekproef van bakterieë, het die MIK resultate nie betekenisvolle weerstandbiedenheid teen enige van die 10 geselekteerde antimikrobiese middels getoon nie.

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CHAPTER 1

1. INTRODUCTION

Antimicrobial drugs have been used in animals since shortly after introduction its in human medicine. It has been of great benefit to animals in terms of alleviation of suffering and to man in helping to meet the growing demand for animal protein and in controlling the agents of potentially serious zoonoses (1). However, through the increasing use of antimicrobials in humans, animals, fish and in agriculture, an antimicrobial resistance problem has been created that is rapidly moving internationally to the forefront of public health concerns (2). These health concerns have generated a lot of attention worldwide with numerous governmental and non-governmental organizations being involved (3).

The role and impact of the use of antimicrobial drugs in animals or in the development of bacterial resistance have not clearly been delineated. It is therefore necessary that their possible role in the development of antimicrobial resistance is considered and guidelines for the prudent use of antibiotics be established. Today, antimicrobials are essential for controlling bacterial infection and are among the most regularly used drugs in veterinary medicine. Antimicrobial drugs represent the largest portion of pharmaceutical sales internationally, both in volume and dollar value of any drugs used in animal production. It constitutes for example the main therapeutic class on the European veterinary drug market, particularly in France. The veterinary drug market in that country is the biggest within the European Union and the use of antimicrobial drugs in bovines represents 75% of the entire market (3). In South Africa it constitutes the fourth largest market sector in Veterinary Drugs (Agricultural and Veterinary Chemical Association of South Africa statistics).

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To safeguard the efficacy of antimicrobial drugs in veterinary medicine and to minimize possible public health risks, it is necessary to limit the emergence of resistance and the spread of resistant bacteria by implementing a veterinary antibiotic policy in which *inter alia* systematic monitoring for the development of resistance and prudent use play essential roles (1, 4). The monitoring of antimicrobial resistance in bacteria from animal sources in South Africa is in its infancy. The veterinary profession in this country recognizes antimicrobial resistance as an emerging problem and wants to address antimicrobial resistance by developing and implementing a National Antimicrobial Strategy for the use of antimicrobials in animals which *inter alia* will include the need for a standardized monitoring programme. It has taken the first step by establishing a Veterinary Antibiotic Workgroup in South Africa. This Workgroup is in the process of developing guidelines for prudent use as well as the establishment of a national veterinary antimicrobial resistance surveillance programme. The ultimate goal for national microbial strategies would be to prolong the efficacy of existing and new antimicrobial agents that are desperately needed to control both human and animal disease and to minimize zoonotic pathogens in humans (5).

The objectives of the study were to establish a repeatable, standardized laboratory procedure that can be used for monitoring the development of antimicrobial resistance in bacteria isolated from animals and food of animal origin in South Africa, and to make recommendations for the practical implementation of a surveillance programme that can provide information nationally.

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CHAPTER 2

2. LITERATURE REVIEW

2.1 Use of antimicrobial drugs in animals

Antimicrobial drugs are products that affect bacteria by inhibiting their growth or by killing them outright. These drugs are used to treat bacterial disease in humans and since their discovery have prevented countless deaths worldwide (6). Many bacterial diseases occurring in animals are potentially fatal whilst others cause pain and distress. Appropriate use of antimicrobial drugs will cure certain sick animals and speed up the recovery of others, while many improve the welfare of treated animals, reducing the spread of infection to other animals. In the case of zoonotic diseases the spread to humans may also be reduced (7).

Antimicrobial drugs are health management tools that are licensed to be used for supporting good husbandry practices aimed at the prevention or treatment of disease, as well as for production enhancement (8). Their application in veterinary practice since the 1950's has assisted in promoting the health of livestock and companion animals (9). The use of antimicrobial drugs has also enhanced production efficiencies that have contributed to the availability of a reasonably priced and plentiful food supply (6).

In parallel with the introduction of antimicrobial use in human medicine from the mid – 1930's, veterinary use provided similar control in both farm animals and domestic pets. This contributed greatly to animal welfare and allowed improvement in livestock production. In human medicine, concerns quickly arose that an overreliance on antimicrobial drugs was contributing to the selection of antimicrobial resistant

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strains of bacteria (10). Historically many of the antimicrobials, that are important in human therapy, have, because of their efficacy, also been used in veterinary medicine (11).

2.2 Historical perspective of antimicrobial resistance

The modern use of chemotherapy against microorganisms began with the introduction of the sulfonamides in the 1930's, the use of penicillin in the 1940's, and the discovery of streptomycin in the mid 1940's (12). The first resistance mechanism was recorded in 1940 by Abraham and Chain who isolated and characterized an enzyme from *E.coli* that was capable of hydrolyzing penicillin (13). Kirby reported a similar penicillinase enzyme in 1944 in *Staphylococcus aureus* (13). Thus, even before the widespread use of penicillin, resistance had already been detected in both Gram-negative and Gram-positive organisms. This indicated that resistance was not only a consequence of the use of antimicrobial drugs, but an integral part of a bacterium's own defense system enhancing it's ability to survive in hostile environments (13, 14). In 1959, resistance to multiple drugs was recognised in strains of *Shigella dysenteriae* and it was soon discovered that all the resistant traits of that organism could be transferred to recipient bacteria. The implications of transferable resistance raised concern in the scientific community (13)

In the 1960's the cephalosporins and semisynthetic, penicillinase-stable penicillins were developed and the problem of resistant *S. aureus* infections was thought to be largely resolved. However, multiple resistance emerged first in *S. aureus* and then in a variety of Gram-negative organisms during the late 1960's and early 1970's (13, 14,

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15). These events required physicians to alter their treatment for these infections, thus making the threat of resistance a greater reality (13).

However, the development and availability of more antimicrobial drugs, including cephalosporins in the early 1980's and the fluoroquinolones in the 1990's dampened fears of outbreaks of untreatable infections (13). Many of these antimicrobial drugs had a broad spectrum of activity and were bacteriocidal at low concentrations. Yet, resistance to these classes of drugs also appeared. By the 1990's, scientists realized that there was a potential resistance mechanism among organisms for every antimicrobial drug, including vancomycin an antimicrobial to which resistance seemed unlikely. In some cases now, organisms acquire resistance to certain antimicrobial drugs almost as soon as they are marketed. Unfortunately there are fewer new and unique antimicrobial drugs that are being developed by the pharmaceutical industry and expectations for the introduction of new antimicrobial classes have diminished (12, 13, 15). Some scientists have referred to the 1990's as the beginning of the post-antimicrobial era, a frightening prospect for the future (12).

The factors favouring the development of antimicrobial resistance are many-fold, but are mainly based on the selective pressure from the use of antimicrobial drugs and the presence of resistance genes (16, 17). The medical profession is probably largely responsible for this selective pressure, although agricultural and veterinary use also contributes to the resistance problem. The addition of antimicrobial drugs to feed or water, for growth promotion or prophylaxis in intensively fed animals, is having an unquantified effect on resistance levels (2, 7, 18).

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2.3 Antimicrobial resistance

A bacterial isolate is classified as resistant when it is not inhibited by the minimal concentration of the antimicrobial drug that inhibits the growth of the susceptible members of that species (MIC)(2, 20, 21, 22).

The excessive and increasing use of antimicrobial drugs in humans, animals and agriculture have led to the selection and extensive dissemination of resistant bacteria (2, 16, 23). The greater the use of antimicrobial drugs, the greater the selective pressure exists for the development of resistant bacterial populations. Moreover, some of these resistant bacteria are capable of transferring genetic elements to sensitive bacteria, rendering the recipient organisms resistant to antimicrobials they have never encountered (11).

A microorganism may have either intrinsic or acquired resistance to an antimicrobial agent. Intrinsic resistance or inherent indicates natural resistance to an antimicrobial drug possessed by the majority of the population of a specific bacterial genus (18). This form of resistance is a stable genetic property encoded in the chromosomal DNA and shared by all members of the genus. This can be seen in Gram-negative bacteria that are inherently resistant to a number of important antimicrobials that are effective against Gram-positive bacteria (18). Resistance to antimicrobials existed even before antimicrobial drugs. This intrinsic or natural form of resistance is not a major source of concern. However, the vast majority of drug resistant organisms have instead emerged as a result of acquired resistance (24). This is the reason why most research and discussions focus on acquired resistance that may develop after the constant exposure of a bacterial population to antimicrobial drugs (23).

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Acquired resistance occurs when there is a change in the bacterial DNA so that a new phenotypic trait can be expressed (12, 25). Bacteria can acquire resistance through genetic mechanisms, for example a mutation in the bacterial host chromosomal DNA or by acquisition of new DNA, of either chromosomal or extrachromosomal origin, that carries the information for antimicrobial resistance (25). Due to the flexibility and rapid multiplication rates of bacterial populations, bacteria have the ability to respond to environmental changes and can therefore adapt to the toxicity of antimicrobial drugs (26). Thus acquired resistance can be seen as an inherent risk associated with any use of antimicrobial medication in any species.

2.4 The nature of antimicrobial resistance

In the case of certain antimicrobial drugs it is possible to define the resistance mechanisms against them. Bacterial isolates can also be examined for the presence of such mechanisms, and if found to possess them, can be regarded as resistant. Despite their versatility, bacteria have a limited number of mechanisms of acquired antimicrobial resistance. However, an organism may use more than one of these mechanisms to protect it from antimicrobial drugs (27).

2.4.1 Acquired Resistance

The acquired ability of a bacterium to multiply and grow in the presence of an antimicrobial drug indicates that there are differences in the genetic makeup of resistant and sensitive organisms (26). Genetic mechanisms of resistance to antimicrobial drugs can occur either from new mutations in the bacterial genome or through the acquisition of genes coding for resistance (28). Mutations can cause chromosomal genes that usually code for antimicrobial sensitivity to start coding for resistance. However, cellular mechanisms exist to replicate DNA accurately and to

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correct errors and repair damage to the chromosomes. Therefore, the frequency of mutation in a given gene is very low (a rate of one per million to one per billion cells) (18, 25, 29).

Bacteria have evolved diverse mechanisms to transfer resistance traits to other members of their own species and to other species. Genetic traits for antimicrobial resistance are coded for in two places in bacteria: chromosomal and extrachromosomal (18, 25, 29). New chromosomal or plasmid DNA (extrachromosomal) containing resistance genes can be transferred from one bacterium to another by conjugation, transduction and transformation (25, 29, 30).

Conjugation is the mechanism of action by which DNA is transferred from one bacterium to another. It occurs when there is physical contact between two cells and a portion of DNA passes from one bacterium to another via small proteinaceous appendages called pili. Both the donor and recipient cell end up with a copy of the portion of DNA that contains the genetic determinant for antimicrobial resistance (19). Conjugation is a vital mechanism for spreading antimicrobial resistance, since it can occur in a broad range of bacterial species and DNA that encodes for multiple drug resistance can be transferred to these different species (25). Conjugation occurs widely in Gram-negative bacteria and less frequently in Gram-positive bacteria (25, 26). The mechanism of conjugation has the ability to transfer resistance genes across genus and species lines, thereby posing as a major clinical problem (12, 30).

The ability to transfer genes that confer drug resistance by cellular conjugation is due to the presence of plasmids and transposons in the bacterial cell. Plasmids are

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extrachromosomal, self-replicating circular DNA elements of bacteria that represent a reasonably stable, but dispensable gene pool in bacteria (18). Plasmids can be either non-conjugative or conjugative; the latter can move from one bacterium to another (30, 31). Bacterial plasmids carry a number of determinants that may cause their bacterial host to survive better in an adverse environment or permit their bacterial hosts to compete more successfully with other microorganisms of the same or different species. One type of plasmid, called R-factor, encodes for antimicrobial resistance to one or more antimicrobial drugs (19, 31). R-factor plasmids are responsible for most of the resistance in Gram-negative bacteria of clinical significance (331. The resistance determinants associated with R-factors can confer resistance to many drugs including the tetracyclines, the sulphonamides, the aminoglycosides, ß-lactams, chloramphenicol and trimethoprim (26).

Certain genes, if they are flanked by so-called insertion sequences, can jump from different DNA sites within a bacterial cell. A gene with an insertion sequence at each side is termed a transposon (jumping gene) and can jump to different locations on chromosomal DNA or from plasmid to plasmid or from chromosome to plasmid (19, 25, 26). Conjugative transposons are probably responsible for at least as much transfer of resistance as plasmids. They are especially prevalent among Gram-positive bacteria but possess a very broad bacterial host range. Conjugative transposons can transfer not only among species within the Gram-positive group or within the Gram-negative group, but also between Gram-positive and Gram-negative bacteria (30). The different combinations and permutations of these exchange mechanisms give bacteria countless resources for transferring and propagating resistance (25). Because the range of

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bacteria to which bacterial plasmids can spread is often limited, transposons are important in spreading resistance genes across such boundaries (18).

Genetic elements that acquire and exchange exogenous DNA are called integrons. This acquiring and exchanging of exogenous DNA, other wise known as gene cassettes, takes place via a site-specific recombination mechanism. The function of these gene cassettes is to result in antimicrobial drug resistance and are characterised by a target recombination sequence. This sequence is an *attC* site that is usually associated with a single reading frame. It is this reading frame that codes for the antimicrobial resistance. Multidrug resistance among Gram-negative bacteria is thought to be due to various combinations of these integrons occurring in a single bacterium (32).

In transduction, bacterial resistance genes of plasmid or chromosomal origin are carried from one bacterium to another by bacteriophages (phages; bacterial virus) (19, 25, 26). The transfer of genetic information between bacteria by transduction via a bacteriophage occurs in Gram-positive and Gram-negative bacteria (26). However, bacteriophages have a very narrow bacterial host range and are thus not so effective in disseminating resistance within a bacterial population (25).

In transformation, bacteria acquire DNA from their environment, for example after cell lysis, when the DNA is integrated into their genomes. As in transduction, the compatibility range between donor and recipient is narrow and is confined essentially to Gram-positive bacteria (18, 25, 26). The role of transformation in the transfer of

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resistance between bacteria is not very well known, but it appears to be of marginal importance (19, 26).

2.4.2 Biochemical mechanisms of resistance

Bacterial resistance genes are expressed phenotypically as biochemical interference of an antimicrobial's mode of action and reduced activity against the strain with the resistant trait. The biochemical mechanisms of both intrinsic and acquired resistance operate in a similar way. The biochemical mechanisms that bacteria exhibit to protect themselves from antimicrobials can be divided into five basic categories (12, 18, 25, 26, 33):

1) Drug inactivation (enzymatic inactivation)
2) Prevention of the antimicrobial from reaching its target
3) Modification of the antimicrobial target site
4) Metabolic bypass
5) Tolerance

2.4.2.1 Drug inactivation

A resistant bacterium synthesizes enzymes that are capable of chemically transforming the antimicrobial into an inert/inactive product. Such inactivation or modification may occur as a result of enzymes that are coded within plasmids, or coded within chromosomes and are present at all times, or that can be produced when the genes are stimulated or induced to do so. The most renowned of these enzymes are the beta-lactamases. Beta-lactamase act on the beta-lactam bond of certain antimicrobials, such as the penicillins and cephalosporins, thus inactivating them (12, 18, 25, 27, 33, 34).

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2.4.2.2 Preventing antimicrobials to reach its target

Once an antimicrobial has reached a cell, it still has to penetrate and find its site of action, or target. Bacteria can prevent this in several ways. The bacterial cell may synthesize a protein that will pump the antimicrobial out (efflux) and thus extrude it. Increased efflux of antimicrobials via an energy-requiring membrane pump is a well-recognised mechanism for resistance to a wide range of antimicrobial drugs, especially the tetracyclines (12, 18, 33).

Bacteria can also limit access of an antimicrobial drug to a site by virtue of their membrane characteristics, as in the case of ß-lactams and aminoglycosides. The complex make-up of Gram-negative bacteria ensures that they are naturally less sensitive than Gram-positive bacteria to a variety of antimicrobial drugs. This is due to the porins in the outer membrane of Gram-negative bacteria, that limits access to the cytoplasmic membrane. Mutations changing the structure of the porins can influence the uptake of the antimicrobial drugs. Mutations affecting permeability have been shown, for example, to produce resistance to chloramphenicol, tetracycline and streptomycin (15, 25, 33, 34).

Another method is through mutations in the genes that code for the synthesis of specific proteins in the transport process that may responsible for antimicrobial resistance (26). When penetration through the membrane is due to a specific transport mechanism, as for example tetracyclines and aminoglycosides, a specific carrier protein may be involved. An alteration of the carrier protein is a likely mechanism of resistance (19).

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2.4.2.3 Modification of the antimicrobial target site

Bacterial mutations may alter the target of the drug or inactivate it reversibly or irreversibly, so that the antimicrobial no longer binds to it (12, 18, 25). For example single step mutations in RNA polymerase and DNA gyrase, the enzymes targeted by rifampin and fluoroquinolones, respectively, have led to the emergence of resistance to these drugs (12, 19, 25). Resistance to other antimicrobial drugs has also been shown, e.g. ß-lactams, tetracyclines, macrolides and others (12, 25).

2.4.2.4 Metabolic bypass

Another mechanism by which bacteria may protect themselves from antimicrobial drugs is through the production of an alternative target (usually an enzyme) that is resistant to inhibition by the antimicrobial while continuing to produce the original sensitive target. This allows bacteria to survive in the face of selection: the alternative enzyme bypasses the effect of the antimicrobial (18, 26, 34). For example, bacteria can produce a new dihydrofolate reductase that is not inhibited by dihydropyrimidines such as trimethoprim, e.g. dihydropteroate synthetase that is not susceptible to potentiated sulfonamides, thus developing resistance to these antimicrobials (25).

2.4.2.5 Tolerance

B-lactam antimicrobials and other cell-wall inhibitors such as vancomycin and bacitracin, posses the ability to rapidly kill and in many cases lyse susceptible bacteria. Other types of antimicrobial drugs only inhibit multiplication but do not cause an unalterable inactivation of the cell (25, 35). The term antimicrobial tolerance is used to describe this new type of bacterial response to antimicrobial treatment (35). Tolerance has been considered present when the minimum bactericidal concentration (MBC) is significantly greater (generally 32-fold) than the minimum inhibitory

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concentration (MIC), where the MBC is defined as the concentration of an antimicrobial drug killing 99.9% of the inoculum. The term tolerance thus refers to bacteria in which a characteristically bactericidal-bacteriolytic response to antimicrobials is changed in the direction of bacteriostasis (35). This type of resistance may be due to the lack of autolytic enzymes, particularly in streptococci, and is also seen when beta-lactams bind to transpeptidase that results in growth inhibition but not cell killing (25).

2.5 Dispersal of resistance genes or resistant bacteria

Antimicrobial resistance is often an emotive and controversial issue. The potential role of the use of antimicrobial drugs in veterinary medicine, as well as the implications of the transfer of antimicrobial resistant bacteria to man, subsequent infection and treatment, is one of great concern (36). The potential role of antimicrobial use in veterinary medicine and its contribution to resistance transfer remains to be fully understood and a substantial amount of research still has to be done (24, 36).

There are multiple dispersal routes of resistant microorganisms, both commensal and pathogenic, namely:

- Animals and their faeces
- Food of animal origin that may have been contaminated during processing
- Fruit or vegetables that may come from a contaminated environment
- Contaminated water
- Human beings (24)
- Hospital environments containing a large reservoir of resistant bacteria (36);

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- The intestinal flora of intensively farmed animals, such as poultry, calves and pigs (36, 37).

The main source of resistance in the human population is the use of antimicrobials in human medicine. However, the direct contact with animals and the consumption of contaminated food of animal origin are recognised to be the main routes of transfer of resistance genes and resistant bacteria from animals to humans. In addition, while much attention has been focused on the transfer of resistant bacteria from food animals to man it must be kept in mind that human and animal populations constitute overlapping reservoirs of resistance, as shown in Figure 1 (17, 24).

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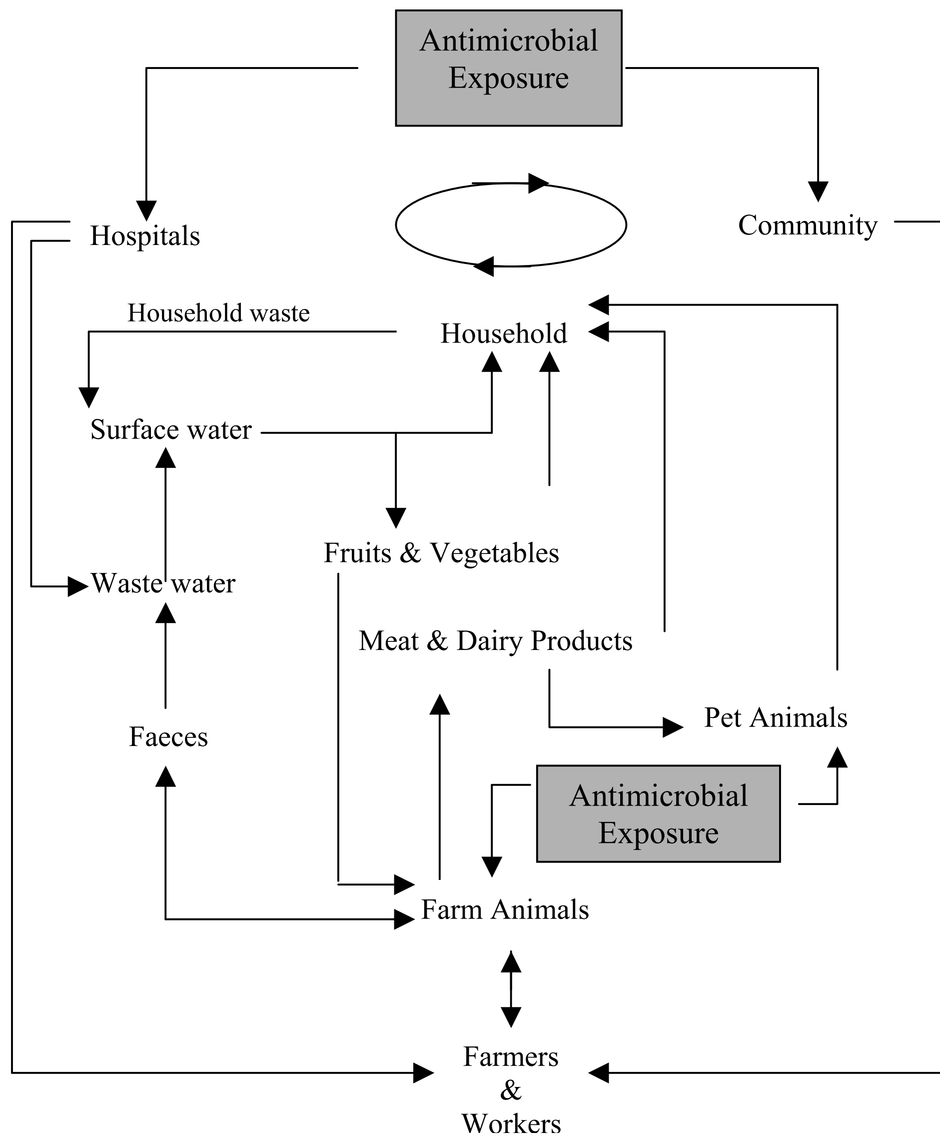

Figure 1.1: Overlapping reservoirs of antimicrobial resistance

There are four possible routes of transfer by which the use of antimicrobials in animals could pose a risk to human health:

1) During slaughter and/or food preparation, foodstuff is contaminated with resistant zoonotic bacteria. When this food is ingested it causes an infection that requires antimicrobial treatment that may be unsuccessful. These zoonotic bacteria may also transfer resistance to bacteria in the human gut.

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2) Antimicrobial resistant bacteria non-pathogenic to humans are selected in the animal; the foodstuff is contaminated and ingested. The bacteria can then transfer their resistance genes to other bacteria in the human gut.
3) Antimicrobials remain as residues in animal products which allows for the selection of antimicrobial resistant bacteria in the consumer of the food (36).
4) Transfer from animals to workers and then further transfer from the workers to the human population. This may also be due to exposure of workers to antimicrobial drugs mixed for example into feed.

2.6 Transfer of resistant bacteria from animals to humans

2.6.1 Zoonotic bacteria

Food infected with Gram-negative bacteria, such as *Salmonella*, *Yersinia* spp. and *Campylobacter* spp., is one of the main routes of resistance transfer from animals to humans. *Salmonella* provides evidence for the spread of antimicrobial resistance over the entire time scale of antimicrobial application in human and veterinary medicine since 1948 (37, 38).

Animals can infect humans with salmonella through direct contact with the infected animal or animal faeces (28, 37, 38, 39). However, the most important source of human infection is via food products of animal origin. Asymptomatic *Salmonella* infections and carriers are common in food animals kept under intensive animal husbandry practices. The intestinal tracts of these animals contaminate the meat and meat products with *Salmonella* during slaughtering. Humans can then become infected with the intestinal bacteria via consumption of meat, eggs or other animal products (28, 29, 37). The animal-food-human spread of *Salmonella* must be regarded

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as an important contribution to the spread of antimicrobial resistant bacteria from farm animals (37).

The continuing pandemic of human infections with *Salmonella enteriditis* is associated with the consumption of raw or lightly cooked shell eggs and egg containing products. The second most common *Salmonella* serovar in humans is *Salmonella typhimurium*, which is prevalent in the porcine, ovine and bovine meat industries (37, 38). It originates from environmental sources, contaminated feeds and animal to animal transmission of infection (37). Increasing isolation of multiple resistant *S. typhimurium* strains in Europe and in the USA, causing foodborne infections in humans, is being reported (40). During these *Salmonella* infections the same phage type with identical resistance profiles were isolated from animal and human infections. Especially worrying is the epidemic spread of the multiple resistant *S. typhimurium* phage type DT 104 in Europe and in USA since 1994 (11, 37, 40). This strain was first recognised in human and bovine cases in the UK in the late 1980's and has now been reported from the USA, Canada, Denmark, Germany, France and Austria (37, 39, 41). The DT 104 strain is resistant to most antimicrobials originally used for enteric infections in animals. Additional resistance has also been acquired against fluoroquinolones and trimethoprim (38, 40). An outbreak of 25 human cases with fluoroquinolone resistant *S. typhimurium* DT 104 has recently been described in Denmark. Molecular data clearly indicated that the primary source was a Danish swine herd (38).

The most important reservoir for human *Campylobacter* infections is poultry products (38). Following the introduction of fluoroquinolones for use in poultry, there has been

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a steep rise in the emergence of fluoroquinolone resistant *Campylobacter jejuni* isolated in poultry, poultry meat and from infected humans. However, prior to the use of fluoroquinolones in poultry, no resistant strains were reported in humans with no previous exposure to quinolones (29, 39, 42, 42). Two cases of ciprofloxacin resistant *C. jejuni* infections in patients, who had ingested chicken in Europe, were reported in Australia and are found with increasing frequency in the USA and Europe (29, 42). By contrast, in Australia one of several countries where fluoroquinolones have not been approved for use in food producing animals, ciprofloxacin resistant *Salmonella* and *Campylobacter* remain uncommon (42). It is now becoming very clear that the introduction of fluoroquinolones has resulted in the enrichment of quinolone resistant *Campylobacter* isolates from animals and human patients in many parts of the world (37). Some studies using molecular markers have shown a link between human and animal isolates of susceptible *Campylobacter*. One study conducted in the USA has confirmed links for fluoroquinolone-resistant *Campylobacter* (37, 41).

2.6.2 Indicator bacteria

The intestinal flora of humans and animals constitute an enormous reservoir or source of resistance genes for potentially pathogenic bacteria. It is important to compare the prevalence of resistance in various populations to discover the possible transfer of resistant bacteria from humans to animals and *vice versa* (37, 38).

Escherichia coli is used as an indicator species in surveillance programmes to determine the antimicrobial resistance status of the enteric microflora of both farm animals and humans. The advent of *E.coli* strains pathogenic for animals and humans has initiated studies of their antimicrobial resistance that might pose a threat to effective antimicrobial treatment (37, 38). *Escherichia coli* has been used as a model

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to study the resistance levels of bacteria from persons involved in animal handling such as pig and turkey farmers, abattoir workers and veterinarians. Compared to the overall community, persons with a high level of exposure to farm animals have a considerably higher percentage of antimicrobial resistant *E. coli* in their intestinal microflora. This was seen in prospective studies on chicken and turkey farms several years ago, but still holds true in retrospective studies under field conditions (29, 36, 37, 38). This suggests that there is transfer of resistant strains from animals to humans. The extent of transfer seems to be associated with the prevalence of resistance in the animal population, that positively correlates with the amounts of antimicrobial drugs to which the animal population is exposed (37, 38).

Escherichia coli has also been used as an indicator to show that use of streptothricin as a porcine growth-promoting agent in the former East Germany between 1983 and 1990 resulted in the development of an antimicrobial resistant transposon (28, 29). After its introduction two years later resistant isolates of *E.coli* were found in porcine guts and meat products and subsequently in the intestinal microflora of the pig farmer and their families, as well as in patients with urinary tract infections and the general public within the municipality. By 1990 the same transposon had been detected in *Shigella* and other human enteric bacteria (28, 29, 37, 38). Since this antimicrobial drug has not been used in human medicine, it is possible to presume that growth-promoting antimicrobial drugs can induce bacterial resistance in the animal population. Transfer via enterobacteria into the human population without any specific selection pressure may therefore occur (37).

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2.6.3 Disturbance of colonisation resistance

Another effect of the use of antimicrobial drugs is the disturbance of the colonisation resistance, also known as the "gut-barrier", of the intestinal flora of animals exposed to certain antimicrobial drugs. With reduced colonisation resistance, the minimal infection or colonisation dose of pathogenic or resistant bacteria is significantly lower. When this occurs these animals excrete these bacteria over a longer period of time as well as in higher numbers compared to animals with an intact intestinal flora. This not only enhances dissemination of salmonella or resistant bacteria within a group of animals, but also increases the contamination of carcasses with these bacteria during slaughter. This effect has been demonstrated for most broad-spectrum antimicrobial drugs and also for certain growth promoters such as avoparcin, as well as to a lesser extent virginiamycin and tylosin. The "gut barrier" in humans may also be adversely affected by antimicrobial residues in food consumed by humans (38).

2.7 An overview of current perceptions and approaches to antimicrobial resistance

Actions to mitigate the problem of antimicrobial resistance include the development of new antimicrobial drugs, better infection control and greater conservation of excisting agents (43). However, the number of new antimicrobial classes of drugs is decreasing and our ability to control outbreaks of infectious diseases through antimicrobial use alone is slowly diminishing (13). One pressing problem is the paucity of data to measure the impact of resistance on public health or the effect of interventions to prevent its emergence and spread (43). The whole aspect of antimicrobial resistance has come to forefront of public health concern and has raised a lot of attention all over the world. It is seen as a growing problem and drastic measures have to be taken to curb antimicrobial resistance.

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2.7.1 International meetings

The World Health Organization (WHO) convened a meeting in October 1997 in Berlin Germany. The title of this meeting was "The medical impact of the use of antimicrobials in food animals". At this meeting they recommended that the use of antimicrobial drugs as production enhancers should be terminated if the particular antimicrobial drug is also used for human medical purposes, or if it is known to be selective for cross-resistance to antimicrobial drugs in human medicine. It was also resolved that monitoring of antimicrobial resistance in food animals and food of animal origin should be introduced and that the WHO should take the lead in coordinating national and international efforts as part of the WHO Programme on Resistance Monitoring (39).

In June 1998, the WHO hosted another meeting in Geneva, Switzerland. It was entitled "The Use of Quinolones in Food Animals and Potential Impact on Human Health". The experts requested during this meeting that the WHO, in conjunction with the Food and Agriculture Organization of the United Nations (FAO) and the Office International des Epizooties (OIE), should work together to gather data, standardize testing methods and develop prudent use guidelines for antimicrobial drugs in food animals. It was also agreed that the indiscriminate use of fluoroquinolones in food-producing animals should be reduced (41, 44).

Another conference in 1998, that addressed the problem of antimicrobial resistance, was held in Copenhagen, Denmark during September. It was entitled "The Microbial Threat". This conference focused on the problem of increasing antimicrobial resistance in humans and the implications thereof to human health. At the end of this meeting the organisers had consolidated the recommendations into a single document

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namely "The Copenhagen Recommendations" which they suggested be used as a future action plan for Europe. Among the many points made in the Copenhagen document, the creation of a European surveillance system for antimicrobial resistance based on national systems, as well as the development of prudent use guidelines were considered high priorities (45, 46).

A European Scientific Conference entitled "The Use of Antimicrobials in Animals – Ensuring the Protection of Public Health" was held at the headquarters of the OIE in Paris in March 1999. At the conference the participants focused on means of implementing strategies and actions to contain bacterial resistance resulting from the use of antimicrobial drugs in animals. Recommendations were made for the development of a risk analysis model and the implementation of guidelines for the prudent use of antimicrobial drugs in animals. They also proposed the establishment of a European antimicrobial resistance monitoring system for animal bacteria and that this system should build on existing national monitoring systems (4).

2.7.2 Scientific reports and discussion documents

The use of antimicrobial drugs in animals and the potential spill over of resistance genes to humans, which may lead to treatment failures, has been addressed by a number of committees, bodies and organizations worldwide. In the UK, this topic has been addressed in several reports, notably those of the Netherthorpe Committee in 1962, the Swann Committee in 1969, and the Lamming Committee in 1992. All of these reports focused on the same underlying and recurrent theme, namely the potential threat of finding resistant bacteria in food animals and the consequent need for prudent use of antimicrobial drugs in livestock production (11). It was stated that the Swann Report for the UK Joint Houses of Parliament first recognised that

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important therapeutic antimicrobial drugs in humans or animals should not be used in animal feeds, especially as growth promoters. They suggested that the use of antimicrobial drugs without prescription in animal feed should be limited to antimicrobial drugs that have little or no application as therapeutic agents (10).

The European member countries of the Office International des Epizooties (OIE) decided at the 65th General Session of the OIE International Committee in May 1997 to launch an investigation into "The role of international trade of animals, animal products and animal feed in the spread of antimicrobial resistance and the means to control the spread of resistance factors of infective agents". This report was prepared by the OIE's Collaborating Centre for Veterinary Drugs, Agence Nationale du Médicament Vétérinaire (ANMV) and Centre national d'études vétérinaires et alimentaires (CNEVA), Fougeres, France. The findings were presented at the Conference of the OIE Regional Commission for Europe in Prague, Czech Republic during September 1998. This report essentially represents an analysis of the information obtained from answers to questionnaires, that had been designed to obtain the factual information necessary to obtain an objective evaluation of the current situation in Europe and to make conclusions that are based on reliable technical data (47).

In line with global trends, Australia's Commonwealth Government also undertook action to address the growing problem of antimicrobial resistance. In December 1997, the Joint Expert Technical Advisory Committee on Antimicrobial Resistance (JETACAR) was established to examine antimicrobial resistance issues (48). The terms of reference of this committee were to investigate the link between the use of

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antimicrobial drugs in food-producing animals and the spread of resistance to humans, the development of evidence-based recommendations for the prudent use of antimicrobial drugs in food-producing animals, and the correct management thereof (49). The JETACAR report was published in October 1999. It contained recommendations for a resistance management programme involving animals and humans (10, 48). They also agreed that the use of antimicrobial drugs in animals could affect human health, but that before any actions are taken, that decisions are based on sound science and risk analysis. In October 2000 the Commonwealth Government tabled a positive response to the JETACAR report and proposed the establishment of an interdepartmental implementation group to oversee and coordinate their antimicrobial strategy (48, 49).

One of the responsibilities of the US Food and Drug Administration's Center for Veterinary Medicine is to evaluate issues relating to the use of antimicrobial drugs in both humans and animals, and to develop policies to protect public health (6). With regard to antimicrobial drug use in animals, the FDA, prepared a document in 1998 entitled, " Evaluation of the Human Health Impact of the Microbial Effects of Antimicrobial New Drugs Intended for Use in Food-Producing Animals". This document addressed the potential human health impact of the microbial effects associated with all uses of all classes of new antimicrobial animal drugs intended for use in food-producing animals (50). In December 1998, the FDA released a discussion document entitled "A Proposed Framework for Evaluating and Assuring the Human Safety of the Microbial Effects on Antimicrobial New Animal Drugs Intended for Use in Food-Producing Animals". The document focused on the issue of

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the use of antimicrobial drugs in food-producing animals and the potential spill over of resistant bacteria to humans (6).

The Working Group of the Advisory Committee on the Microbiological Safety of Food (ACMSF) compiled a report that was published in 1999 in the United Kingdom. It was entitled " Microbial Antibiotic Resistance in Relation to Food Safety". The terms of reference of this document was to estimate the risk of transfer of antimicrobial resistant microorganisms from the food-chain to humans, and to consider the necessity for any actions to protect public health. An underlying question that the ACMSF has taken into consideration was whether the administration of antimicrobial drugs to animals contributes to the selection pressure on the bacterial population that may be transferred to humans in food and cause incurable human infections. This question has been the subject of debate for the past 40 years and is the primary discussion topic of the report. The report also discussed the fact that the administration of antimicrobial drugs to animals results in the acceleration of the rate of antimicrobial resistance emerging in humans. The greater and more frequent the exposure, the greater the selective pressure. However, the magnitude to which antimicrobial drugs given to animals contribute to the overall problem of bacterial antimicrobial resistance in humans remains uncertain (11).

The objectives of the Danish Integrated Antimicrobial Resistance Monitoring and Research Programme (DANMAP) are to provide data on trends in the occurrence of antimicrobial resistance, to monitor the use of antimicrobial drugs, and to see if a comparison can be made between the use of antimicrobial drugs and the occurrence of resistance. This programme represents a close collaboration between veterinary, food

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and health authorities in order to provide comparable data for food animals, food and humans. The results of these monitoring programmes are reported on an annual basis and are known internationally as the DANMAP reports (51).

Lastly, the International Committee of the Office International des Epizooties (OIE) decided in May 1999, to establish an OIE *ad hoc* Expert Group. The mandate of this group was to address antimicrobial resistance originating from the use of antimicrobial drugs in veterinary medicine and the associated public health risks. The findings and recommendations included in the September 1998 ANMV/CNEVA report to the OIE Regional Commission for Europe, as well as the inputs from the OIE Standards Commission had considerably influenced the decision to establish this *ad hoc* group. The OIE *ad hoc* Expert Group was requested to create guidance documents for all OIE member countries for the following issues:

- Risk analysis methodology for managing the potential impact on public health of antimicrobial resistant bacteria of animal origin
- The responsible and prudent use of antimicrobial drugs in veterinary medicine
- Monitoring the quantity of antimicrobial drugs used in animal husbandry
- Harmonization of national antimicrobial resistance monitoring programmes in animals and animal-derived foods
- Standardization and harmonization of antimicrobial susceptibility testing methodologies.

The intention of these guidelines was not to be prescriptive, but to set up a framework for member countries that could be used to generate scientific based information (49).

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When these guidelines are used, results can be obtained that can be compared internationally and assist in the risk analysis process.

2.8 Surveillance and monitoring of antimicrobial resistance

During the recent past there have been many calls for action to halt the well-documented increase in occurrence of antimicrobial resistance. The current awareness of governments and the public health care concerns have prompted action to tackle the antimicrobial resistance problem (50). A key element in the fight against antimicrobial resistance and a better understanding of the magnitude of this problem is the surveillance and monitoring of antimicrobial resistance (29, 52). Better surveillance of resistance is urgently needed to understand the interaction between the use of antimicrobial drugs and the development of resistance. Once correlation between use and resistance has been established for a particular country or area, surveillance data can be used as "information for action" to reduce unnecessary prescribing and extend the usefulness of existing antimicrobial drugs as well as to monitor the impact of management strategies (43). The collection of data and information forms an integrate part of risk assessment, that will be followed by risk management when using the data for scientific decision-making for the control of bacterial resistance to antimicrobial drugs (14, 20, 53, 54).

2.8.1 Definition of monitoring and surveillance

In the International Animal health Code, the OIE defines surveillance in animal health as 'the continuous investigation of a given population to detect the occurrence of disease for control purposes, which may involve testing of a part of the population'. The OIE definition of monitoring 'constitutes on-going programmes directed at the detection of changes in the prevalence of disease in a given population and in its

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environment'. In the context of this guideline, "disease" can be substituted with "antimicrobial resistance" (55).

2.8.2 Existing surveillance and monitoring programmes

A questionnaire prepared by the OIE indicated that only 16 of the 35 European member countries have official antimicrobial resistance monitoring programmes, while only 9 include monitoring of food of animal origin. Moreover, only 7 out of 16 countries indicated that they coordinate programmes in the veterinary and human fields (47). A similar study was done by the Commission of European Communities, Agriculture and Fisheries (FAIR) on the existence of antimicrobial resistance surveillance programmes in 13 European countries. They found that human and animal bacterial isolates were monitored in most of these countries but only in one was there regular monitoring of isolates from food and healthy animals (56). A few of the current established surveillance and monitoring programmes will be mentioned below.

In 1996, the Centers for Disease Control and Prevention (CDC), the U.S. Department of Agriculture (USDA), and the Food and Drug Administration (FDA) established the National Antimicrobial Resistance Monitoring programme (NARMS) to prospectively monitor changes in antimicrobial susceptibilities of zoonotic enteric pathogens. Specimens are collected from human and animal clinical specimens, from healthy farm animals, and from carcasses of food-producing animals at slaughter plants. This program was proposed to ensure the continued effectiveness of the fluoroquinolones after its approval for use in food-producing animals in the United States. Veterinary testing is conducted on non-typhoid *Salmonella* as the indicator organism. Monitoring

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of veterinary *Campylobacter* spp. and *E. coli* 0157 as well as a pilot study monitoring human and poultry *Enterococcus* isolates have also began in 1998 (5, 57, 58, 59).

In France, two bacterial monitoring programmes are currently running, the first being active since 1969. The latter programme incorporates the resistance monitoring of *Salmonella* isolated not only from sick animals and healthy carriers but also from food and feed or the environment. The second programme consists of resistance monitoring of the main bovine pathogenic bacteria in calves, which include *Salmonella* and *E. coli* for digestive tract infections, *Pasteurella multocida* and *Mannheimia haemolytica* for infections of the respiratory tract and *Staphylococcus* spp. and *Streptococcus* spp. for mammary infections (1, 60). Results obtained in both programmes constitute the basis of predictive epidemiology necessary to set up a policy for the sensible use of antimicrobials in veterinary medicine (60, 61).

Antimicrobial resistance has been monitored in *Salmonella* isolates from animals in England and Wales since 1970. Because of this long-standing monitoring system, comparison of the results of earlier years, with the latest results can indicate the current resistance trends. During the years in existence the monitoring programme also included new antimicrobial drugs that were introduced into veterinary practice. This provided an unique opportunity to study the development and epidemiology of antimicrobial resistance (62).

In Denmark the use of the glycopeptide growth promoter, avoparcin, has been associated with the occurrence of vancomycin resistant enterococci in production animals. Following these observations, the Danish Ministry of Agriculture and

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Fisheries decided to ban avoparcin and establish a surveillance programme to monitor resistance to antimicrobial drugs used for growth promotion and therapy in Denmark. This continuous monitoring of the occurrence of antimicrobial resistance in bacteria was established in 1995 (63, 64, 65).

The programme, Danish Integrated Antimicrobial Resistance Monitoring Programme (DANMAP) establishes close collaboration between veterinary, food and health authorities in order to provide comparable data for food animals, food and humans. In this programme indicator and zoonotic bacteria and animal pathogens are continuously isolated from broilers, cattle, pigs and fish and tested for susceptibility to antimicrobial agents for therapy and growth promotion. *Enterococcus faecium* and *E. faecalis* are used as indicator bacteria for resistance to antimicrobial agents that are active against Gram-positive bacteria, and *Escherichia coli* as indicator for Gram-negative organisms. *Salmonella enterica, Campylobacter coli/jejuni* and *Yersinia enterocolitica* are included as zoonotic agents. Human pathogens are also included in the programme and include *Salmonella*, C*ampylobacter* spp. and *Y. enterocolitica*. Strains are collected from diagnostic submissions from general practices and from hospitals. A number of hospital laboratories also provide results of susceptibility testing for *Streptococcus pneumoniae*, *Staphylococcus aureus,* and *E. coli* and coagulase-negative staphylococci. The Danish surveillance programme is widely believed to be the best and most comprehensive of all (63, 64, 65).

The European Community has funded the implementation of a European antimicrobial resistance surveillance system (EARSS). One of the EARSS's goals is to collect comparable and quantitative data through assessing antimicrobial resistance

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and analyzing regional differences to facilitate new guidelines for use of antimicrobials. This programme commenced in 1998 focusing on *Streptococcus pneumoniae* and *Staphylococcus aureus.* An Antimicrobial Resistance Monitoring network is being established by the World Health Organization (WHO) and EARSS will be incorporated into this network (43).

Another veterinary monitoring programme that has been established is the Spanish surveillance network. The Spanish network officially named ”Red de Vigilancia de Resistencias Antibióticas en Bacterias de Origen Veterinario” (Network of surveillance of antimicrobial resistance in bacteria of veterinary origin), using the acronym VAV, was formed in 1996. The VAV network was designed to cover the three critical areas of veterinary responsibility namely bacteria from sick animals, bacteria from healthy animals and bacteria from food animals. Initially only bacteria from sick animals, using *E. coli* as the indicator bacterium was included in the programme. Surveillance of *E. coli* and *Enterococcus faecium* from healthy pigs was implemented in 1998 and data collection of *Salmonella* was commenced in poultry slaughterhouses in 1999. The surveillance of bacteria from food animals is the only matter that still has to be addressed and implemented (66).

Sweden also established a resistance monitoring programme especially for bacteria of animal origin. The Swedish Veterinary Antimicrobial Resistance Monitoring (SVARM) programme’s first annual report was published in 2000. Intestinal bacteria of healthy animals, zoonotic bacteria and animal pathogens are included in the programme (67).

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In South Africa, the Antibiotic Workgroup, an interdepartmental group recognized by the Departments of Health and Agriculture, took the initiative to establish a national veterinary antimicrobial resistance surveillance and monitoring programme. The terms of reference of the group are:

1) The implementation of an antimicrobial resistance monitoring and surveillance programme
2) The establishment of efficient and effective registration procedures of veterinary medicinal products containing antimicrobial products
3) That the veterinary profession is adequately represented with regard to the registration of veterinary medicinal products
4) The establishment of operational laboratories at a national and regional level capable of monitoring the levels of antimicrobial resistance, the quality of veterinary medicinal products containing antimicrobial drugs and the presence of residues in feed and animal products
5) The efficient control of imported veterinary products so that counterfeit and or substandard products can be seized
6) That the administration of veterinary medicinal products containing antimicrobial drugs is under the control of a veterinarian
7) The establishment and dissemination of technical guidelines for the responsible and prudent use of antimicrobial drugs in veterinary medicine

Two other groups in South Africa namely, The Antibiotic Surveillance Forum (ASF) from the private sector and The Antibiotic Study Group (ASG) from the academic sector are active in the medical field. Their terms of reference are similar to those of

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the Antibiotic Workgroup, but focus on bacteria from humans rather than from animals.

2.8.3 Key elements of a surveillance programme

Surveillance of resistance requires laboratory facilities organised in a network within which data on patterns of resistance can be shared for analysis and interpretations. In many developing countries and in countries whose economics are in transition, laboratory facilities and information networks will need considerable strengthening before reliable resistance surveillance programmes could be established (54). This is the reason why only a few countries have established resistance surveillance programmes, mainly for the most important foodborne zoonotic bacteria, while in other countries, surveillance and monitoring programmes are only in the early stages of development (39).

Surveillance programmes are made up of several elements, that form an intricate part in the whole process of data gathering and processing. In order to compare the results of the different surveillance programmes the following elements or factors should be considered bacteria to be included, antimicrobial drugs to be tested, animal species from which specimens should be collected, sampling strategies, standardized susceptibility testing, quality control and the recording and reporting of results.

2.8.4 Bacteria to be included

The three most important classes of organisms to be included in a surveillance programme are:

1) Veterinary pathogens
2) Zoonotic bacteria

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3) Commensal bacteria (indicator bacteria)

Where possible, information should be gathered on the source of the isolates, such as species or where the sample was taken from, as well as data on antimicrobial treatment history and exposure to antimicrobial drugs (3, 39, 55, 68).

2.8.4.1 Veterinary pathogens

Veterinary pathogens represent an important group of bacteria to be monitored since pathogenic bacteria are the main targets of antimicrobial treatment in animals and undergo the greatest selective pressure (3, 68). The main focus of surveillance of this group of bacteria should be the development of an early warning system for the detection of new, emerging or developing patterns of resistance that may pose a health threat for humans or animals. Veterinary pathogens that are routinely tested in different countries include *Staphylococcus aureus*, *Streptococcus spp.*, *Salmonella* serovars, *Mannheimia haemolytica*, *Pasteurella multocida* and *Actinobacillus pleuropneumoniae*. The choice of organisms to be included in a programme depends on the animal production systems and the animal disease situation in a country (55, 68).

2.8.4.2 Zoonotic Bacteria

The development and spread of antimicrobial drug resistant zoonotic bacteria has direct public health implications. The objective therefore is to evaluate the risks involved through the analysis of the prevalence of zoonotic bacteria in food animals, the monitoring of the emergence of resistant clones of bacteria and the epidemiology of bacterial resistance in countries (3, 55, 68). This will permit the establishment of an early warning system, aimed at identifying new resistance phenotypes, the detection of new mechanisms of resistance, and the prevention of epidemic spread. It will also

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give an indication of the correlation of the prevalence of antimicrobial resistance in these bacteria in reflection to the consumption of antimicrobial drugs in humans and animals (68). Surveillance should be primarily be focused on *Salmonella* and *Campylobacter* spp. but can be extended to other zoonotic bacteria such as *Yersinia enterocolitica* and *Listeria monocytogenes* (3).

2.8.4.3 *Indicator bacteria*

Indicator or commensal bacteria represent a reservoir of bacterial strains and antimicrobial resistance genes that could be transferred to both human and animal pathogens. Studying these organisms will permit comparison of resistance parameters in different animal species and different regimens of antimicrobial use (3, 68). Indicator bacteria that should be focused on for surveillance are *E. coli* and *Enterococcus* spp. especially *Enterococcus faecalis* and *Enterococcus faecium* (3, 55, 68).

2.8.5 Animal species from which specimens should be collected

Sampling should focus on the major food-producing species in which the presence or potential transfer of zoonotic organisms are most likely to be significant. Categories that should be considered for sampling include cattle and calves, slaughter pigs, broiler chickens and/or other poultry and farmed fish (39, 55).

2.8.6 Specimen collection

Specimens for surveillance programmes can either be collected from healthy animals at abattoirs, or bacterial pathogens isolated from specimens routinely submitted to a veterinary diagnostic laboratory (55). The specific group or population to be represented by the specimen may be collected randomly, systematically or stratified

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within the population of concern. If a correct sampling strategy is selected it will present specimen estimates that are accurate for the population of interest. When a suitable sampling strategy has been chosen, calculating a statistically based specimen size will allow programme monitors to determine the accuracy of the prevalence estimates that will be obtained from the collected specimens (56).

2.8.7 Antimicrobial drugs to be tested

The test panel of antimicrobial drugs that should be included in a surveillance programme should consist of all the antimicrobial classes commonly used in both human and veterinary medicine. However, for some classes of antimicrobial drugs cross-resistance exists, therefore only one antimicrobial need to be chosen as a representative of the class. The number of antimicrobial drugs to be tested may have to be limited according to the financial resources of a country (39, 55, 65).

2.8.8 Standardised susceptibility testing

Standardised susceptibility testing is discussed under laboratory methodologies.

2.8.9 Quality control/assurance

Quality control/assurance is discussed under laboratory methodologies.

2.8.10 Recording of results

The capturing of primary, non interpreted data is important in order to allow for the assessment of the data in response to various questions including those that may arise in the future. Results should be entered into an appropriate data base and recorded quantitatively, for example as distribution of MICs in µg/ml or inhibition zone diameters in millimeter. The recording of results forms a vital part of any surveillance

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and monitoring programme because it could indicate the development of reduced sensitivity to bacterial strains or substrains at an early stage when they are compared with the specific microbial population being worked with (55, 65, 68).

2.8.11 Reporting of results

Timely and comprehensive reporting of surveillance results to all interested parties is very important. This can be done by compiling an annual report that include information on the structure of the monitoring system and on the chosen laboratory methods. A report would also include summary data, presented as percentages of resistant, intermediate or susceptible strains and quantitative data (3). Other information that can be of value as a further step in the development of the programme, is statistics on the number of animals, antimicrobial use data and antimicrobials authorized for use. If it is feasible, trends in occurrence of resistance should also be related to antimicrobial usage data and also the disease situation present in the country (39, 55).

2.9 Methodology

2.9.1 Antimicrobial susceptibility testing methodologies

The rapid increase and dissemination of antimicrobial resistance among many bacterial pathogens have resulted in the use of antimicrobial susceptibility testing as a standard procedure. Soon after the introduction of antimicrobial drugs for treatment of bacterial disease, antimicrobial susceptibility testing was instigated in countries around the world. This was primarily driven by the need to identify suitable antimicrobials for successful clinical use (69). Reproducibility of antimicrobial susceptibility testing methods is an important factor when accurate and consistent data are required. Thus, laboratories should use standardized sensitivity testing methods

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and adopt quality control measures to ensure the reporting of reliable and reproducible susceptibility data (69, 70). Additionally, data generated from sensitivity tests can be documented to observe shifts in resistance patterns under the selective effects of antimicrobial drugs and to study the trends in resistance of various strains of bacteria each year (39, 71, 72, 73).

Antimicrobial sensitivity tests are designed to provide reproducible results with a strong correlation to *in vivo* efficacy (70). The main goal of *in vitro* antimicrobial susceptibility testing is to foresee how a bacterial pathogen will respond to the antimicrobial agent *in vivo*. This usually provides information that designates a bacterium as resistant, intermediate (moderately sensitive) or susceptible (sensitive) to the action of a particular antimicrobial (69, 73). Resistant implies that the bacterium is unlikely to respond to treatment with the specific antimicrobial. Susceptible indicates that the antimicrobial drug would be successful in treating the bacterial infection. Intermediate implies that an intermediate or indeterminate response is likely, except perhaps in special, defined circumstances, such as when high levels of the drug can be used, or the drug is concentrated at the site of infection (96, 73, 74).

Antimicrobial sensitivity tests may be classified in two groups, namely qualitative and quantitative. Results are quantitative if zone diameters or MICs are reported and qualitative when they are reported as susceptible, intermediate or resistant. The advantage of determining quantitative susceptibilities of an organism is that it offers the potential to relate this information to knowledge of drug concentrations in body tissue (76). This data can be obtained through a number of methods, including broth dilution, agar dilution and disk diffusion (39). A wide variety of antimicrobial

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susceptibility testing methodologies are being used in microbiological laboratories but only these three methods have shown to give reproducible and repeatable results (69).

2.9.2 Dilution techniques

Dilution susceptibility tests are quantitative tests used to determine the minimal concentration of an antimicrobial required to inhibit or kill bacteria *in vitro*. In dilution methods, specific amounts of the antimicrobial, prepared in decreasing concentration in broth or agar, are inoculated with a standardised suspension of the bacteria to be tested (71). The lowest concentration of the drug that inhibits the visible growth of an organism is the minimal inhibitory concentration (MIC) (69, 71, 74, 76). The MIC method is also the most quantitative method used to measure bacterial susceptibilities to a drug *in vitro*. It has the advantage of being a single reference point for drugs that are usually bacteriostatic, as well as for those that can be bactericidal.

The broth dilution method can be performed in either tubes (macrodilution) or in microtitration plates (microdilution). Microdilution tests provide MIC's that are nearly identical to those that are obtained with macrodilution tests. If large numbers of strains and multiple agents have to be tested the broth microdilution method is usually used because it offers efficiency of technical time and materials compared to the macrodilution method (77). Sensitivity testing by the MIC method has improved during the past few years through establishment of standards and quality control procedures by the National Committee for Clinical Laboratory Standards (NCCLS), 940 West Valley Road, Suite 1400, Wayne, Pennsylvania 19087 USA (71, 74, 78, 79). Microdilution trays can be prepared in any laboratory but are also commercially available in a frozen or freeze-dried state. One of the commercially available systems

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for the microdilution method is the well-known Sensititre Susceptibility System, approved by the NCCLS (71, 74, 78, 79).

Agar dilution tests are similar to the broth dilution method, with the substitution of liquid for semi-solid media. It involves the incorporation of an antimicrobial drug into an agar medium in a geometrical progression of concentrations followed by the application of a defined bacterial inoculum to the surface of the plate. The agar dilution method also results in the accurate determination of a MIC (69).

2.9.2.1 Disk diffusion method

Rather than preparing dilutions of each drug, antimicrobial susceptibility tests may be done with agar diffusion methods, the two best known examples being the Kirby-Bauer and Stokes methods (74). The latter test however, is now generally regarded as obsolete. A constant concentration of antimicrobial in the form of discs or tablets are placed on a seeded agar medium and allowed to diffuse into the surrounding medium. This exposes the test organism to a continuous gradient of drug concentrations, with diminishing concentrations at increasing distances from the disk. The diameter of each zone of inhibition is measured to the nearest millimeter with a ruler, sliding caliper or electronic instrument. The zone diameters for individual antimicrobial drugs are translated in terms of susceptible, intermediate or resistant categories by referring to an interpretive chart (80).

The disk diffusion method provides a qualitative or at best semi-quantitative indication of susceptibility (79, 81). The MIC of an organism can be extrapolated from inhibitory zone diameters, and these MIC values have been used to define breakpoints to describe bacteria as either susceptible or resistant (74, 82). The disk-

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(agar) diffusion method is satisfactory only when a microorganism is either very susceptible or very resistant (78, 81). Disk diffusion is technically straightforward to perform, reproducible and does not require expensive equipment. Although disk diffusion is the easiest and most cost-effective method for antimicrobial susceptibility methods, there are many aspects of this method that must be standardised (69).

2.9.2.2 Agar diffusion versus MIC testing

There has been some controversy about the best and the most reliable methods for susceptibility testing of antimicrobial drugs (83). Agar disc diffusion and MIC testing have been regarded as the two reference methodologies to choose from (82). In 1982, the dilution and disk diffusion susceptibility test results were examined and reported for the College of American Pathologists Survey. They discovered that the microdilution technology was slowly gaining in popularity and consequently the number of laboratories that were using the standardized disk test was slowly decreasing (78). It has been suggested that MIC testing is preferred, rather than agar disk diffusion tests, to determine the antimicrobial susceptibilities of veterinary pathogens. This is, because of the qualitative nature of the latter test method and lack of zone size interpretive criteria available for veterinary pathogens in the different animal species (70, 74).

Perhaps one of the most useful features of MIC testing is that the data can be used to describe resistance. This is especially important in a surveillance programme when minor shifts or trends in resistance need to be detected (79). There is currently not a worldwide consensus on what laboratory method to use. According to the OIE guidelines, in order to standardise and harmonise methodologies all over the world, the best antimicrobial susceptibility data to be collected should be quantitative rather

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than qualitative for comparison purposes (69). Dilution testing is favoured over disk diffusion when considering surveillance and monitoring of antimicrobial resistance, but there is general consensus that disk diffusion can reach the same objectives if dilution tests cannot be preformed for practical reasons.

2.9.2.3 Breakpoints in in-vitro antimicrobial sensitivity testing

The designations sensitive, resistant or intermediate, common to almost all methods of clinical laboratory testing, are distinguished by the use of an *in vitro* breakpoint antimicrobial drug concentration. In the disc diffusion method, the determination is indirect, but nonetheless ultimately related to these designations. The only clear property of breakpoint antimicrobial drug concentrations is that it is largely arbitrary, based on consensus decisions related to pragmatic considerations. Breakpoints seek to achieve two objectives that may be irreconcilable: therapeutic relevance and laboratory reproducibility (73, 84).

The antimicrobial susceptibility (or resistance) of a strain cannot be measured directly but must be deduced from the *in vitro* activity of the antimicrobial drug. Among the various methods available, MIC determinations are the most widely used to assess *in vitro* activity for clinical isolates. To convert MIC values into susceptible, intermediate or resistant categories, i.e., to assess whether it is possible to treat an infection by a given antimicrobial drug, reference is made to the critical values recommended by national committees such as the British Society for Antimicrobial Chemotherapy (BSAC), or the National Committee for Clinical Laboratory Standards (NCCLS) (84). The values are established on the basis of bacteriological, pharmacological and clinical criteria (84, 86).

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The differences between the critical values recommended by the various committees arise from the contrasting definitions of susceptibility and resistance. Despite these differences a consensus has been reached in Europe for the establishment of critical values based on the three criteria mentioned below:

1) MIC distribution of bacterial populations belonging to different species and harboring genetically and biochemically characterized resistance mechanisms
2) Pharmacokinetics, at usual and maximum dosages, using the different routes of administration
3) Correlation between the clinical and bacteriological results for the therapeutic indications assigned by the different Ministries of Health (70, 84).

That breakpoints vary considerably between the different guidelines seems to be the rule and not the exception. Even the methods of reporting breakpoints used by different parties differ to the degree that it makes comparison problematic. To agree upon a single set of guidelines seems to be difficult because of local differences in antimicrobial drug legislation and a number of uncertainties regarding drug dose, drug concentration, MIC and effects of antimicrobial drugs. Nonetheless, international standardization is needed and should be considered as an important issue for international collaboration and harmonization (85).

2.9.3 Standardization of antimicrobial susceptibility testing methodologies

There are at least six standardized methodologies used, but the majority of veterinary diagnostic laboratories rely on the National Committee for Clinical Laboratory Standards' (NCCLS) recommended testing procedures using both the agar disk diffusion and broth microdilution techniques (70, 72, 78). The NCCLS procedures are well documented and offer high reproducibility and a rigorous quality control. Most

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European clinical diagnostic laboratories use NCCLS methods routinely. The majority of European, International and Pharmaceutical Surveillance Systems use NCCLS guidelines, as do most laboratories in North and South America and also some in Japan and Australia (82).

2.9.4 Quality control/assurance

The goals of a quality control programme are to monitor the following:

1) The precision and accuracy of the susceptibility test procedure
2) The performance of reagents and the viability of the microorganisms used in the test
3) The performance of the personnel who carry out the tests and interpret the results

Strict adherence to the standardised techniques in conjunction with quality control of media and reagents is necessary for the generation of reliable and reproducible antimicrobial susceptibility data. It is important that the appropriate quality control reference bacteria are included to ensure standardisation (69, 87).

2.10 Possible benefits from this research project

In South Africa, a veterinary antimicrobial resistance monitoring programme has not been established and there is a paucity of literature on this subject. Implementation of such a programme may lead to the following :

1) A network for surveillance that will ensure the early detection of the emergence of resistance in bacteria of veterinary and human public health importance.
2) Information on resistance trends that may be utilized to encourage and advise role players on the responsible (prudent) use of antimicrobials in animal production, thereby prolonging its effective use both for animal and human health.

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3) A national standard operating procedure (SOP) that will ensure reproducible results.
4) Cooperation with similar programmes in public health.
5) Local and descriptive data on the extent and temporal trends of antimicrobial resistance in selected veterinary pathogens, from production animal populations, on an annual basis.
6) Collaboration among the national and international laboratories involved in surveillance programmes.

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CHAPTER 3

3. MATERIALS AND METHODS

3.1 Specimens

Specimens for the isolation of bacteria and stored isolates were obtained from the bacteriology laboratory of the Department of Veterinary Tropical Diseases, and Poultry Reference Centre of the Faculty of Veterinary Science, University of Pretoria; the Onderstepoort Veterinary Institute; and Du Buisson and Partners, Medical Pathologists, Pretoria. In addition, lung specimens from various cattle feedlots in South Africa were collected by representatives from Intervet SA (Pty) Ltd for the purpose of isolating strains of *Mannheimia (Pasteurella) haemolytica.* Human strains of *Enterococcus faecalis* were also collected due to the low isolation rate of this bacterial species in animals.

Pure strains of overnight growth of each organism were inoculated into Brain Heart Infusion broth (CA Milsch), transferred to sterile 2ml Simport vials and stored at -70°C. The isolates stored for testing included the following:

Zoonotic pathogens: *Salmonella.*

Indicator bacteria: *E. coli, Enterococcus faecium, E. faecalis*

Veterinary pathogens: *Mannheimia (Pasteurella) haemolytica*

The 30 isolates of each bacterium collected for susceptibility testing are indicated in Annexure I. The source and animal/human from which they were isolated are also indicated.

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3.2 Antimicrobial drugs

Table 3.1: Antimicrobial drugs selected for use in a surveillance programme.

NUMBER	ANTIMICROBIAL	SUPPLIER
1	Enrofloxacin	Bayer
2	Oxytetracycline	Pfizer
3	Gentamicin	Virbac
4	Florfenicol	Schering-Plough
5	Amoxycillin	Intervet
6	Cephalexin	Virbac
7	Neomycin	Pharmacia Animal Health
8	Tilmicosin	Elanco
9	Trimethoprim/Sulfamethoxazole	Virbac
10	Sulfadimethoxine	Burchem

3.3 Reference strains

The reference strains were obtained from the bacteriology laboratory of the Department of Veterinary Tropical Diseases and the South African Bureau of Standards. The following reference strains were used:

Escherichia coli ATCC 25922

Pseudomonas aeruginosa ATCC 27853

Enterococcus faecalis ATCC 29212

Staphylococcus aureus ATCC 29213

3.4 Experimental design

The specimens were collected as systematic random specimens; however, formal randomization was not carried out.

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Specimens were streaked onto Blood and MacConkey agar (CA Milsch). The bacterial growth was examined for purity and viability. Isolates that were contaminated were purified before further tests were attempted. Isolated organisms were batched and tested once the required number was obtained. All isolates were identified and labeled as follows:

- Specimen number
- Date of specimen collection
- Animal/human from which it originated
- Source from which it was isolated

The contributing laboratories did the initial identification of their isolates. To ensure that the correct identification was made, specific tests were done on the respective isolates.

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Susceptibility to the therapeutic agents was determined with the microdilution method and expressed as minimum inhibitory concentrations (MICs). The standardized method according to the NCCLS was used. Susceptibility tests were done twice for each individual organism, including the reference strains as a quality control measure.

Results were compared with the NCCLS minimum inhibitory concentration breakpoints. The tested isolates could then be classified according to their MICs as susceptible, intermediate or resistant (87).

3.5 Bacterial identification

The following tests were done to confirm the identity of isolates:

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1) *Salmonella:* Are non-lactose fermenters and produce pale colonies on MacConkey agar. Colonies characteristic for *Salmonella* on the selective media was inoculated, singly into a triple sugar iron (TSI) agar slope and lysine decarboxylase broth. The typical reaction for *Salmonella* in TSI agar is a red (alkaline) slant, yellow (acid) butt and superimposed (black) H_2S production. The test for lysine decarboxylase is positive (purple) (88).

2) *E coli*: Is a Gram-negative rod. The majority of *E. colis* produce bright pink colonies because of fermentation of lactose in MacConkey agar. The IMViC test (indole +/ MR +/ VP-/ citrate -) is a quick presumptive method for identifying *E. coli*, as almost no other lactose–fermenting member of the Enterobacteriaceae gives the combination of results for these tests (88).

3) *Mannheimia (Pasteurella) haemolytica*: To differentiate between *Mannheimia haemolytica* and *Pasteurella multocida,* the criteria depicted in Table 3.2 were used.

Table 3.2: Parameters to differentiate between *Mannheimia haemolytica* and *Pasteurella multocida*

TESTS	***Mannheimia haemolytica***	***Pasteurella multocida***
Indole	-	+*
MacConkey agar	Small pink colonies	No growth
Blood agar	Beta haemolysis	No haemolysis
Odour	None	Sweetish
Oxidase	-*	-*
Catalase	+	+
Motility	-	-
H_2S production	-*	-*
Acid from glucose	+	+
Fermentation of sugars in TSI	Medium yellow throughout No gas	Same No gas

*Rare exceptions

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4) *Enterococcus faecium:* Enterococci tolerate the bile salts in MacConkey agar and appear as small pinpoint colonies on this medium. They are facultative anaerobes, catalase-negative, oxidase-negative, Gram-positive and belong to Lancefield Group D. For the differentiation of *Enterococcus faecium* and *Enterococcus faecalis* the following parameters were used: lactose, arabinose, sorbitol, mannitol and growth in 6.5% NaCl + (88). The criteria are indicated in Table 3.3.

Table 3.3: Parameters used to differentiate between *Enterococcus faecalis* and *Enterococcus faecium.*

TESTS	*Enterococcus faecalis*	*Enterococcus faecium*
Lactose	+	+
Arabinose	-	+
Sorbitol	+	-
Mannitol	+	+
Growth in 6.5% NaCl	+	+
Inulin	-	-/+

3.6 Experimental procedure

3.6.1 Microdilution method

Sterile, plastic, microdilution plates with round wells (Sterilab), each containing 0.05 ml of broth were used. The plates containing the antimicrobial drugs for testing were prepared in-house and are described in subsequent section (40, 84, 89).

3.6.1.1 Preparation of antimicrobial stock solutions

a) Weighing antimicrobial drug powders

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Powders were weighed on an analytical balance and dissolved to yield the required concentration based on balanced activity or potency of the respective antimicrobial drugs (87, 89). The following formula was used to determine the amount of diluent needed for a standard solution:

$$\text{Volume (ml)} = \frac{\text{Weight (mg)} \times \text{Assay Potency } (\mu g/mg)}{\text{Concentration } (\mu g/ml)}$$

More than 100 mg of each antimicrobial powder was weighed. It is advisable to weigh accurately a portion of the antimicrobial powder in excess of what is required and then calculate the volume of diluent needed to obtain the desired concentration. The antimicrobial powders were stored according to the directions of the manufacturer.

b) Preparing stock solutions

Antimicrobial stock solutions were prepared at concentrations of 1,000µg/ml or 5120 µg/ml. The concentration used depended on the highest dilution tested for each antimicrobial drug in its dilution range (87, 89).

Some of the antimicrobial drugs had to be dissolved in solvents other than water. In those cases only enough solvent was used to solubilize the antimicrobial powder to give a translucent solution. It was then further diluted to the final stock concentration with water or the appropriate diluent. The solvents and diluents used for preparation of the antimicrobial powders for this study are shown in Table 3.4.

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Table 3.4: Solvents and diluents used in the preparation of stock solutions of antimicrobial drugs requiring solvents other than water

ANTIMICROBIAL DRUG	SOLVENT	DILUENT
Enrofloxacin	½ volume water, then add 1mol/L NaOH to dissolve	Water
Oxytetracycline	Water	Water
Gentamicin	Water	Water
Florfenicol	95% Ethanol	Water
Amoxycillin	Phosphate buffer, pH 6.0, 0.1 mol/L	Phosphate buffer, pH 6.0, 0.1 mol/L
Neomycin	Water	Water
Tilmicosin	95% Ethanol	Water
Trimethoprim/ Sulfamethoxazole	0.05 mol/L lactic or hydrochloric acid, 10% of final volume	Water
Sulfadimethoxine	½ Volume of hot water and minimal amount of 2.5 mol/L NaOH to dissolve	Water
Cephalexin	Phosphate buffer, pH 6.0, 0.1 mol/L	Water

Aliquots of a 1000µl of the stock solution were dispensed into sterile 2ml Eppendorf tubes, sealed, and stored at –70 ºC. Stock solutions of most antimicrobial drugs will remain stable for at least six months at –20 ºC and longer at –70 ºC, in concentrations of 1,000 µg/ml or greater (84, 87, 89). Vials were removed when needed and used the same day. Any unused drugs were discarded at the end of the day.

3.6.1.2 <u>Dilution of antimicrobial drugs</u>

The two-fold dilution schemes that were used were determined by the published breakpoints for the different antimicrobial drugs, and are indicated in Table 3.6. The antimicrobial stock solutions were diluted as follows:

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Table 3.5: Scheme for preparing dilutions of antimicrobial drugs to be used in broth dilution susceptibility tests.

ANTIMICROBIAL SOLUTION:						
STEP	CONC	SOURCE	VOL[a] +	CAMHB[b] VOL[a] =	FINAL CONC	LOG_2
1	5120µg/ml	Stock	1 ml	9 ml	512µg/ml	9
2	512	Step 1	1	1	256	8
3	512	Step 1	1	3	128	7
4	512	Step 1	1	7	64	6
5	64	Step 4	1	1	32	5
6	64	Step 4	1	3	16	4
7	64	Step 4	1	7	8	3
8	8	Step 7	1	1	4	2
9	8	Step 7	1	3	2	1
10	8	Step 7	1	7	1	0
11	1	Step 10	1	1	0.5	-1
12	1	Step 10	1	3	0.25	-2
13	1	Step 10	1	7	0.125	-3

a. The volumes selected were multiples of these figures, depending upon the number of tests to be performed.

b. Cation adjusted Mueller-Hinton Broth

c. This scheme was obtained from the NCCLS Document M31-A, Vol. 19 No. 11, June 1999.

The concentration range of each antimicrobial used in the dilution scheme is shown in Table 3.6 (47, 57, 67, 75, 84, 87, 89).

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Table 3.6: Dilution scheme for antimicrobial drugs used in this study.

COLUMN	ANTIMICROBIAL	CONCENTRATION RANGE (µg/ml)
1	Enrofloxacin	0.03 – 4
2	Oxytetracycline	0.5 – 64
3	Gentamicin	0.25 – 32
4	Florfenicol	0.125 – 16
5	Amoxycillin	0.5 – 32
6	Neomycin	1 – 128
7	Tilmicosin	05 – 64
8	Trimethoprim/ Sulfamethoxazole (1/19)	0.125/304– 16/2.4
9	Sulfadimethoxine	4 – 512
10	Cephalexin	0.125 – 8

a) If the inoculum was added manually by means of a microtitre pipette, a 1:2 dilution of the final drug concentration was allowed. This was made possible by preparing double strength solutions for each antimicrobial agent concentration.

b) From each antimicrobial concentration in the dilution range, aliquots of 50µL were dispensed to the corresponding wells in each plate.

c) Each microdilution plate also included two growth control wells that did not contain any antimicrobial solution, but only the bacterial suspension to check the viability of the test organism.

3.6.1.3 *Broth media*

1. Cation-adjusted Mueller-Hinton broth (CAMHB) (CA Milsch) was used.
2. To achieve reproducible results for the MICs of tetracycline for all bacteria, Mueller Hinton broth contained the correct concentration of the divalent cations Ca^{++} and Mg^{++}. The Mueller-Hinton broth received from the manufacturer did not contain the correct concentrations, and cation adjustments were made.

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3. According to the NCCLS guidelines, Mueller-Hinton broth should contain 20 – 25 mg of Ca^{++}/L and 10 – 12.5 Mg^{++}/L. To obtain this, the following procedures were followed to have the correct divalent cation concentration:

a) For the preparation of the magnesium stock solution, 8.36 g of $MgCl_2.2H_2O$ was dissolved in 100ml deionized water. This solution contained 10 mg of Mg^{++}/ml.

b) For the preparation of the calcium chloride stock solution, 3.68 g of $CaCl_2.2H_2O$ was dissolved in 100 ml deionized water. This solution contained 10 mg Ca^{++}/ml.

c) Stock solutions were sterilized by membrane filtration and stored at 4°C.

d) With stirring, 0.1 ml of chilled Ca^{++} and Mg^{++} stock solution per litre of broth was added for each desired increment of 1mg/L in the final concentration of Ca^{++} and Mg^{++} in the adjusted Mueller-Hinton broth. This medium was designated cation adjusted Mueller-Hinton broth (CAMHB).

4. Broth preparation was as follows: 21g of Mueller-Hinton broth was dissolved in 1 litre of distilled water, autoclaved for 15 minutes at 121°C, and chilled overnight at 4°C.

5. The pH of each batch of Mueller-Hinton broth was checked with a pH meter when the medium was prepared. The correct pH of 7.2 - 7.4 at room temperature (25°C) was obtained for each batch of broth (84, 87, 89).

***Note:**

Enhanced growth and greater consistency was obtained when testing ***Mannheimia haemolytica*** by supplementing the Mueller-Hinton broth with 0.1 ml inactivated bovine serum prior to inoculation. Heating for 30 minutes at 56ºC inactivated the serum.

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3.6.1.4 *Preparation and storage of microdilution plates*

The following procedures were followed when preparing the microdilution plates:

1. As the plates were filled, they were stacked in groups of 5 plates and covered with an adhesive seal. In this way, each tray fitted on top of the other tightly enough to provide a cover that minimizes evaporation and contamination.
2. Each stack was then sealed in a plastic bag.
3. The freshly filled plates were stored at -70ºC until required for use. This treatment ensured maximum stability of the antimicrobial solutions. Self-defrosting units were avoided because a fluctuation in temperature during the defrost cycle can occur. This fluctuation may be significant enough to thaw and refreeze the antimicrobial agents, thus contributing to their rapid deterioration.
4. Prior to inoculation, the trays were thawed at room temperature for approximately 1 hour before use.
5. Unused thawed plates were discarded and never refrozen.
6. Storage at -70ºC can significantly increase the shelf life to at least 2 months. Randomly selected plates were stored at -70ºC for a period of two months and retested with the reference strains (89, 90, 91).

3.6.1.5 *Inoculation procedures*

a) Preparation of inoculum

A spectrophotometer was used to standardize the inoculum.

1. Four or five well isolated colonies with the same morphological appearance were picked with a wire loop from an overnight agar plate.
2. The colonies were emulsified in a tube containing 5 ml of Mueller-Hinton broth.
3. The spectrophotometer was set at a wavelength of 550 nm and referenced with uncontaminated Mueller-Hinton broth.

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4. 2 ml of each suspension was introduced and the optical density (OD) determined.
5. Generally, an OD of 0.10 to 0.12 corresponds according to bacterial species, to 1 to $3x10^8$ CFU/ml. Optic densities of approximately 0.11 was determined for each isolate (84).
6. When the optical density reading was more then the expected value of 0.11, the following formula was used to dilute the suspension accurately with Mueller-Hinton broth:

$$\frac{\text{OD x Volume (5 ml)}}{0.11} - \frac{\text{Volume (5 ml)}}{1}$$

= X ml (amount of Mueller-Hinton broth to add)

7. When the optical density reading was less then the expected value of 0.11, more of the single isolated colonies of the overnight agar plate were inoculated in the 5 ml suspension.
8. After the suspensions were diluted with broth or concentrated with more bacteria the OD's were determined until the desired reading was obtained.
9. For this system that used an inoculum volume of 0.05 ml to inoculate 0.05 ml of broth, a 1:100 dilution of the above-mentioned suspensions was made with Mueller-Hinton broth to yield an inoculum of 10^6CFU/ml. When this inoculum was added to the wells, the 1:2 dilution of the 10^6CFU/ml inoculum resulted in a final inoculum concentration of 5×10^5 CFU/ml (5×10^4 CFU/ml) and also halved the antimicrobial concentration in each well (84, 87, 89).
10. Colony forming units of inoculum suspensions were determined weekly to ensure that the final inoculum concentration routinely obtained approximated 5×10^5 CFU/ml. This was accomplished by removing a 0.01 ml aliquot from the growth control well or tubes immediately after inoculation and diluting it in 10ml of 0.9% saline.

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11. After mixing, a 0.1 ml aliquot was spread over the surface of a suitable agar medium. Following incubation, the presence of approximately 50 colonies indicated an inoculum density of 5 x 10^5 CFU/ml.
12. As a purity check, a loopfull of the 10^5 CFU/ml suspension was streaked onto an agar plate for every sample and incubated overnight to detect mixed cultures and to provide freshly isolated colonies in case re-testing was required (84, 87, 89).

b) Inoculation of broth

Each well of a freshly prepared or thawed plate was inoculated with 50 µl of diluted inoculum within 15 minutes after it was standardized. To prevent drying, each tray was sealed in a plastic bag before incubation (87, 89).

3.6.1.6 Incubation

Plates were incubated at 35°C for 16 to 20 hours in an aerobic incubator in stacks of not more than four trays to ensure that even incubation temperatures were kept. The incubation chamber was kept sufficiently humid to avoid evaporation but not too humid to lead to condensation (84, 87, 89, 92).

3.7 Quality control

Reference organisms were tested every time a new batch of microdilution plates were prepared. When the MICs of the reference strains did not fall between the required ranges according to the requirements of the NCCLS, the plates were discarded. The results of these tests were compared with the expected values given by the NCCLS (in Table 4, Document M31-A, Vol. 19 NO. 11) June 1999, for accuracy. These results were recorded on a quality control record sheet.

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Other control procedures that were included during testing included growth control, purity control and inoculum control as explained in sections 3.6.1.2 and 3.6.1.5 (87, 89).

3.8 Repeatability

Susceptibility tests were done twice for each individual organism to determine the interplate variation. Plates were thawed and tested eight weeks after freezing to determine the influence of storage temperature on the repeatability of the test.

3.9 Data Analysis

All stored and recorded data were analysed by statisticians form the Department of Research Support (Ms. R Owen) and Department of Statistics (Ms. A Neethling) of the University of Pretoria. The statistical package SAS, version 8.2 was used. Descriptive calculations were made that included the determination of two-way frequency testing, as well as the determination of means, standard deviations and minimum and maximum values.

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CHAPTER 4

4. RESULTS

4.1 Specimens

A total of 120 isolates were collected and included 30 strains of *E. coli*, 30 strains of *Salmonella*, 30 strains of *Mannheimia haemolytica*, 26 strains of *Enterococcus faecalis* and 4 strains of *Enterococcus faecium*. The description of the 120 isolates are given in Annexure I.

4.2 MIC test results

The MIC range of each organism tested against each antimicrobial drug is indicated in Table 4.1. The individual MIC values in µg/ml for replicate tests for *Salmonella*, *E. coli*, *Enterococcus faecalis*, *E. faecium* and *Mannheimia haemolytica* are given in Annexure II.

The percentage of MIC values in the dilution ranges for each antimicrobial drug tested against each bacterium, as well as the repeat tests, are shown in Tables 4.2 - 4.5. These tables give an indication of the comparison between the two MIC test results, as well as the distribution of the MICs in each dilution range, for each antimicrobial drug. Thus, the overall scatter of MICs could be evaluated. The shaded areas depict the dilution range of each antimicrobial drug and the occurrence of the isolates for each dilution. When the results were lower or higher than the concentration tested, the MIC percentage was shown outside the shaded area. Thus, the distribution of the isolates for each dilution range against each antimicrobial drug could be observed.

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Table 4.1: Minimum Inhibitory Concentrations (MIC) recorded for antimicrobial drugs tested against the different isolates

ANTIMICROBIAL	ORGANISM	MIC RESULTS IN µg/ml
Enrofloxacin	*Salmonella* *E. coli* *M. haemolytica* *E. faecalis, E.faecium*	<0.03 – 2 <0.03 - >4 <0.03 – 0.5 0.125 - >4
Oxytetracycline	*Salmonella* *E. coli* *M. haemolytica* *E. faecalis, E.faecium*	2 - >64 4 - >64 <0.5 - >64 <0.5 - >64
Gentamicin	*Salmonella* *E. coli* *M. haemolytica* *E. faecalis, E.faecium*	0.5 - >32 0.5 – 8 0.5 – 8 1 – 32
Florfenicol	*Salmonella* *E. coli* *M. haemolytica* *E. faecalis, E.faecium*	4 - >16 4 - >16 0.5 – 4 1 – 4
Amoxycillin	*Salmonella* *E. coli* *M. haemolytica* *E. faecalis, E.faecium*	<0.5 - >32 <0.5 - >32 <0.5 – 16 <0.5 – 4
Neomycin	*Salmonella* *E. coli* *M. haemolytica* *E. faecalis, E.faecium*	<1 – 32 <1 – 128 2 – 32 8 - > 128
Tilmicosin	*Salmonella* *E. coli* *M. haemolytica* *E. faecalis, E.faecium*	64 - >64 64 - >64 <0.5 – 32 <0.5 - >64
Trimethoprim/ Sulfamethoxazole	*Salmonella* *E. coli* *M. haemolytica* *E. faecalis, E.faecium*	<0.125 - >16 <0.125 - >16 <0.125 <0.125 – 1
Sulfadimethoxine	*Salmonella* *E. coli* *M. haemolytica* *E. faecalis, E.faecium*	<0.125 - >512 32 - >512 <4 – 256 <4 - >512
Cephalexin	*Salmonella* *E. coli* *M. haemolytica* *E. faecalis, E.faecium*	4 - >8 4 - >8 <0.125 – 4 0.5 - >8

Table 4.2: Distribution of MICs for *Salmonella*

ANTIMICROBIAL DRUGS	REPEAT	DISTRIBUTION (%) OF MICs (µg/ml) [2] (n=30)																
		<0.03	0.03	0.06	0.125	0.25	0.5	1	2	4	8	16	32	64	128	256	512	>512
Enrofloxacin	1	3.3		40.0	40.0	3.3	6.7	3.3	3.3									
	2			70.0	10.0	13.3	6.7											
Oxytetracycline	1								6.7	36.7	6.7			3.3	46.7			
	2								30.0	23.3	3.3			6.7	36.7			
Gentamicin	1						46.7	26.7	13.3			3.3	3.3	6.7				
	2						30.0	60.0					3.3	6.7				
Florfenicol	1									3.3	56.7	20.0	20.0					
	2										43.3	36.7	20.0					
Amoxycillin	1					36.7		43.3						20.0				
	2					30.0		50.0						20.0				
Neomycin	1						70.0		26.7				3.3					
	2						70.0		26.7				3.3					
Tilmicosin	1													10.0	90.0			
	2													10.0	90.0			
Trimethoprim/Sulfa[1]	1			56.7		16.7	13.3	6.7	3.3					3.3				
	2			53.3		16.7	10.0	16.7						3.3				
Sulfadimethoxine	1													3.3	36.7	23.3	3.3	33.3
	2														30.0	33.3	3.3	33.3
Cephalexin	1									33.3	63.3	3.3						
										26.7	73.3							

[1]Concentration of trimethoprim/sulfamethoxazole given, was tested in a concentration ratio 1/19; [2] Hatched fields denote range of dilutions tested for each antimicrobial drug. MICs above or below the range are given as the concentration closest to the range.

Table 4.3: Distribution of MICs for *E. coli*

ANTIMICROBIAL DRUGS	REPEAT	DISTRIBUTION (%) OF MICs (µg/ml) [2] (n=30)																
		<0.03	**0.03**	**0.06**	**0.125**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**64**	**128**	**256**	**512**	**>512**
Enrofloxacin	1	46.7	6.7	16.7	10.0	3.3	6.7	3.3			6.7							
	2	53.3		20.0	10.0	3.3	6.7				6.7							
Oxytetracycline	1					3.3					3.3		3.3	6.7	83.3			
	2									3.3	3.3		3.3		90.0			
Gentamicin	1						10.0	26.7	36.7	20.0	6.7							
	2						10.0	46.7	26.7	16.7								
Florfenicol	1									3.3	43.3	43.3	10.0					
	2										16.7	70.0	13.3					
Amoxycillin	1					3.3		3.3	16.7	33.3	6.7			36.7				
	2								23.3	36.7	3.3			36.7				
Neomycin	1						30.0		30.0	6.7	3.3	3.3	16.7	6.7	3.3			
	2						26.7		20.0	20.0	3.3	13.3	6.7		10.0			
Tilmicosin	1													10.0	90.0			
	2													30.0	70.0			
Trimethoprim/Sulfa[1]	1			40.0		16.7	10.0	10.0						23.3				
	2			33.3		20.0	3.3	10.0	3.3					30.0				
Sulfadimethoxine	1												3.3	6.7	13.3	26.7	3.3	46.7
	2												6.7	3.3	20.0	10.0	3.3	56.7
Cephalexin	1									10.0	63.3	26.7						
										3.3	70.0	26.7						

[1]Concentration of trimethoprim/sulfamethoxazole given, was tested in a concentration ratio 1/19; [2] Hatched fields denote range of dilutions tested for each antimicrobial drug. MICs above or below the range are given as the concentration closest to the range.

Table 4.4: Distribution of MICs for *Enterococcus faecalis, E. faecium*

ANTIMICROBIAL DRUGS	REPEAT	DISTRIBUTION (%) OF MICs (µg/ml) [2] (n=30)																
		<0.03	0.03	0.06	0.125	0.25	0.5	1	2	4	8	16	32	64	128	256	512	>512
Enrofloxacin	1					26.7	50.0	16.7			6.7							
	2				3.3	13.3	60.0	16.7			6.7							
Oxytetracycline	1					3.3				3.3			50.0	13.3	30.0			
	2					3.3			3.3			10.0	40.0	13.3	30.0			
Gentamicin	1							3.3		10.0	36.7	33.3	6.7	10.0				
	2								3.3	3.3	43.3	23.3	13.3	13.3				
Florfenicol	1							13.3	46.7	40.0								
	2							3.3	50.0	46.7								
Amoxycillin	1					90.0		6.7		3.3								
	2					90.0		6.7	3.3									
Neomycin	1										3.3	20.0	23.3	13.3	3.3	36.7		
	2										13.3	16.7	16.7	16.7	6.7	30.0		
Tilmicosin	1					3.3		3.3			10.0	36.7	13.3	3.3	30.0			
	2					3.3		3.3			16.7	33.3	10.0		33.3			
Trimethoprim/Sulfa[1]	1			80.0		10.0	10.0											
	2			80.0		10.0	10.0											
Sulfadimethoxine	1								3.3					10.0	30.0	30.0	23.3	3.3
	2								3.3					6.7	33.3	26.7	23.3	6.7
Cephalexin	1					3.3	3.3				10.0	83.3						
						3.3	3.3				3.3	90.0						

[1]Concentration of trimethoprim/sulfamethoxazole given, was tested in a concentration ratio 1/19; [2] Hatched fields denote range of dilutions tested for each antimicrobial drug. MICs above or below the range are given as the concentration closest to the range.

Table 4.5: Distribution of MICs for *Mannheimia haemolytica*

ANTIMICROBIAL DRUGS	REPEAT	DISTRIBUTION (%) OF MICs (µg/ml) [2] (n=30)																
		<0.03	0.03	0.06	0.125	0.25	0.5	1	2	4	8	16	32	64	128	256	512	>512
Enrofloxacin	1	66.7		13.3	3.3	6.7	10.0											
	2	73.3		10.0	6.7		6.7				3.3							
Oxytetracycline	1					20.0		46.7	10.0		6.7			10.0	6.7			
	2					16.7		30.0	30.0			6.7	6.7	6.7	3.3			
Gentamicin	1						6.7	10.0	70.0	3.3	10.0							
	2						3.3	26.7	50.0	13.3	6.7							
Florfenicol	1						30.0	50.0	16.7	3.3								
	2						10.0	60.0	26.7	3.3								
Amoxycillin	1					90.0		3.3	3.3			3.3						
	2					93.3			3.3			3.3						
Neomycin	1								33.3	46.7	3.3	10.0	6.7					
	2								23.3	53.3	10.0	10.0	3.3					
Tilmicosin	1					6.7		3.3	10.0	53.3	16.7	10.0						
	2					6.7		3.3	10.0	50.0	23.3	3.3	3.3					
Trimethoprim/Sulfa[1]	1			100														
	2			100														
Sulfadimethoxine	1								20.0		3.3	16.7	10.0	20.0	20.0	10.0		
	2								20.0		10.0	3.3	10.0	20.0	36.7			
Cephalexin	1			3.3			6.7	53.3	33.3	3.3								
				3.3			3.3	40.0	50.0	3.3								

[1]Concentration of trimethoprim/sulfamethoxazole given, was tested in a concentration ratio 1/19; [2] Hatched fields denote range of dilutions tested for each antimicrobial drug. MICs above or below the range are given as the concentration closest to the range.

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4.3 Repeatability of test results

Table 4.6 shows the repeatability in percentage of the duplicate test results, accepting a difference in MIC values corresponding to one log_2-dilution step. The data indicated a high degree of correlation between the two sets of test results. All the antimicrobial drugs, except for oxytetracycline, tested against *Enterococcus faecalis* and *E. faecium* gave a repeatability percentage of 90% and higher. An overall agreement of 96.5% was seen between the 120 specimens and panel of antimicrobial drugs. The frequency and percentage of these results as well as deviations greater or smaller than a one log_2-dilution step are indicated in Annexure III. The distribution of test results that were lower or exceeded one serial dilution were evenly spread.

Table 4.6: Repeatability of duplicate analysis

ANTIMICROBIAL	PERCENTAGE OF REPEAT ANALYSES WITHIN ONE DILUTION			
	Salmonella	***E. coli***	***Mannheimia haemolytica***	***E. faecalis, E. faecium***
Enrofloxacin	93.33	100.0	90.0	100.0
Oxytetracycline	93.33	93.33	90.0	86.67
Gentamicin	96.67	93.33	100.0	96.67
Florfenicol	96.67	100.0	100.0	100.0
Amoxycillin	90.0	90.0	96.67	100.0
Neomycin	100.0	96.67	100.0	93.33
Tilmicosin	100.0	100.0	100.0	93.33
Trimethoprim/Sulfa	96.67	90.0	100.0	100.0
Sulfadimethoxine	100.0	90.0	96.67	96.67
Cephalexin	100.0	100.0	100.0	100.0

The total frequencies and percentages of results producing equals, one log_2-dilution variation and more than one log_2-dilution was also calculated. These results are indicated in Annexure IV. The results indicated that the average percentage of 72.39% of the MIC results for all four bacteria were equal to each other and 24.18% gave a

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deviation of one log_2-dilution step. When the deviation of one log_2-dilution was taken into consideration the antimicrobial drug gentamicin gave the highest occurrence of deviation (48.33%) and florfenicol the second highest deviation (40.83%.) The rest of the antimicrobial drugs had a total percentage less than 28%. A total percentage of 3.42% corresponding to more than one doubling dilution was found and was most prominent for oxytetracycline (9,17%), whilst the other antimicrobial drugs showed an occurrence of less than 6%. If discrepancies of one log_2 were not allowed the repeatability will decrease from 96.5% to 72.39%.

4.4 Occurrence of resistance to antimicrobial drugs

Resistant isolates for each antimicrobial drug are shown in Table 4.7, and are also shown graphically in Figure 4.1 for each bacterium tested against the ten selected antimicrobial drugs.

Salmonella, *E. coli, E. faecalis* and *E. faecium* was totally resistant against tilmicosin. It was effective against 96.7% of *Mannheimia haemolytica* strains with only 3.33% strains resistant. Oxytetracycline was poorly effective against *Salmonella*, *E. coli, E. faecalis* and *E. faecium* but not *Mannheimia haemolytica*. *Enterococcus faecalis* and *E. faecium* showed a high resistance against gentamicin. A high occurrence of resistance was also shown against sulfadimethoxine for *Salmonella*, *E. coli, E. faecalis* and *E. faecium*; amoxycillin for *E. coli* and *Salmonella* and neomycin for *Enterococcus faecalis* and *E. faecium*. The lowest occurrence of resistance, when considering all five organisms, was seen with enrofloxacin.

Table 4.7: Distribution (%) of resistant isolates for each antimicrobial drug

ANTIMICROBIAL DRUGS	*Salmonella* (n=30)		*E.coli* (n=30)		*Enterococcus faecalis/faecium* (n=30)		*Mannheimia haemolytica* (n=30)	
	BREAKPOINT[1]	**R (%)**	**BREAKPOINT**	**R (%)**	**BREAKPOINT**	**R (%)**	**BREAKPOINT**	**R (%)**
Enrofloxacin	≥ 2	3.3	≥ 2	6.7	≥ 2	6.7	≥ 2	3.3
Oxytetracycline[2]	≥ 16	46.7	≥ 16	93.3	≥ 16	93.3	≥ 16	23.3
Gentamicin	≥ 16	13.3	≥ 16	0.0	≥ 16	50.0	≥ 16	0.0
Florfenicol	–	–	–	–	–	–	–	–
Amoxycillin[3]	≥ 32	20.0	≥ 32	40.0	≥ 16	0.0	≥ 32	0.0
Neomycin[4]	≥ 64	0.0	≥ 64	10.0	≥ 64	53.3	≥ 64	6.7
Tilmicosin	≥ 32	100.0	≥ 32	100.0	≥ 32	46.7	≥ 32	3.3
Trimethoprim/ Sulfamethoxazole	$\geq 4/76$	3.3	$\geq 4/76$	30.0	$\geq 4/76$	0.0	$\geq 4/76$	0.0
Sulfadimethoxine	≥ 512	36.7	≥ 512	60.0	≥ 512	30.0	≥ 512	0.0
Cephalexin[5]	≥ 32	3.3	≥ 32	26.7	≥ 32	90.0	≥ 32	0.0

[1]Isolates with MIC values higher than the given figures are considered resistant; these values were derived from NCCLS, Table2, Document M31-A, Vol. 19 NO. 11, June 1999; [2] The published breakpoints of tetracycline was used as the reference for oxytetracycline; [3] The published breakpoints of ampicillin was used as the reference for amoxycillin; [4] The published breakpoints of kanamycin was used as the reference for neomycin; [5] The published breakpoints of cephalothin was used as the reference for cephalexin.

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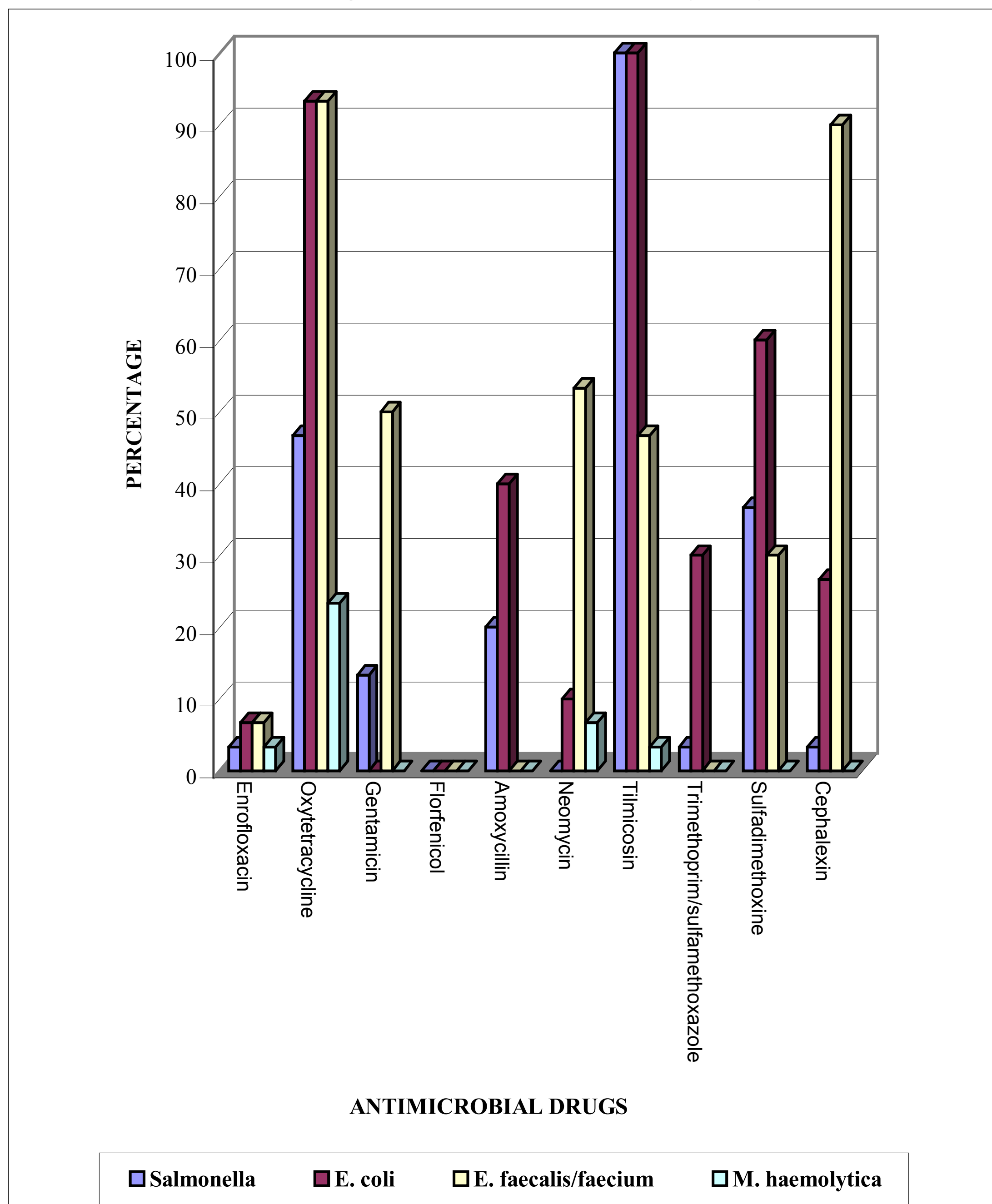

Figure 4.1: Percentage antimicrobial resistance observed against the bacterial strains tested

4.5 Effect of storage

The results in Table 4.8 represent the data collected after testing the reference strains on plates that were stored at –70°C for a 2 month period. The aim was to see if the plates were still stable and that the correct quality control ranges were still achievable

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after this time. The results indicated that plates containing pre-prepared antimicrobial drugs could be frozen at -70°C for at least two months.

Table 4.8: Results of reference strains tested after plates were stored for two months

DRUGS	***Staphylococcus aureus*** **ATCC 29213**		***Enterococcus faecalis*** **ATCC 29212**		***Escherichia coli*** **ATCC 25922**		***Pseudomonas aeruginosa*** **ATCC 27853**	
	Control Limits[a]	Results[b]	Control Limits	Results	Control limits	Results	Control limits	Results
1	0.03-0.12	0.06	0.12-1	0.25	0.008-0.03	<0.03	1-4	4
2	0.25-1	0.5	8-32	8	0.5-2	2	8-32	8
3	0.12-1	0.5	4-16	16	0.25-1	1	0.5-2	2
4	2-8	2	2-8	2	2-8	8	>16	>16
5	0.25-1	0.5	0.5-2	0.5	2-8	4	–	–
6	1-4	2	16-64	16	1-4	2	–	–
7	1-4	1	≥32	32	≥64	>64	>64	>64
8	≤0.5/9.5	0.5	≤0.5/9.5	<0.125	≤0.5/9.5	<0.125	8/152-32/608	32
9	32-128	32	32-128	32	8-32	8	–	–
10	0.12-0.5	0.5	–	–	4-16	4	–	–

[a] Accepted quality control ranges of MICs (µg/ml) for reference strains, derived from NCCLS, Table 4, Document M31-A, Vol.19 No. 11, June 1999; [b] Results (µg/ml) after storage for 2 months at –70°C.
1) enrofloxacin; 2) oxytetracycline; 3) gentamicin; 4) florfenicol; 5) amoxycillin; 6) neomycin; 7) tilmicosin; 8) trimethoprim/sulfamethoxazole; 9) sulfadimethoxine; 10) cephalexin.

4.6 Reading and interpretation of viewer results

The test results were read using a viewer that displays the underside of the wells and are shown graphically in Figure 4.2. Occasionally the appearance of certain wells did not conform to the criteria described for the testing procedures. These wells made it difficult to read and interpret the results. They sometimes appeared as fading end-points, but this was considered normal when testing sulfonamides. Other discrepancies were due to contamination or mixed cultures. Skips, where no growth occurred, were also noticed, but when only a single well in a sequence was skipped,

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the results were still acceptable. Plates were discarded and re-tested where discrepancies could affect the test results. After reading the plates, the MICs were recorded as the lowest concentration of the relevant antimicrobial that inhibited visible growth.

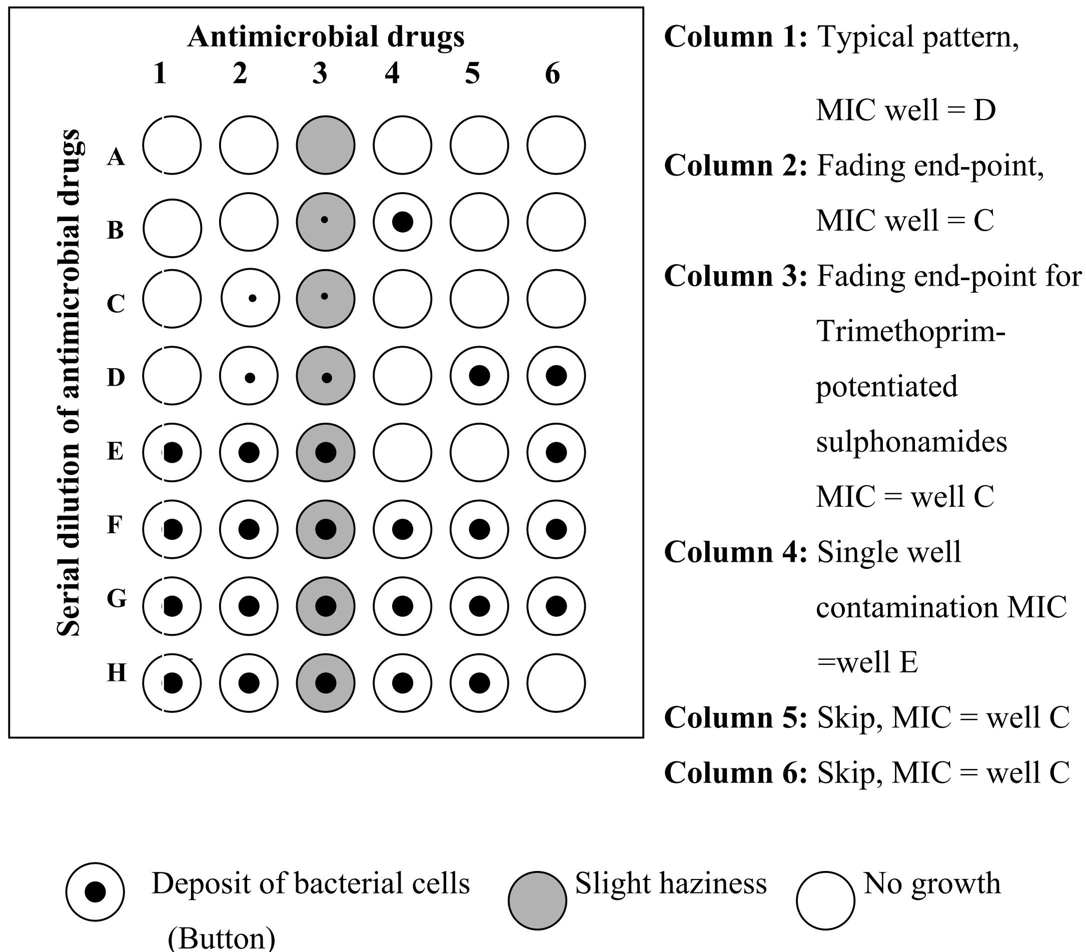

Figure 4.2: Examples of the microbial growth patterns observed during this study. A-H indicate the different dilution concentrations, in descending order, of the antimicrobial drug being tested. The rows 1-6 can be used for the simultaneous testing of different antimicrobial drugs. Where buttons of bacteria are visible, the bacteria are still viable, but where buttons are no longer visible the antimicrobial drug proved to be effective.

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CHAPTER 5

5. DISCUSSION

The bacteria collected and tested during this study were based on three categories of bacteria: indicator or commensal bacteria (*E. coli* and *Enterococcus faecalis, E. faecium*), zoonotic bacteria (*Salmonella*), and veterinary pathogens (*Mannheimia haemolytica*). Indicator bacteria are representative of intestinal tract bacteria of different animal species and are prone to acquire resistance. Zoonotic bacteria were included because they can develop resistance in the animal reservoir that may ultimately compromise therapy when treating infected humans. Veterinary pathogens are also important since they are the main targets of antimicrobial treatment in animals and undergo the greatest selective pressure (55).

The *Salmonella* isolates used in this study were not serotyped as the aim of the study was to standardise the methodology. However, for surveillance and monitoring purposes serovars of epidemiological importance, such as *S. typhimurium* and *S. enteritidis*, should be included. All *Salmonella* isolates should be serotyped and if possible, phage-typed according to standard methods used. Very few isolates of *Enterococcus faecium*, *E. faecalis* were obtained from the caecum of chickens and cattle faeces. Human isolates were therefore also included. For a veterinary antimicrobial resistance surveillance and monitoring programme the inclusion of human isolates should be avoided because they can give a skewed indication of the actual resistance problem in animals.

Antimicrobial drugs to be recommended for inclusion in a future South African surveillance programme and use during this study, were chosen on the basis of local

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marketing authorization and use. They represent all antimicrobial classes used in human and veterinary medicine in South Africa.

When using 96-well microtitre plates, up to seventeen antimicrobial drugs can be tested simultaneously. However, the ten antimicrobial drugs chosen were regarded as sufficiently representative for South African purposes. The dilution ranges for each antimicrobial drug were based on antimicrobial drug ranges used in several surveillance programmes as well as the prescribed ranges of the NCCLS (57, 67, 84, 87).

The microdilution minimal inhibitory concentration (MIC) antimicrobial susceptibility test was chosen because it overcomes several limitations of the disk diffusion test. It is a quantitative determination of the degree of susceptibility, not dependent on subjective interpretation, measurement of zones of growth inhibition or extrapolation of MIC values from zone sizes. The precise amount of antimicrobial drug required to inhibit bacterial growth can also be determined (91). The MIC is generally a reproducible quantitative characteristic of the bacterial isolate that can be measured readily (74). This was confirmed in the current study.

Before reading and recording of MICs, the growth control wells must be examined for organism viability, inoculum subcultures must be checked for contamination, colony counts must be performed to confirm appropriate inoculum size, and the accuracy of the MICs obtained must be compared to the quality control strains. All these quality control measures were used for the confirmation of the results.

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The guidelines of the National Committee for Clinical Laboratory Standards (NCCLS) were used as the reference method for determining the minimum inhibitory concentrations (45). It is the quantitative method used by most countries for the determination of susceptibility of bacteria. The National Committee for Clinical Laboratory Standards has also developed protocols for susceptibility testing of bacteria of animal origin and the determination of the interpretive criteria. In addition, the OIE has endorsed the use of NCCLS standards and guidelines (55).

The MIC distribution results of each isolate against the ten antimicrobial agents obtained in the current study was generally similar to those reported in related studies (67, 79, 93, 94, 95). However, different MIC distributions were obtained for neomycin against *E. coli* and *Salmonella.* Certain of these results fell within comparable ranges, but a high percentage of MICs were also found at a dilution of <1µg/ml. A particular large deviation was found with sulfadimethoxine against *Mannheimia haemolytica*. The reported highest MIC value in this study is >256 µg/ml whilst the greatest distribution of MICs were obtained at a dilution of <64 µg/ml and 128 µg/ml. This could be due to the use of samples from diagnostic submissions in this study, whereas comparative studies made use of specimens taken from healthy animals.

In this study results were evaluated by direct comparison between the two sets of test results. From a practical point of view, the overall results of microwell susceptibility testing are considered to be satisfactory if the results of independent tests vary no more than plus or minus one serial dilution. The results of the two replicate tests in this project revealed agreement within one dilution for most organisms tested. If one

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wants to determine whether repeatability is adequate, it is considered necessary to calculate the coefficients of variation (SD of replicates divided by mean of replicates) (96). However, to do these calculations continuous data is required and not discrete data as was the case in this study. This is due to the fact that the results are also considered to be concordant when the independent tests vary with one serial dilution. Using the standard method of the NCCLS, with all the quality control measures, the results showed 90% comparability, except for oxytetracycline that showed 86.67% comparability.

Stability was evaluated for plates prepared in-house and frozen for a period of two months. The results of these tests correlated with the accepted quality control ranges for the ATCC reference strains recommended by the NCCLS. Thus, the microtitre plates containing the antimicrobial drug concentrations can be safely stored at -70ºC for at least two months following preparation.

Most specimens included in this study were collected from diagnostic submissions and the results must therefore be interpreted with caution. These types of submissions tend to include specimens from severe and/or recurrent clinical cases, that may include therapeutic failures. Thus, the prevalence of resistant strains may be overestimated and may not reflect the resistance situation in the animal population (67). The number of specimens included was also relatively small and cannot be considered as representative of resistance patterns for the bacteria tested.

When reading the plates, the MIC is recorded as the lowest concentration of an antimicrobial drug that inhibits the visible growth. However, when reading the plates

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manually certain discrepancies can occur which may lead to the inaccurate interpretation of the test results. To avoid this, the following points should be considered:

a) Fading end-points

Most organism/antimicrobial combinations give distinct end-points but with some combinations there is a gradual fading of growth over 2 to 3 wells. The end-points should be taken as the first wells showing no growth except when the results for trimethoprim and/or sulphonamides are read. For these drugs the MIC is read as an 80 – 90% decrease in growth compared with the growth of the microorganism in the control well. When indistinct end-points are widespread on a particular plate the test should be repeated.

b) Contamination

Contamination may result in a single button of growth in a well with the wells on either side showing no growth. Such a single-well contamination can be ignored, but if multiple-well contamination occur, the test should be repeated.

c) Skips

A skip is a well showing no growth with the wells on either side showing growth. A single skip can be ignored. However, the skipped well should not be read as the MIC but always read as the lowest well above the skipped well where there was constantly no growth. If there were multiple skipped wells in a column, the result in that column should be ignored and the test repeated.

d) Mixed cultures

Mixed cultures are assumed when two end-points are seen, such as a distinct button of cells followed by several wells of diffuse growth with the button no longer visible. If a mixed culture is detected the test result is discarded and the test repeated

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CHAPTER 6

6. CONCLUSIONS AND RECOMMENDATIONS

Antimicrobial drug use in animals, humans and agriculture, and especially misuse, is an important selecting factor in bacterial antimicrobial resistance. For this reason, application of prudent use of antimicrobial drugs is critical in the prevention of emergence and spread of antimicrobial resistant microorganisms. An important component of prudent use is the ongoing monitoring of the resistance status of bacteria. The lack of such data, also in the veterinary field has consistently resulted in the recommendation that national surveillance programmes for antimicrobial resistance should be established as one of the outcomes of meetings and reports produced in recent years by many international organizations (55, 66).

The structure of a veterinary antimicrobial surveillance monitoring programme should of necessity have a different emphasis when compared with similar programmes for humans. The amount of data regularly produced by human hospitals is uncommon in the veterinary field and a variety of animal species must be included. Veterinary antimicrobial surveillance and monitoring programmes should aim to detect the level of resistance in bacteria isolated from animals and animal-derived foods, to evaluate the risks of the use of antimicrobial drugs in animals and to quantify the impact of these findings on human health (66).

When implementing a veterinary surveillance programme in South Africa, it is recommended that participating laboratories should be representative of the major food animal farming areas. A central national coordinating laboratory should form the

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core structure and all data recorded should be accumulated, processed, stored and published by this laboratory.

For a programme of this nature to be successful and render reproducible and comparative results, harmonization of laboratory methodologies should take place. The standardized methodologies and interpretive criteria developed by the NCCLS and applied in the current study are recommended.

Isolates collected and tested by the participating laboratories should be representative of the following three groups: indicator bacteria, zoonotic bacteria, and veterinary pathogens. As the surveillance programme progresses, additional bacterial species in the respective groups can be included that can enhance the detection of the emergence of resistance.

Specimen collection can include diagnostic submissions that are readily available, but should also include field samples taken from healthy animals, food of animal origin and abattoirs. Specimens can be collected either weekly, monthly, or otherwise as required. All specimens collected should preferably be from food producing animals, and sampling done randomly to avoid bias. The OIE guidance document only specifies which bacterial species should be included but is not prescriptive of the exact number of specimens to be collected. Thus, it is recommended that an optimal number of specimens as determined by available resources be collected and included to give a true reflection and valid statistical results. The OIE has recommended certain bacterial species, that could be included in surveillance programmes and are grouped under the following three major categories of organisms:

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1) Veterinary pathogens: *Pasteurella spp., Haemophilus somnus, Actinobacillus pleuropneumoniae, E. coli, Salmonella, Staphylococcus aureus, Streptococcus spp, Streptococcus suis, Aeromonas spp.* and *Brachyspira spp.*
2) Zoonotic bacteria: *Salmonella typhimurium, Salmonella enteriditis, Campylobacter jejuni, Campylobacter coli* and an enterohaemorrhagic *E. coli* such as 0 157.
3) Commensal/Indicator bacteria: *E. coli* and enterococci especially *E. faecium* (55).

For a national surveillance programme in South Africa, it is recommended that the bacterial species tested in this study be used. Additional organisms and types of specimens can be included when available resources make it possible.

The antimicrobial drugs included in this study are recommended for the initial phase of the programme. Other drugs can be considered for inclusion at later stages. It would be ideal to develop antimicrobial panels for each group of bacteria to be tested. Antimicrobial panels can be designed specifically for Gram-negative and Gram-positive bacteria or for each bacterial species tested in the surveillance programme (22, 55, 64, 66).

To make such a programme more cost effective and ensure more accurate results, it is suggested that all the plates required for testing be produced at the central coordinating laboratory. These plates can then either be distributed to the laboratories involved, or these laboratories could submit all cultures for testing to the central laboratory. The plates must be kept at a temperature of between –20°C and –70°C when being distributed and therefore makes transporting of the plates more difficult.

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Data recording, computing and reporting are essential for a suitable monitoring system. Each laboratory should therefore be linked to a central database. The database should include information on the sampling population, time of isolation, origin of sample, down to a specific herd, flock or animal, animal species and age. Results should be entered into a suitable national database and recorded quantitatively, for example as distributions of MICs in µg/ml. In addition identification of isolates as resistant, intermediate or sensitive will also prove helpful in the interpretation of results or data (64).

Information on the consumption of antimicrobial drugs for different animal species is also needed to assess the impact on the occurrence of resistance and to determine where and for which infections most antimicrobial drugs are used. Programmes collecting data on the consumption of antimicrobial drugs are therefore also urgently needed. The hope is that cautious, rational, quantitatively and qualitatively adjusted use of antimicrobial drugs in human and veterinary medicine will at least slow down the emergence of resistance and the problems associated with this phenomenon (12, 22).

At present, information on antimicrobial resistance in animals is lacking in most countries. An organized surveillance and monitoring programme for antimicrobial resistance in South Africa will contribute to the detection of emerging resistance problems at the earliest possible stage.

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ANNEXURE I: Isolates examined

E. coli

SAMPLE NO.	SOURCE	ANIMAL
13	Sinus	Poultry
14	Peritoneum	Poultry
15	Liver	Bovine
16	Liver	Bovine
17	Pericardium	Poultry
18	Faeces	Bovine
20	Organs	Poultry
21	Peritoneum	Poultry
22	Pericardium	Poultry
23	Joint	Poultry
24	Pericardium	Poultry
26	Organs	Poultry
27	Intestine	Porcine
28	Lung	Poultry
29	Faeces	Bovine
30	Abdominal cavity	Poultry
31	Trachea	Poultry
32	Peritoneum	Poultry
33	Airsac	Poultry
34	Airsac	Poultry
35	Liver	Poultry
36	Organs	Porcine
37	Kidney	Porcine
38	Meat	Ovine
39	Organs	Bovine
40	Spleen	Ovine
41	Intestine	Bovine
42	Faeces	Bovine
43	Intestine	Porcine
44	Kidney	Porcine

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ANNEXURE I: cont.

Salmonella

SAMPLE NO.	SOURCE	ANIMAL
1	Egg	Poultry
2	Egg	Poultry
3	Organs	Bovine
4	Faeces	Porcine
5	Liver	Bovine
6	Foetus	Bovine
7	Intestine	Bovine
9	Faeces	Poultry
10	Lung	Bovine
11	Intestine	Goat
12	Intestine	Bovine
45	Organs	Bovine
46	Faeces	Poultry
47	Meat	Bovine
49	Cloaca	Poultry
50	Faeces	Poultry
51	Faeces	Poultry
52	Meat	Poultry
53	Urachus	Bovine
54	Unknown	Poultry
55	Liver	Bovine
56	Intestine	Poultry
57	Lung	Bovine
58	Meat	Poultry
59	Meat	Poultry
60	Meat	Porcine
61	Intestine	Poultry
62	Faeces	Ovine
63	Intestine	Bovine
64	Unknown	Poultry

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ANNEXURE I: cont.

Enterococcus faecalis/ Enterococcus faecium

SAMPLE NO.	SOURCE	ANIMAL
65	Urine	Human
66	Urine	Human
67	Urine	Human
68	Urine	Human
69	Urine	Human
70	Urine	Human
71	Urine	Human
72	Urine	Human
73	Urine	Human
74	Urine	Human
75	Urine	Human
76	Unknown	Poultry
77	Joint	Poultry
78	Urine	Human
80	Urine	Human
82	Urine	Human
83*	Foetus	Bovine
84	Urine	Human
85	Faeces	Bovine
86*	Lung	Bovine
87	Urine	Human
88	Urine	Human
89*	Throat swab	Canine
90	Urine	Human
91*	Foetus	Equine
92	Urine	Human
93	Urine	Human
94	Urine	Human
95	Urine	Human
96	Urine	Human

* *Enterococcus faecium*

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ANNEXURE I: cont.

Mannheimia haemolytica

SAMPLE NO.	SOURCE	ANIMAL
97	Tracheal aspirate	Bovine
98	Tracheal aspirate	Bovine
99	Organs	Ovine
100	Tracheal aspirate	Bovine
101	Tracheal aspirate	Bovine
102	Tracheal aspirate	Bovine
103	Tracheal aspirate	Bovine
104	Lung	Ovine
105	Tracheal aspirate	Bovine
106	Tracheal aspirate	Bovine
107	Lung	Ovine
108	Lung	Bovine
109	Lung	Bovine
110	Tracheal aspirate	Bovine
111	Tracheal aspirate	Bovine
112	Kidney	Bovine
113	Lung	Ovine
114	Lung	Bovine
115	Lung	Ovine
116	Tracheal aspirate	Bovine
117	Lung	Bovine
118	Lung	Ovine
119	Tracheal aspirate	Bovine
120	Tracheal aspirate	Bovine
122	Lung	Bovine
123	Lung	Ovine
124	Organs	Ovine
126	Lung	Bovine
127	Lung, liver, intestine	Bovine
128	Lung	Ovine

ANNEXURE II: MIC values for *Salmonella, E. coli, Enterococcus* and *Mannheimia haemolytica* determined for all isolates and replicates

MICs for S*almonella*

ISOLATES		MICs (µg/ml)									
ISOLATE NO	**REPEAT**	**Ef**	**Oxy**	**Gm**	**Ff**	**Ac**	**Nm**	**Til**	**Tri/Sul[1]**	**Sfm**	**Cep**
1	1	0.06	4	0.5	16	1	<1	>64	<0.125	128	4
	2	0.06	2	0.5	16	1	<1	>64	<0.125	128	8
2	1	0.25	>64	0.5	8	1	<1	>64	0.25	128	8
	2	0.25	>64	1	8	1	<1	>64	0.25	256	8
3	1	0.06	4	1	8	<0.5	<1	>64	<0.125	128	8
	2	0.06	2	1	16	<0.5	<1	>64	<0.125	128	4
4	1	0.125	4	0.5	4	<0.5	2	>64	0.25	256	8
	2	0.125	2	1	8	<0.5	2	>64	0.25	256	4
5	1	0.06	2	2	8	<0.5	<1	>64	<0.125	128	8
	2	0.06	2	1	8	<0.5	<1	>64	<0.125	128	8
6	1	0.06	4	0.5	8	<0.5	<1	64	<0.125	128	4
	2	0.06	2	1	8	<0.5	<1	64	<0.125	128	4
7	1	0.06	>64	0.5	>16	>32	<1	>64	1	>512	8
	2	0.06	>64	0.5	>16	>32	<1	>64	1	>512	8
9	1	0.06	4	1	8	1	<1	>64	0.25	128	8
	2	0.06	2	1	16	1	<1	>64	0.25	256	8
10	1	<0.03	4	0.5	8	1	2	64	<0.125	128	4
	2	0.06	8	0.5	16	1	2	64	<0.125	128	4
11	1	0.125	>64	2	16	1	<1	>64	0.5	>512	4
	2	0.25	>64	1	16	1	<1	>64	1	>512	8
12	1	0.5	>64	1	>16	>32	<1	>64	2	>512	8
	2	0.25	>64	1	>16	>32	<1	>64	1	>512	8
45	1	0.125	2	0.5	8	1	<1	>64	<0.125	128	4
	2	0.06	2	1	8	1	<1	>64	<0.125	128	8
46	1	0.06	>64	0.5	8	>32	<1	64	<0.125	64	4
	2	0.06	4	1	16	1	<1	>64	<0.125	128	8
47	1	0.125	>64	0.5	>16	<0.5	<1	>64	<0.125	>512	8
	2	0.125	>64	0.5	>16	<0.5	<1	>64	1	>512	8
49	1	1	>64	1	8	>32	2	>64	>16	512	4
	2	0.5	>64	1	8	>32	2	>64	>16	512	8

[1]Concentration of trimethoprim/sulfamethoxazole was tested in a ratio of 1/19; concentration of trimethoprim is given.

Ef: Enrofloxacin; Oxy: Oxytetracycline; Gm: Gentamicin; Ff: Florfenicol; Ac: Amoxycillin; Nm: Neomycin; Til: Tilmicosin;

Tri/Sul: Trimethoprim/Sulfamethoxazole; Sfm: Sulfadimethoxine; Cep: Cephalexin.

ANNEXURE II: cont.

MICs for *Salmonella* (cont.)

ISOLATES		MICs (µg/ml)									
ISOLATE NO	REPEAT	Ef	Oxy	Gm	Ff	Ac	Nm	Til	Tri/Sul[1]	Sfm	Cep
50	1	0.06	4	1	8	<0.5	2	64	<0.125	256	8
	2	0.06	4	1	8	<0.5	2	>64	<0.125	256	8
51	1	0.125	4	32	8	1	2	>64	<0.125	>512	8
	2	0.06	4	>32	16	1	2	>64	<0.125	>512	8
52	1	0.5	8	>32	8	<0.5	2	>64	0.25	>512	8
	2	0.25	4	>32	8	<0.5	2	>64	0.25	>512	8
53	1	0.125	>64	>32	>16	<0.5	2	>64	1	>512	>8
	2	0.06	>64	1	>16	>32	2	>64	1	>512	8
54	1	0.125	>64	2	>16	<0.5	<1	>64	0.25	128	4
	2	0.06	4	1	8	<0.5	<1	>64	0.25	256	8
55	1	0.125	64	2	16	1	<1	>64	0.125	256	8
	2	0.06	64	1	16	1	<1	>64	<0.125	256	8
56	1	0.125	4	1	16	1	2	>64	<0.125	256	4
	2	0.06	4	1	16	1	2	>64	<0.125	256	4
57	1	0.125	>64	1	>4	>32	<1	>64	0.5	>512	8
	2	0.06	64	1	>16	>32	<1	>64	0.5	>512	8
58	1	0.125	>64	0.5	16	1	<1	>64	0.5	>512	4
	2	0.06	>64	0.5	16	1	<1	64	0.5	>512	4
59	1	0.06	>64	0.5	8	1	32	>64	0.5	>512	8
	2	0.06	>64	0.5	8	1	32	>64	0.5	>512	8
60	1	0.06	>64	0.5	8	1	<1	>64	<0.125	128	8
	2	0.06	>64	0.5	8	1	<1	>64	<0.125	128	8
61	1	0.125	>64	16	16	<0.5	<1	>64	<0.125	256	8
	2	0.125	>64	32	>16	<0.5	<1	>64	<0.125	256	8
62	1	0.06	8	0.5	8	1	<1	>64	<0.125	256	8
	2	0.06	4	1	8	1	<1	>64	<0.125	256	4
63	1	0.06	4	1	8	<0.5	<1	>64	<0.125	128	8
	2	0.06	2	0.5	8	1	<1	>64	<0.125	128	4
64	1	2	2	0.5	8	>32	<1	>64	<0.125	256	8
	2	0.5	2	0.5	16	>32	<1	>64	<0.125	256	8

[1]Concentration of trimethoprim/sulfamethoxazole was tested in a ratio of 1/19; concentration of trimethoprim is given.

Ef: Enrofloxacin; Oxy: Oxytetracycline; Gm: Gentamicin; Ff: Florfenicol; Ac: Amoxycillin; Nm: Neomycin; Til: Tilmicosin;

Tri/Sul: Trimethoprim/Sulfamethoxazole; Sfm: Sulfadimethoxine; Cep: Cephalexin.

ANNEXURE II: cont.

MICs for *E. coli*

ISOLATES		MICs (µg/ml)									
ISOLATE NO	REPEAT	Ef	Oxy	Gm	Ff	Ac	Nm	Til	Tri/Sul[1]	Sfm	Cep
13	1	<0.03	>64	4	8	4	2	>64	0.25	256	8
	2	<0.03	>64	2	16	4	2	>64	0.25	256	8
14	1	<0.03	>64	2	8	>32	32	>64	<0.125	512	>8
	2	<0.03	>64	4	16	>32	32	>64	<0.125	512	8
15	1	0.03	64	4	8	>32	64	>64	>16	>512	8
	2	<0.03	>64	2	16	>32	128	>64	>16	>512	8
16	1	<0.03	>64	8	16	4	2	>64	0.25	>512	>8
	2	<0.03	>64	4	16	4	4	>64	0.25	>512	>8
17	1	<0.03	>64	8	16	4	32	>64	<0.125	256	8
	2	<0.03	>64	1	16	>32	32	>64	<0.125	128	8
18	1	<0.03	64	1	8	>32	64	>64	>16	>512	>8
	2	<0.03	>64	1	8	>32	128	64	>16	>512	8
20	1	0.06	>64	2	8	4	8	64	<0.125	64	8
	2	0.06	>64	2	16	4	4	64	<0.125	32	8
21	1	>4	>64	4	>16	>32	<1	>64	<0.125	256	8
	2	>4	>64	4	>16	>32	<1	>64	<0.125	256	>8
22	1	0.03	>64	1	8	4	2	>64	0.125	256	4
	2	<0.03	>64	2	16	4	2	64	<0.125	128	8
23	1	0.06	>64	4	16	2	32	>64	<0.125	64	>8
	2	0.06	>64	4	16	2	16	>64	<0.125	64	8
24	1	>4	>64	2	16	>32	2	>64	>16	>512	8
	2	>4	>64	4	16	>32	2	>64	>16	>512	8
26	1	0.06	>64	4	8	2	<1	64	>16	>512	8
	2	0.06	>64	2	8	2	<1	64	>16	>512	8
27	1	0.125	>64	0.5	16	<0.5	32	>64	1	>512	8
	2	0.125	>64	2	16	2	16	>64	1	>512	8
28	1	<0.03	>64	2	8	1	16	>64	0.5	>512	8
	2	<0.06	>64	1	8	2	16	>64	0.25	>512	4
29	1	<0.03	>64	2	16	>32	128	>64	<0.125	256	8
	2	<0.06	>64	1	16	>32	128	>64	>0.125	256	8

[1]Concentration of trimethoprim/sulfamethoxazole was tested in a ratio of 1/19; concentration of trimethoprim is given.

Ef: Enrofloxacin; Oxy: Oxytetracycline; Gm: Gentamicin; Ff: Florfenicol; Ac: Amoxycillin; Nm: Neomycin; Til: Tilmicosin; Tri/Sul: Trimethoprim/Sulfamethoxazole; Sfm: Sulfadimethoxine; Cep: Cephalexin.

ANNEXURE II: cont.

MICs for *E. coli* (cont.)

ISOLATES		MICs (µg/ml)									
ISOLATE NO	REPEAT	Ef	Oxy	Gm	Ff	Ac	Nm	Til	Tri/Sul[1]	Sfm	Cep
30	1	1	>64	1	16	>32	2	>64	<0.125	128	8
	2	0.5	>64	1	16	>32	2	>64	<0.125	>512	>8
31	1	0.5	>64	2	8	2	<1	>64	1	>512	8
	2	0.25	>64	1	16	2	<1	64	1	>512	8
32	1	0.5	>64	2	8	8	<1	>64	0.5	>512	>8
	2	0.5	>64	1	16	8	<1	>64	1	>512	>8
33	1	<0.03	>64	2	8	>32	<1	>64	0.25	256	8
	2	<0.03	4	1	16	4	<1	>64	0.25	128	8
34	1	0.06	>64	2	16	4	4	>64	0.25	128	8
	2	0.06	>64	2	16	4	4	>64	0.25	128	8
35	1	<0.03	>64	2	16	4	<1	64	<0.125	128	8
	2	<0.03	>64	1	16	4	<1	64	<0.125	128	8
36	1	<0.03	>64	4	16	4	2	>64	<0.125	32	>8
	2	<0.03	>64	2	16	4	4	>64	<0.125	32	>8
37	1	<0.03	>64	1	16	4	2	>64	0.5	>512	8
	2	<0.03	>64	0.5	8	2	2	64	0.25	>512	8
38	1	0.125	>64	1	16	>32	<1	>64	<0.125	256	>8
	2	0.06	>64	1	16	>32	8	64	2	>512	>8
39	1	<0.03	>64	2	8	>32	32	>64	>16	>512	4
	2	<0.03	>64	1	16	>32	16	>64	>16	>512	8
40	1	<0.03	8	1	8	4	2	>64	<0.125	128	4
	2	<0.03	8	0.5	16	4	2	>64	<0.125	128	8
41	1	0.06	>64	1	>16	>32	2	>64	>16	>512	>8
	2	0.06	>64	1	>16	>32	4	>64	>16	>512	>8
42	1	<0.03	32	0.5	>16	8	<1	>64	0.25	256	8
	2	<0.03	32	1	>16	4	<1	64	>16	>512	>8
43	1	0.125	>64	1	16	2	4	>64	1	>512	8
	2	0.125	>64	1	>16	4	4	>64	0.5	>512	8
44	1	0.25	>64	0.5	4	2	<1	>64	>16	>512	8
	2	0.125	>64	0.5	8	2	<1	>64	>16	>512	8

[1]Concentration of trimethoprim/sulfamethoxazole was tested in a ratio of 1/19; concentration of trimethoprim is given

Ef: Enrofloxacin; Oxy: Oxytetracycline; Gm: Gentamicin; Ff: Florfenicol; Ac: Amoxycillin; Nm: Neomycin; Til: Tilmicosin; Tri/Sul: Trimethoprim/Sulfamethoxazole; Sfm: Sulfadimethoxine; Cep: Cephalexin.

ANNEXURE II: cont.

MICs for *Enterococcus faecalis, E. faecium*

ISOLATES		MICs (µg/ml)									
ISOLATE NO	REPEAT	Ef	Oxy	Gm	Ff	Ac	Nm	Til	Tri/Sul[1]	Sfm	Cep
65	1	0.5	>64	4	2	<0.5	16	32	<0.125	128	>8
	2	0.5	64	8	2	<0.5	8	16	<0.125	128	>8
66	1	0.5	>64	4	2	<0.5	16	8	<0.125	128	>8
	2	0.5	64	8	4	<0.5	16	8	<0.125	128	>8
67	1	1	64	1	2	<0.5	32	16	<0.125	128	>8
	2	0.5	>64	2	4	<0.5	16	8	<0.125	128	>8
68	1	1	32	8	2	<0.5	32	16	<0.125	512	>8
	2	0.5	32	16	2	<0.5	8	8	<0.125	512	>8
69	1	0.5	32	8	2	<0.5	16	16	<0.125	512	>8
	2	0.5	32	16	2	<0.5	16	>64	<0.125	512	>8
70	1	0.5	32	>32	2	<0.5	16	>64	<0.125	128	>8
	2	0.5	32	>32	2	<0.5	16	>64	<0.125	128	>8
71	1	0.5	32	16	2	<0.5	32	16	<0.125	128	>8
	2	0.5	16	16	2	<0.5	64	8	<0.125	128	>8
72	1	0.5	32	32	4	<0.5	16	16	<0.125	<4	>8
	2	0.5	32	32	4	<0.5	8	16	<0.125	<4	>8
73	1	0.5	32	16	2	<0.5	>128	>64	<0.125	128	>8
	2	0.25	32	8	4	<0.5	>128	>64	<0.125	128	>8
74	1	0.5	32	8	4	<0.5	>128	>64	<0.125	128	>8
	2	0.5	>64	8	4	<0.5	>128	>64	<0.125	128	>8
75	1	0.5	>64	8	4	<0.5	>128	>64	<0.125	128	>8
	2	0.5	>64	8	4	<0.5	>128	>64	<0.125	128	>8
76	1	0.5	>64	8	4	<0.5	64	16	<0.125	512	>8
	2	0.5	>64	16	4	<0.5	32	16	<0.125	512	>8
77	1	0.5	>64	16	4	<0.5	64	32	<0.125	512	>8
	2	1	>64	16	4	<0.5	64	32	<0.125	512	>8
78	1	0.25	32	8	2	<0.5	32	16	<0.125	512	>8
	2	0.5	32	8	4	<0.5	32	16	<0.125	512	>8
80	1	0.25	64	16	2	<0.5	32	16	<0.125	256	>8
	2	0.5	32	8	2	<0.5	32	16	<0.125	256	>8

[1]Concentration of trimethoprim/sulfamethoxazole was tested in a ratio of 1/19; concentration of trimethoprim is given.

Ef: Enrofloxacin; Oxy: Oxytetracycline; Gm: Gentamicin; Ff: Florfenicol; Ac: Amoxycillin; Nm: Neomycin; Til: Tilmicosin;
Tri/Sul: Trimethoprim/Sulfamethoxazole; Sfm: Sulfadimethoxine; Cep: Cephalexin.

ANNEXURE II: cont.

MICs for *Enterococcus faecalis, E. faecium* (cont.)

ISOLATES		MICs (µg/ml)									
ISOLATE NO	REPEAT	Ef	Oxy	Gm	Ff	Ac	Nm	Til	Tri/Sul[1]	Sfm	Cep
82	1	0.25	64	32	2	<0.5	16	16	<0.125	256	8
	2	0.25	32	16	2	<0.5	16	16	<0.125	256	>8
83	1	0.25	4	4	1	<0.5	8	<0.5	<0.125	128	0.25
	2	0.25	2	4	1	<0.5	8	<0.5	<0.125	128	0.25
84	1	>4	>64	>32	4	<0.5	>128	>64	0.5	256	.8
	2	>4	>64	>32	4	<0.5	>128	>64	1	256	>8
85	1	0.5	32	16	4	<0.5	64	8	<0.125	256	>8
	2	1	<0.5	8	2	<0.5	64	16	<0.125	256	>8
86	1	1	<0.5	8	1	<0.5	64	1	0.5	>512	0.5
	2	1	64	8	2	<0.5	64	1	0.5	>512	0.5
87	1	1	>64	8	4	<0.5	>128	>64	<0.125	512	>8
	2	1	>64	8	4	<0.5	>128	>64	<0.125	512	>8
88	1	0.25	32	16	2	4	32	8	<0.125	256	>8
	2	0.5	16	8	2	2	32	8	<0.125	256	>8
89	1	0.5	>64	8	4	<0.5	>128	64	0.25	256	8
	2	0.5	>64	8	2	<0.5	32	16	0.25	>512	>8
90	1	0.25	32	16	4	<0.5	32	16	<0.125	256	>8
	2	0.125	64	32	4	<0.5	64	16	<0.125	256	>8
91	1	1	32	8	4	1	128	32	0.25	256	>8
	2	1	32	8	4	1	128	32	0.5	256	>8
92	1	0.5	32	8	2	<0.5	>128	16	<0.125	64	8
	2	0.5	32	16	2	<0.5	128	16	<0.125	128	8
93	1	0.25	>64	16	4	1	>128	32	<0.125	64	>8
	2	0.5	>64	32	4	1	>128	32	<0.125	64	>8
94	1	0.25	32	>32	1	<0.5	>128	>64	0.5	64	>8
	2	0.25	32	>32	2	<0.5	>128	>64	0.5	64	>8
95	1	>4	32	16	1	<0.5	>128	>64	0.25	256	>8
	2	>4	32	32	2	<0.5	>128	>64	0.25	256	>8
96	1	0.5	64	16	2	<0.5	>128	>64	<0.125	512	>8
	2	0.5	16	>32	2	<0.5	>128	>64	<0.125	512	>8

[1]Concentration of Trimethoprim/Sulfamethoxazole was tested in a ratio of 1/19; concentration of Trimethoprim is given.

Ef: Enrofloxacin; Oxy: Oxytetracycline; Gm: Gentamicin; Ff: Florfenicol; Ac: Amoxycillin; Nm: Neomycin; Til: Tilmicosin; Tri/Sul: Trimethoprim/Sulfamethoxazole; Sfm: Sulfadimethoxine; Cep: Cephalexin.

ANNEXURE II: cont.

MICs for M*annheimia haemolytica*

ISOLATES		MICs (µg/ml)									
ISOLATE NO	REPEAT	Ef	Oxy	Gm	Ff	Ac	Nm	Til	Tri/Sul[1]	Sfm	Cep
97	1	0.125	1	2	0.5	<0.5	4	4	<0.125	256	2
	2	>4	16	4	1	<0.5	4	4	<0.125	128	2
98	1	0.25	1	2	1	<0.5	16	4	<0.125	32	1
	2	0.125	2	2	2	<0.5	8	4	<0.125	64	1
99	1	0.06	1	1	2	1	32	16	<0.125	<4	2
	2	0.06	2	1	2	<0.5	32	16	<0.125	<4	2
100	1	0.5	8	8	1	2	16	8	<0.125	128	1
	2	0.5	16	8	1	2	16	4	<0.125	128	2
101	1	0.5	>64	8	2	<0.5	32	16	<0.125	32	1
	2	0.5	64	4	1	<0.5	16	8	<0.125	64	2
102	1	0.25	1	2	1	<0.5	4	8	<0.125	128	1
	2	<0.03	1	2	2	<0.5	4	8	<0.125	128	2
103	1	<0.03	2	2	1	<0.5	4	2	<0.125	16	1
	2	<0.03	2	2	2	<0.5	4	4	<0.125	32	1
104	1	<0.03	1	2	1	<0.5	4	<0.5	<0.125	<4	<0.125
	2	<0.03	1	1	1	<0.5	2	<0.5	<0.125	<4	<0.125
105	1	<0.03	64	2	0.5	<0.5	2	8	<0.125	8	0.5
	2	<0.03	32	2	1	<0.5	2	8	<0.125	8	0.5
106	1	0.5	8	2	1	<0.5	8	4	<0.125	16	2
	2	<0.03	1	2	2	<0.5	4	8	<0.125	8	1
107	1	0.06	1	4	1	<0.5	16	4	<0.125	<4	2
	2	0.06	1	2	1	<0.5	16	4	<0.125	<4	2
108	1	<0.03	<0.5	2	0.5	<0.5	4	4	<0.125	16	2
	2	<0.03	<0.5	1	1	<0.5	2	2	<0.125	64	2
109	1	<0.03	1	2	0.5	<0.5	4	4	<0.125	64	2
	2	0.03	2	2	1	<0.5	2	2	<0.125	32	2
110	1	<0.03	1	2	1	<0.5	4	8	<0.125	128	1
	2	<0.03	1	2	1	<0.5	2	4	<0.125	128	1
111	1	<0.03	64	1	0.5	<0.5	4	4	<0.125	16	1
	2	<0.03	32	1	1	<0.5	4	4	<0.125	8	1

[1]Concentration of trimethoprim/sulfamethoxazole was tested in a ratio of 1/19; concentration of trimethoprim is given.

Ef: Enrofloxacin; Oxy: Oxytetracycline; Gm: Gentamicin; Ff: Florfenicol; Ac: Amoxycillin; Nm: Neomycin; Til: Tilmicosin;
Tri/Sul: Trimethoprim/Sulfamethoxazole; Sfm: Sulfadimethoxine; Cep: Cephalexin.

ANNEXURE II: cont.

MICs for *Mannheimia haemolytica* (cont.)

ISOLATES		MICs (µg/ml)									
ISOLATE NO	REPEAT	Ef	Oxy	Gm	Ff	Ac	Nm	Til	Tri/Sul[1]	Sfm	Cep
112	1	<0.03	1	2	0.5	<0.5	4	1	<0.125	<4	2
	2	<0.03	1	2	0.5	<0.5	4	1	<0.125	<4	2
113	1	<0.03	1	2	1	<0.5	2	<0.5	<0.125	<4	1
	2	<0.03	1	4	1	<0.5	2	<0.5	<0.125	<4	1
114	1	<0.03	2	1	2	<0.5	2	8	<0.125	256	2
	2	<0.03	2	2	1	<0.5	4	8	<0.125	128	2
115	1	<0.03	<0.5	2	1	<0.5	2	4	<0.125	256	2
	2	<0.03	<0.5	2	1	<0.5	4	8	<0.125	128	2
116	1	<0.03	2	2	1	<0.5	2	4	<0.125	64	1
	2	<0.03	2	1	1	<0.5	4	4	<0.125	64	1
117	1	<0.03	1	2	1	<0.5	4	4	<0.125	64	1
	2	<0.03	1	1	0.5	<0.5	8	4	<0.125	64	2
118	1	0.06	>64	0.5	4	16	4	4	<0.125	64	1
	2	0.125	>64	1	2	16	4	4	<0.125	64	1
119	1	<0.03	1	2	2	<0.5	2	16	<0.125	64	1
	2	<0.03	2	2	4	<0.5	4	32	<0.125	128	1
120	1	<0.03	<0.5	8	1	<0.5	4	2	<0.125	64	2
	2	<0.03	<0.5	8	1	<0.5	8	4	<0.125	128	1
122	1	<0.03	<0.5	2	1	<0.5	4	4	<0.125	128	1
	2	<0.03	2	2	2	<0.5	4	4	<0.125	128	1
123	1	<0.03	<0.5	2	0.5	<0.5	2	4	<0.125	32	0.5
	2	<0.03	<0.5	1	1	<0.5	4	2	<0.125	32	1
124	1	<0.03	<0.5	0.5	0.5	<0.5	2	4	<0.125	<4	1
	2	<0.03	<0.5	0.5	1	<0.5	2	4	<0.125	<4	2
126	1	<0.03	1	2	1	<0.5	4	4	<0.125	128	1
	2	<0.03	2	2	2	<0.5	4	4	<0.125	128	2
127	1	<0.03	64	2	0.5	<0.5	2	2	<0.125	128	1
	2	<0.03	64	4	0.5	<0.5	4	4	<0.125	128	2
128	1	0.06	1	2	2	<0.5	2	4	<0.125	16	4
	2	0.06	1	2	1	<0.5	4	8	<0.125	16	4

[1]Concentartion of trimethoprim/sulfamethoxazole was tested in a ratio of 1/19; concentration of trimethoprim is given.

Ef: Enrofloxacin; Oxy: Oxytetracycline; Gm: Gentamicin; Ff: Florfenicol; Ac: Amoxycillin; Nm: Neomycin; Til: Tilmicosin; Tri/Sul: Trimethoprim/Sulfamethoxazole; Sfm: Sulfadimethoxine; Cep: Cephalexin.

ANNEXURE III: Repeatability of duplicate test results

Repeatability of a MIC test on a panel of *Salmonella* strains for ten selected antimicrobial drugs

	ANTIMICROBIAL DRUGS									
	Ef	**Oxy**	**Gm**	**Ff**	**Ac**	**Nm**	**Til**	**Tri/Sul**	**Sfm**	**Cep**
	GREATER THAN ONE SERIAL DILUTION									
FREQUENCY	0	2	1	1	1	0	0	0	0	0
PERCENTAGE	0	6.67	3.33	3.33	3.33	0	0	0	0	0
	LESS THAN ONE SERIAL DILUTION									
FREQUENCY	2	0	0	0	2	0	0	1	0	0
PERCENTAGE	6.67	0	0	0	6.67	0	0	3.33	0	0
	NO DIFFERENCE									
FREQUENCY	28	28	29	29	27	30	30	29	30	30
PERCENTAGE	93.33	93.33	96.67	96.67	90.0	100.0	100.0	96.67	100.0	100.0

n= 30

Ef: Enrofloxacin; Oxy: Oxytetracycline; Gm: Gentamicin; Ff: Florfenicol; Ac: Amoxycillin; Nm: Neomycin; Til: Tilmicosin;
Tri/Sul: Trimethoprim/Sulfamethoxazole; Sfm: Sulfadimethoxine; Cep: Cephalexin.

Repeatability of a MIC test on a panel of *E. coli* strains for ten selected antimicrobial drugs

	ANTIMICROBIAL DRUGS									
	Ef	**Oxy**	**Gm**	**Ff**	**Ac**	**Nm**	**Til**	**Tri/Sul**	**Sfm**	**Cep**
	GREATER THAN ONE SERIAL DILUTION									
FREQUENCY	0	1	1	0	1	0	0	0	0	0
PERCENTAGE	0	3.33	3.33	0	3.33	0	0	0	0	0
	LESS THAN ONE SERIAL DILUTION									
FREQUENCY	0	1	1	0	2	1	0	3	3	0
PERCENTAGE	0	3.33	3.33	0	6.67	3.33	0	10.0	10.0	0
	NO DIFFERENCE									
FREQUENCY	30	28	28	30	27	29	30	27	27	30
PERCENTAGE	100.0	93.33	93.33	100.0	90.0	96.67	100.0	90.0	90.0	100.0

n= 30

Ef: Enrofloxacin; Oxy: Oxytetracycline; Gm: Gentamicin; Ff: Florfenicol; Ac: Amoxycillin; Nm: Neomycin; Til: Tilmicosin;
Tri/Sul: Trimethoprim/Sulfamethoxazole; Sfm: Sulfadimethoxine; Cep: Cephalexin.

ANNEXURE III: cont.

Repeatability of a MIC test on a panel of *Mannheimia haemolytica* strains for ten selected antimicrobial drugs

	ANTIMICROBIAL DRUGS									
	Ef	**Oxy**	**Gm**	**Ff**	**Ac**	**Nm**	**Til**	**Tri/Sul**	**Sfm**	**Cep**
GREATER THAN ONE SERIAL DILUTION										
FREQUENCY	2	1	0	0	1	0	0	0	0	0
PERCENTAGE	6.67	3.33	0	0	3.33	0	0	0	0	0
LESS THAN ONE SERIAL DILUTION										
FREQUENCY	1	2	0	0	0	0	0	0	1	0
PERCENTAGE	3.33	6.67	0	0	0	0	0	0	3.33	0
NO DIFFERENCE										
FREQUENCY	27	27	30	30	29	30	30	30	29	30
PERCENTAGE	90.0	90.0	100.0	100.0	96.67	100.0	100.0	100.0	96.67	100.0

n= 30

Ef: Enrofloxacin; Oxy: Oxytetracycline; Gm: Gentamicin; Ff: Florfenicol; Ac: Amoxycillin; Nm: Neomycin; Til: Tilmicosin; Tri/Sul: Trimethoprim/Sulfamethoxazole; Sfm: Sulfadimethoxine; Cep: Cephalexin.

Repeatability of a MIC test on a panel of *Enterococcus faecalis, E. faecium* strains for ten selected antimicrobial drugs

	ANTIMICROBIAL DRUGS									
	Ef	**Oxy**	**Gm**	**Ff**	**Ac**	**Nm**	**Til**	**Tri/Sul**	**Sfm**	**Cep**
GREATER THAN ONE SERIAL DILUTION										
FREQUENCY	0	2	0	0	0	2	1	0	0	0
PERCENTAGE	0	6.67	0	0	0	6.67	3.33	0	0	0
LESS THAN ONE SERIAL DILUTION										
FREQUENCY	0	2	1	0	0	0	1	0	1	0
PERCENTAGE	0	6.67	3.33	0	0	0	3.33	0	3.33	0
NO DIFFERENCE										
FREQUENCY	30	26	29	30	30	28	28	30	29	30
PERCENTAGE	100.0	86.67	96.67	100.0	100.0	93.33	93.33	100.0	96.67	100.0

n= 30

Ef: Enrofloxacin; Oxy: Oxytetracycline; Gm: Gentamicin; Ff: Florfenicol; Ac: Amoxycillin; Nm: Neomycin; Til: Tilmicosin; Tri/Sul: Trimethoprim/Sulfamethoxazole; Sfm: Sulfadimethoxine; Cep: Cephalexin.

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ANNEXURE IV: Deviation of duplicate test results

Deviations of the results when comparing the duplicate tests of *Salmonella*

	ANTIMICROBIAL DRUGS									
	Ef	**Oxy**	**Gm**	**Ff**	**Ac**	**Nm**	**Til**	**Tri/Sul**	**Sfm**	**Cep**
	PLUS/MINUS ONE DILUTION									
FREQUENCY	12	11	13	8	0	0	2	2	4	11
PERCENTAGE	41.38	36.67	43.33	26.67	0	0	6.67	6.67	13.33	36.67
	NO DIFFERENCE									
FREQUENCY	16	17	16	21	27	30	28	27	26	19
PERCENTAGE	55.17	56.67	53.33	70.0	90.0	100.0	93.33	90.0	86.67	63.33
	PLUS/MINUS MORE OR LESS THAN ONE DILUTION									
FREQUENCY	1	2	1	1	3	0	0	1	0	0
PERCENTAGE	3.45	6.67	3.33	3.33	10.0	0	0	3.33	0	0

n= 30

Ef: Enrofloxacin; Oxy: Oxytetracycline; Gm: Gentamicin; Ff: Florfenicol; Ac: Amoxycillin; Nm: Neomycin; Til: Tilmicosin; Tri/Sul: Trimethoprim/Sulfamethoxazole; Sfm: Sulfadimethoxine; Cep: Cephalexin.

Deviations of the results when comparing the duplicate tests of *E. coli*

	ANTIMICROBIAL DRUGS									
	Ef	**Oxy**	**Gm**	**Ff**	**Ac**	**Nm**	**Til**	**Tri/Sul**	**Sfm**	**Cep**
	PLUS/MINUS ONE DILUTION									
FREQUENCY	6	2	18	13	4	9	6	4	4	10
PERCENTAGE	20.0	6.67	60.0	43.33	13.33	30.0	20.0	13.33	13.33	33.33
	NO DIFFERENCE									
FREQUENCY	24	26	10	17	23	20	24	23	23	20
PERCENTAGE	80.0	86.67	33.33	56.67	76.67	66.67	80.0	76.67	76.67	66.67
	PLUS/MINUS MORE OR LESS THAN ONE DILUTION									
FREQUENCY	0	2	2	0	3	1	0	3	3	0
PERCENTAGE	0	6.67	6.67	0	10.0	3.33	0	10.0	10.0	0

n= 30

Ef: Enrofloxacin; Oxy: Oxytetracycline; Gm: Gentamicin; Ff: Florfenicol; Ac: Amoxycillin; Nm: Neomycin; Til: Tilmicosin; Tri/Sul: Trimethoprim/Sulfamethoxazole; Sfm: Sulfadimethoxine; Cep: Cephalexin.

ANNEXURE IV: cont.

Deviations of the results when comparing the duplicate tests of *Mannheimia haemolytica*

	ANTIMICROBIAL DRUGS									
	Ef	**Oxy**	**Gm**	**Ff**	**Ac**	**Nm**	**Til**	**Tri/Sul**	**Sfm**	**Cep**
	PLUS/MINUS ONE DILUTION									
FREQUENCY	2	9	12	19	0	16	13	0	11	10
PERCENTAGE	6.67	30.0	40.0	63.33	0	53.33	43.33	0	36.67	33.33
	NO DIFFERENCE									
FREQUENCY	25	18	18	11	29	14	17	30	18	20
PERCENTAGE	83.33	60.0	60.0	36.67	96.67	46.67	56.67	100.0	60.0	66.67
	PLUS/MINUS MORE OR LESS THAN ONE DILUTION									
FREQUENCY	3	3	0	0	1	0	0	0	1	0
PERCENTAGE	10.0	10.0	0	0	3.33	0	0	0	3.33	0

n= 30

Ef: Enrofloxacin; Oxy: Oxytetracycline; Gm: Gentamicin; Ff: Florfenicol; Ac: Amoxycillin; Nm: Neomycin; Til: Tilmicosin;
Tri/Sul: Trimethoprim/Sulfamethoxazole; Sfm: Sulfadimethoxine; Cep: Cephalexin.

Deviations of the results when comparing the duplicate tests of *Enterococcus faecalis, E. faecium*

	ANTIMICROBIAL DRUGS									
	Ef	**Oxy**	**Gm**	**Ff**	**Ac**	**Nm**	**Til**	**Tri/Sul**	**Sfm**	**Cep**
	PLUS/MINUS ONE DILUTION									
FREQUENCY	10	7	15	9	1	7	5	2	1	2
PERCENTAGE	33.33	23.33	50.0	30.0	3.33	23.33	16.67	6.67	3.33	6.67
	NO DIFFERENCE									
FREQUENCY	20	19	14	21	29	21	23	28	28	28
PERCENTAGE	66.67	63.67	46.67	70.0	96.67	70.0	76.67	93.33	93.33	93.33
	PLUS/MINUS MORE OR LESS THAN ONE DILUTION									
FREQUENCY	0	4	1	0	0	2	2	0	1	0
PERCENTAGE	0	13.33	3.33	0	0	6.67	6.67	0	3.33	0

n= 30

Ef: Enrofloxacin; Oxy: Oxytetracycline; Gm: Gentamicin; Ff: Florfenicol; Ac: Amoxycillin; Nm: Neomycin; Til: Tilmicosin;
Tri/Sul: Trimethoprim/Sulfamethoxazole; Sfm: Sulfadimethoxine; Cep: Cephalexin.

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