Antimicrobial resistance patterns and ESBL producers among *Escherichia coli* isolates from dogs

BY

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Declaration

I Dr Samson Manatsa declare the E coli isolate used in the study was collected form Bacteriology Laboratory of the Faculty of Veterinary Science of the University of Pretoria. These were routine clinical sample submitted from the Small Animal Hospital of the Faculty of Veterinary Science of the University of Pretoria. Kirby-Bauer test results used in this study were retrospective result conducted by the Bacteriology laboratory. The MICE test was done by me. Screening of ESBL producing E coli isolates was done by Inqaba Biotech^{TM}, South Africa. Apart from the acknowledgement for the ad and guidance received from my supervisor, this dissertation is a product of my own work. The full dissertation, or part of, has not been, is not being and will not be submitted for another degree at this or any other university.

This dissertation is being Submitted in partial fulfilment of the requirements for the degree Magister Scientiae (Master of Science) Tropical Animal Health) in the Department of Veterinary Tropical Disease, Faculty of Veterinary Science, University of Pretoria

Samson Manatsa 12 March 2020

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Abbreviations

ACA	Amoxicillin / Clavulanic acid
AMP	Ampicillin
ARHAI	Advisory Committee on Antimicrobial Resistance and Healthcare associated Infection
CLSI	Clinical and laboratory standards institute
CTZ	Ceftazidime
CTX	Cefotaxime
DARC	Defra Antimicrobial Resistance Coordination
ESBL	Extended-spectrum beta-lactamase
IPM	Imipenem
MDR	Multi-drug resistance
MDRO	Multi-drug resistant organism
MIC	Minimum Inhibitory Concentration
OMP	Outer membrane proteins
PBP	Penicillin binding proteins
PCR	Polymerase chain reaction
rRNA	ribosomal Ribonucleic acid
3GC	Third Generation Cephalosporins
UVIS	Universal Veterinary Information System
WHO	World Health Organization

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Summary

Emerging extended-spectrum beta lactamase (ESBL) *Escherichia coli* in veterinary and human medicine is a global threat to health delivery. This study investigated the presence and prevalence of ESBL producers from diagnostic isolates. The isolates were characterised through retrospective analysis of existing Kirby-Bauer results to determine the phenotypic resistance prevalence and screen for ESBL producing isolates, to quantify resistance through the M.I.C.E test and detect the TEM gene. A total of 36 isolates out of 37 were ESBL producers and 10 isolates were carrying the TEM gene. Retrospective analysis demonstrated varying resistance prevalence from 5.4 to as high as75.7% against the 15 antibiotics across all 37 isolates. Resistance across all 37 isolates was observed with a prevalence of 78.4% against ampicillin, 54.1% against amoxicillin / clavulanic acid, 21.6% against ceftazidime and 24.6% against cefotaxime but no resistance was observed against imipenem. Resistance was also observed in some isolates which were negative for the TEM gene. Further investigation to identify the other ESBL genes causing the resistance phenotype observed in *E. coli* isolates which tested negative for the TEM gene is needed.

Key Words: antimicrobial resistance, *Escherichia coli*, prevalence, TEM gene

Chapter 1

Literature Review

Background

Antimicrobial resistance, a major worldwide public health threat, develops as result of overuse, underuse and abuse of antibiotics. During the past two decades, antimicrobial resistance has been documented in bacteria isolated in companion animals, food animals and human beings. However, bacterial antimicrobial resistance has been in existence since Alexander Fleming discovered penicillin (Prescott & Baggot, 1993; Tang, *et al*. 2014). Prevalence of multidrug resistant bacteria has increased tremendously, but research has not been forthcoming with new drugs particularly against Gram-negative bacteria. Most countries outside the European Union and the United States of America do not have a functional antimicrobial resistance policy to contain antimicrobial resistance. Microbes have no boundaries hence the importance of international collaboration with regard to surveillance and control (WHO 2014).

According to the World Health Organisation (WHO 2014), there are profound gaps in surveillance and international collaboration. Particularly with respect to data of emerging antimicrobial resistance in pets, livestock, wildlife bacteria and their impact on environmental, human and animal health.

The WHO 2014 assessment report estimates the antimicrobial resistance financial impact in the USA as 34billion dollars plus additional 8 more days of hospitalization or treatment which calls for more cross-sectional collaboration at national and international level. According to Huttner *et al* (2013), Alexander Fleming included the following warning during his acceptance speech of the Nobel Prize: “it is not difficult to make microbes resistant to penicillin in the laboratory by exposing them to concentrations not sufficient to kill them. There is the danger that the ignorant man may easily under-dose himself and, by exposing his microbes to non-lethal quantities of the drug make them resistant”.

Escherichia coli (*E. coli*) a rod shaped Gram-negative bacteria, belongs to order Enterobacterales, along with *Klebsiella*, *Enterobacter* and *Proteus*. Commensal *E. coli,* commonly of low virulence, are part of the normal intestinal flora. The bacteria assist with digestion of food and production of vitamin K. These *E. coli* strains can be grouped in pathogenic and non-pathogenic strains. Pathogenic strains of *E. coli* express virulence genes resulting in intestinal and/or extra-intestinal disease. The pathogenic *E. coli* strains are further classified as zoonotic extra-intestinal or intestinal pathogens (Ewers, *et al*. 2012). Intestinal pathogenic *E. coli* has virulence factors that cause gastrointestinal diarrhoea due to enterotoxigenic, entero pathogenic and enterohaemorrhagic toxins (Shierack *et al*. 2009, Belanger *et al*. 2010). In contrast, extra-intestinal *E. coli* does not cause gastrointestinal infection but are opportunistic pathogens with virulence factors that empower them to infect extra-intestinal tissues. Virulence factors, adhesion genes, iron acquisition systems, toxins, polysaccharide capsules and multi-drug resistance through production of beta-lactamases and extended spectrum beta-lactamases (ESBL), play a crucial role in the pathogenesis of extra-intestinal *E. coli* (Platell, *et al*. 2011). In the dog and cat, tissues affected are typically the upper and lower urinary system, vagina, uterus, and udder (Beutin, 1990; Ewers, *et al*. 2012).

Transmission of *E. coli* strains that cause intestinal infections commonly occur via the faecal-oral route due to contamination of the environment by faecal matter. Intestinal flora may be a source of *E. coli* with the ability to cause canine extra-intestinal infections for example urogenital, mammary and endocardial infections (Beutin, 1990). Dogs have been living in close proximity with humans for centuries hence the high possibility of transmission of pathogen at this human-animal interface. Figure 1 shows the possible routes of transmission between human, food animals, wild animals and companion animals in different habitats.

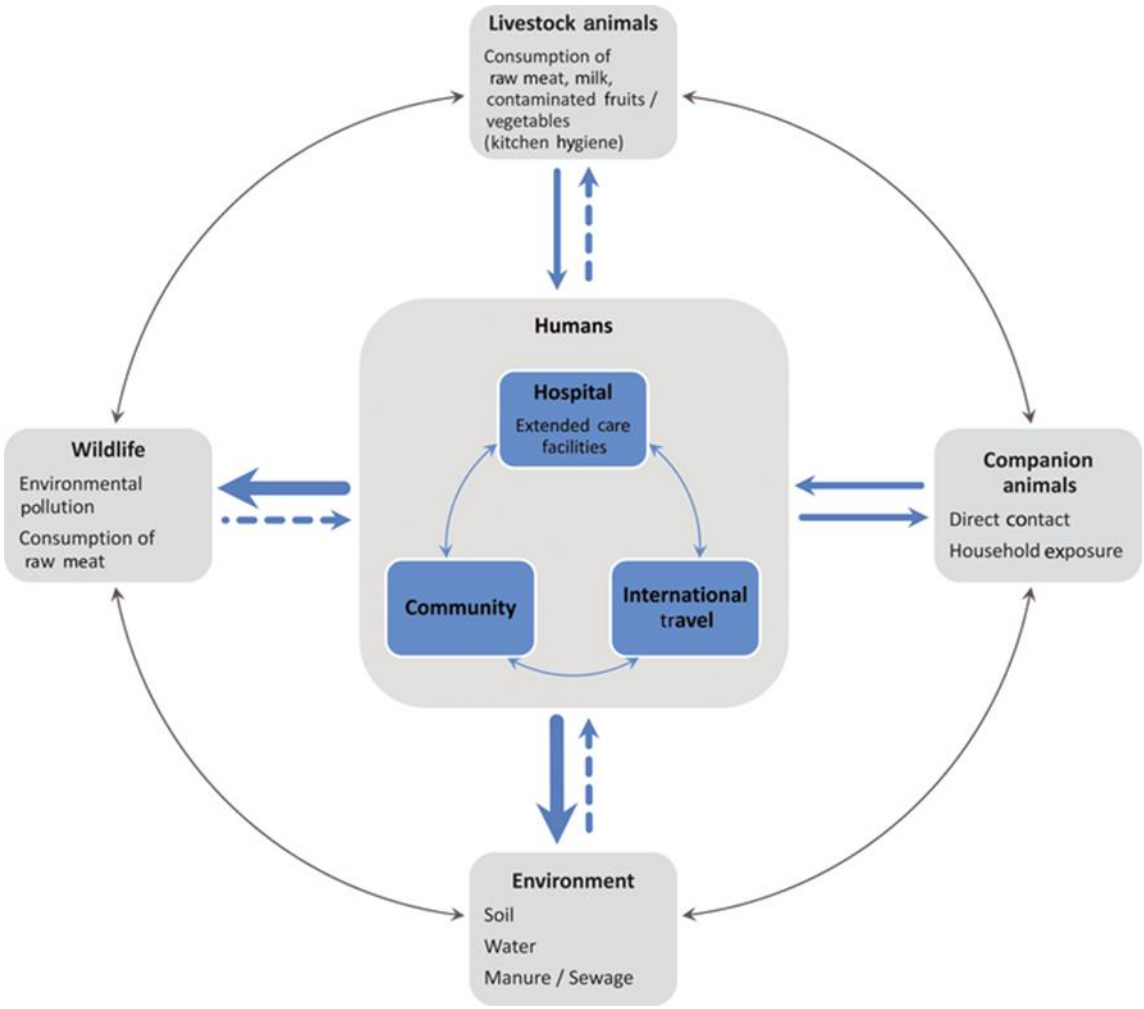


Figure 1: The transmission pathways of antimicrobial resistance between humans, animals and the environment (Ewers, et al 2012).

Mode of action of beta-lactam antimicrobials

Antimicrobials can be classified according to their mode of action (Baquero & Blázquez, 1997; Prescott & Baggot, 1993). In the case of the beta-lactam antibiotics (carbapenems, cephalosporins, penicillins, and monobactams), the mode of action is interference with cell wall synthesis. These antibiotics are bactericidal and their mode of action inhibits the penicillin-binding proteins (PBP) (transpeptidase and peptidoglycan-active enzyme), involved in the final stage of synthesis of the bacterial cell wall. A cell wall is important to maintain the shape of a bacterial cell in a hypertonic environment (Prescott & Baggot 1993;Drawz & Bonomo, 2010).

Mechanisms of antimicrobial resistance

Antimicrobial resistance phenotypically shown by bacteria occurs through a variety of mechanisms but can be categorized as intrinsic and acquired antimicrobial resistance (Tenover 2006; Baquero & Blázquez 1997). Intrinsic resistance is the result of the expression of genes naturally present as part of bacterial chromosomes (Davies & Davies, 2010). Acquired resistance (or horizontal evolution) in *E. coli* occurs mainly through chromosomal point mutation or acquisition of new genes from different strains of the same species or from different bacterial species (Livermore 2002; Tang 2014). Both intrinsic and acquired resistance result in any of the four mechanisms of resistance*, e.g.*

1) The most common mechanism is the production of beta-lactamase enzymes which hydrolyse the beta-lactam ring of beta-lactam antibiotics in the periplasmic space resulting in loss of bactericidal effects of the antibiotic (Prescott & Baggot, 1993).
2) Efflux pumps extrude the antibiotic out of the bacterial cell before it reaches target receptor or reaches lethal intracellular concentration. This mechanism can be acquired or intrinsic. *E. coli* have efflux pumps that are central and instrumental in development and enhancement of multi-drug resistance (Drawz & Bonomo, 2010; Tenovar, 2006).
3) Structural changes of the cell wall as a result of natural transformation and recombination with DNA through point mutation or acquisition of plasmid mediated genes changing the receptor site of PBP. The changes on the receptor site of PBP result in reduced affinity for beta-lactam antibiotics (Tenover 2006).
4) The ability to change cell wall permeability by reducing expression of the outer membrane proteins (OPM). The PBPs are found on the inner cell membrane, therefore the beta-lactam antibiotic has to diffuse through or bind to OMP to traverse porin channels in order to reach the PBPs. Figure 2 illustrates the structure of the Gram-negative bacteria cell wall. Some of the resistance developed by Enterobacterales against carbapenems is through loss of OMPs. This kind of acquired resistance can develop after point mutation or insertion sequences in porin-encoding genes (Drawz & Bonomo 2010; Tenover 2006).

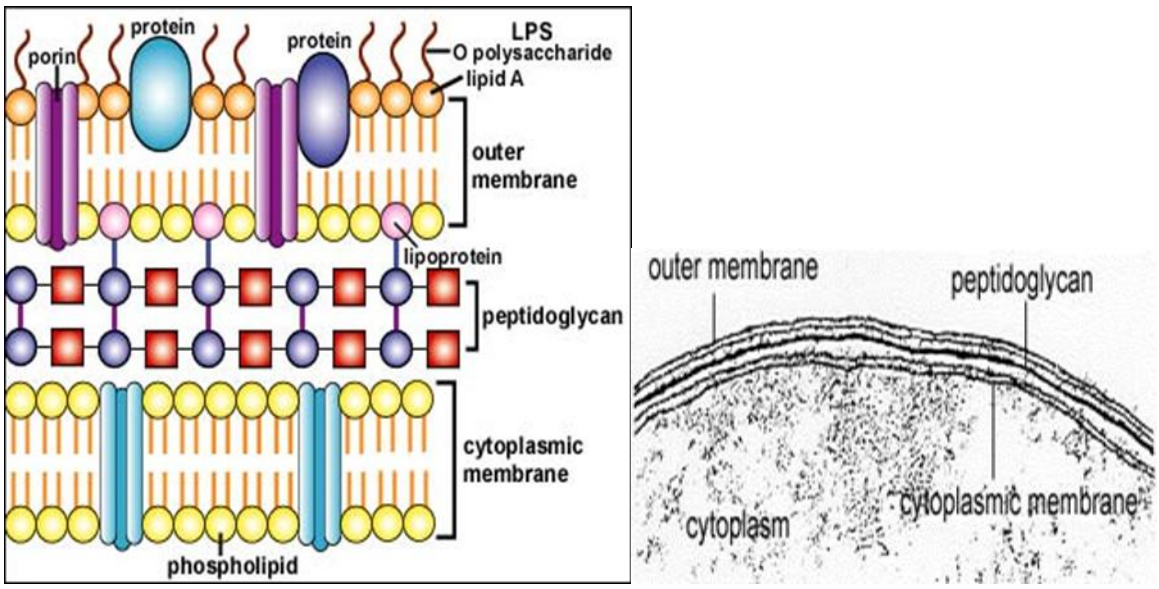


Figure 2. To the left, structure of the Gram-negative cell wall.

The cell wall is made up of the outer membrane (made up of lipopolysaccharides (LPS) and phospholipids) and the inner layer made of peptidoglycan. To the right is the electron microscope picture of the cell wall. (Kaiser. G, 2019)

The development of resistance through genetic exchange or mutation enables the bacteria to adapt to the new environment created by the introduced antibiotic (Platell, *et al.*2011). The predominant antimicrobial evolution in bacteria has been achieved through horizontal gene transfer, mostly plasmid-encoded (Davies & Davies, 2010). Exchange of genetic material in horizontal evolution is through transduction, conjugation and transformation by means of transposons facilitating the acquisition of genes encoding for resistance. Introduction of a gene into a cell by a virus or viral factor is transduction unlike conjugation a process where another bacteria transfer genetic material to another bacteria by contact. Transformation horizontal transfer occurs when there is gene transfer between two bacteria resulting in gene integration into the recipient bacteria genome. Repeated mutations together with exchange of genetic material result in bacteria developing resistance to multiple antimicrobials (Platell, *et al.* 2011).

Environmental stress created by the presence of antibiotic during treatment result in increased chances of mutation rates on the bacterial chromosome resulting in

development of acquired resistance. Exposure to a particular antibiotic will activate the resistance mechanism to that antibiotic (Baquero & Blázquez, 1997). According to Baquero & Blázquez (1997), the mechanisms that increase the rate of mutation are polymerase slippage of rRNA, gene conversion as well as genomic rearrangement or point mutation. A single chromosomal mutation of Enterobacterales does not confer resistance to all antimicrobials but can result in the bacteria developing resistance to one or more antimicrobials, for example a point mutation that results in increased efflux of antimicrobials can affect the efficacy of fluoroquinolones and most beta-lactams (Livermore, 2002). Once *E. coli* develops antimicrobial resistance against a specific antibiotic, exposure to the antibiotic will result in selection for resistant bacteria in a population (Baquero & Blázquez, 1997).

Extended-spectrum beta-lactamase (ESBL) is one of the mechanisms of antimicrobial resistance. In addition, ESBL-producing organisms are often multi-drug resistant. ESBL producing *Escherichia coli* has been documented during the last three decades in both human and veterinary medicine (Huber, *et al*. 2013). A literature review by Ewers *et al.* (2012) indicates a steady increase in reports of ESBL-producing *E. coli* in domesticated and wild animals during the past two decades.

Companion animals are major contributors in the spill over of human ESBL-producing *E. coli* at the human/animal interface as they live in close contact with their owners. *E. coli* play an integral role in the horizontal transfer of ESBL antimicrobial resistance to different bacterial species (Ewers, *et al.* 2012). *E. coli* strains with ESBL antimicrobial resistance has been documented in healthy community cats and dogs as well as hospitalised cats and dogs (Tuerena, *et. al*. 2016).

Development of Extended-spectrum beta-lactamase in *E. coli*.

The emergence of multi drug resistant strains of *E. coli* worldwide presents a serious challenge for government, pet owners and human and animal clinicians due to high morbidity and mortality as result of poor response to treatment. This leads to increased cost of human and animal health care. Antimicrobial resistance has always been there as many antimicrobial substances are produced by bacteria. For bacteria to survive in their habitat with other bacteria they had to be resistant to the

antimicrobial substances produced by other bacteria (Davies & Davies, 2010). Antimicrobial resistance due to antibiotic use has been evolving since the 1950s with the introduction of the first antibiotic. It increased from minor therapeutic resistance to the present day where we are now facing major challenges of multi-drug resistant Gram-negative bacteria like *E. coli* (Baquero & Blázquez, 1997). According to Davies, *et al*. (2010), the enzyme penicillinase was identified before the use of the antibiotic penicillin. The emergence of antimicrobial resistance is a direct consequence of the selective events imposed by the use of antimicrobial resulting in silent selection of resistant bacteria for a long time through under dosage during clinical case management (Baquero & Blázquez, 1997). Sewerage, hospitals and farms provide the ideal environment for the selective events for antimicrobial resistance to take place because of general faecal contamination and use of antimicrobials (Davies & Davies, 2010).

In 1940, the first beta-lactamase enzyme that hydrolysed penicillin was isolated. This was *AmpC* cephalosporinase produced by *E. coli*. Horizontal transfer of genetic material through conjugation, transformation or transduction is the major mechanism that results in development of ESBL antimicrobial resistance (Shaikh, *et al*. 2015). According to Tenover (2006), *E. coli* strains acquired ESBL plasmid-encoded enzymes TEM-, SHV- or CTX-M type that resulted in development of antimicrobial resistance against monobactams and third generation cephalosporins. The acquired antimicrobial resistance results in point mutation on the plasmid-encoded genes for the enzymes TEM-, SHV- or CTX-M. Inhibition of ESBL by clavulanic acid, sulbactam and tazobactam is a principle that is used to classify beta-lactamase and ESBL (Huber, *et al*. 2013).In most cases, ESBL confers resistance phenotype to third generation cephalosporins (3GC), but not to cephamycins (cefoxitin, cefotetan). In addition, it may confer resistance to other antibiotics (e.g. aminoglycosides, quinolones, and cotrimoxazole) leading to bacteria that are classified as multi-drug resistant organisms (MDROs). Multi-drug resistance can be defined as phenotype of resistance pattern observed against at least three classes of antibiotic (Magiorakos et al. 2011, Tenover 2006, Huber, *et al*. 2013, Thenmozhi, *et al.* 2014).

Since they are not susceptible to beta-lactamases and extended spectrum beta lactamases, carbapenems are the last choice in the case of ESBL production and

are critical antimicrobials for treatment of clinical cases of infection by ESBL producing Gram-negative bacteria. Carbapenems' mechanism of action, like penicillin, disrupts cell synthesis of the cell wall by binding to PBP (Shaikh,*et al.* 2015). Therefore, a report of the emergence of resistance phenotype to carbapenems is concerning (Guerra, *et al.* 2014).

Classification of Extended-spectrum beta-lactamase (ESBL)

Most beta-lactamases are penicillinases rather than cephalosporinases. Beta-lactamases are classified according to their hydrolytic activity. The Ambler molecular classification groups the enzymes according to their molecular homology and is the most common. Class A, C and Dare serine-beta-lactamases that need serine on the active site. Class B metallo-beta-lactamase need zinc (Tang, *et al.* 2014). The second classification of beta-lactamases, including ESBL, is the Bush-Jacoby-Medeiros system, which categorises the beta-lactamases according to the function of the substrate and the inhibitory properties into four main groups. Beta-lactamase class TEM-1, TEM-2, and SHV-1 are acquired plasmid-encoded enzymes produced by *E. coli* (Tang,*et al.*2014). Penicillin, ampicillin, oxacillin and cloxacillin are readily inactivated by the TEM-type and OXA-type beta-lactamases (Prescott & Baggot, 1993). TEM-1 was found to be common in *E. coli* isolates of human origin in South Africa (DeFrancesco,*etal.* 2017;Founou,*et al.* 2018).

Distribution of ESBL producing *E. coli* in companion animals

The first report of transferable plasmid mediated resistance was recorded in Europe during the early 1980s upon introduction of cefotaxime. The beta-lactamase, SHV-2 was first documented in Germany and France. This is a variant of the TEM beta-lactamase, which led to the adaptation, and use of the term extended-spectrum beta-lactamase. The first animal ESBL, SHV-12, was identified in an *E. coli* isolate from a dog with a urinary infection in 2000 (Ewers, *et al.* 2012). ESBL variants are mostly found in nosocomial hospital settings in human and animal hospitals. A study by Tuerena *et al.* (2016), in the United Kingdom, reported a prevalence of 14% resistance against amoxicillin by ESBL-producing *E. coli* isolated from dogs that visited a veterinary clinic, which is higher than the prevalence of 6% to 8% seen in dogs that never visited a veterinary clinic. The high prevalence seen in dogs getting veterinary attention was attributed to hospitalization.

Yet another ESBL gene, CTX-M, was first reported in early 1990. Some of the gene subtype encodes carbapenemase resistance. It was identified in ESBL producing coliforms from humans. In most cases CTX-M-producing *E. coli* in humans are acquired through nosocomial infection (Ewers, *et al*. 2012).

Though appearing late in animals, CTX-M-producing *E. coli* has now been documented in food animals such as dairy, pigs, poultry and companion animals such as dogs. Eight out of 107 urinary samples of dogs and cats admitted at the small animal clinic of the University of Zurich due to urinary infection had ESBL-producing *E. coli.* These isolates were resistant to all beta-lactams, fluoroquinolones, nalidixic acid, tetracycline and sulfamethoxazole/trimethoprim. In addition, CTX-M-15 was found in all ESBL-producing *E. coli* (Huber, *et al*. 2013).

Therefore, the use of these drugs for treatment of infection in companion animals and food animals will result in selection for ESBL-producing *E. coli*. It is thought that the introduction of ceftiofur for treatment of mastitis in cattle and septicaemia in poultry, is one of the drivers of selection of ESBL-producing *E. coli* by antibiotics hence the rise in prevalence in dairy and poultry (Report by the Joint Working Group of DARC and ARHAI).

Surveillance of ESBL-producing *E. coli* in companion animals has been neglected for a long time. This is an animal welfare and public health issue because publications indicate sharing of ESBL-producing *E. coli* between companion animals and human beings (Ewers, et al. 2012).

The major drivers of development of antimicrobial resistance are antimicrobial usage of non-prescription drugs and under dosage. Due to the perceived high cost of laboratory testing, veterinarians opt to treat animals without laboratory test guidance. Therefore, under dosage and use of incorrect drugs to treat a sick animal is very common (Shears, 2001).

Detection of ESBL producing *E. coli*

The MICE method is a quantitative susceptibility method developed to determine the minimum inhibition concentration of antimicrobial. The MICE test in the presence of

ESBL-producer indicator can be used as a screening test for ESBL-producing bacteria. In this case the 3rd generation cephalosporin (ceftazidime and cefotazime) and clavulanic acid (amoxicillin/clavulanic acid) were the indicators of the presence of an ESBL-producer.The objective of this study is to determine the prevalence of antimicrobial resistance and ESBL production among *E. coli* isolates from extra-intestinal infections in dogs admitted at the Faculty of Veterinary Science hospital of the University of Pretoria.

Hypothesis

1. The null hypothesis is: There is no antimicrobial resistance or ESBL-producing *E. coli* in dogs.
2. Alternative hypothesis: There is antimicrobial resistance and ESBL-producing *E. coli* in dogs.

Chapter 2

Materials and Methods

Sampling Methods

Over a period of 11months, November 2016 to December 2017, all *E.coli* strains cultured and identified from routine clinical case management of dogs were collected by the bacteriology laboratory of the Faculty of Veterinary Science of the University of Pretoria. The *E. coli* strains were from clinical samples from clinical cases with suspect urinary infection, septicaemia and otitis externa admitted at the small animal hospital at the Faculty of Veterinary Science, University of Pretoria. Thirty-seven isolates were picked from the sixty-three isolates collected over the study period. The isolates were kept at -80ºC at the bacteriology laboratory.

Study Design

This is a cross sectional study using a collection of *E. coli* isolates from diagnostic samples from clinical cases. Selection criteria were as follows: the isolates had to be *E. coli*, an antibiogram had to be included and isolates had to come from a routine clinical case at the small animal hospital at the Faculty of Veterinary Science, University of Pretoria. The animals of origin had to be dogs.

Sample size calculation was based binomial model (Fosgate 2009). Sample size was calculated using the prevalence from literature of 7% at 95% confidence. The sample was calculated from the study population. The study population was made up of isolates collected over study period. The sample size was calculated using the formula below:

$$n = \log \propto \div \log[(1 - p)]$$

Where n is the sample size, $\propto$ is 1- confidence and p is the prevalence(Fosgate 2009).Existing antibiogram data, obtained by Kirby-Bauer testing, was sourced from the laboratory data management system (UVIS) and read into a spread-sheet. The isolates were subjected to further antimicrobial susceptibility testing.

Laboratory Analysis

The *E. coli* isolates were retrieved from the -80^{0}C freezer at the Bacteriology laboratory, thawed and sub-cultured onto Columbia agar with 5% horse blood

(Selectamedia, Thermofischer). Inoculated plates were incubated at 37ºC in normal air for 24 hours.

Disk diffusion results were further investigated and quantified by epsilometer (E-test) method (M.I.C.E., Oxoid, Basingstoke, UK), for minimum inhibitory concentration determination. Phenotypic test, M.I.C.E., was used to test *E. coli* isolates using an antimicrobial susceptibility profile that indicate production of extended-spectrum beta-lactamase (ESBL). The profile consisted of beta-lactam antibiotics: ampicillin (256-0.016µg/ml), amoxycillin/clavulanic acid (256-0.016µg/ml), cefotaxime (256-0.015µg/ml), ceftazidime (32-0.002µg/ml) and imipenem (32-0.002µg/ml). *E. coli* ATCC 25922 was the quality control organism. A suspension equal in turbidity to a 0.5 MacFarland standard was prepared of each *E. coli* isolate. Each suspension was swabbed with a sterile cotton swab onto Mueller Hinton agar (Selectamedia, Thermofischer) in three different directions to obtain an inoculum one-cell layer thick. M.I.C.E test strips were applied onto the inoculated Mueller Hinton Agar manually as per manufacturer's instructions. The plates were incubated at 37^0C overnight in normal air. The next day, the minimum inhibitory concentrations were read from the strip meter at the point of intersection if the inhibition ellipses with the minimum inhibition concentration reading scale in µg/ml.

In addition, each isolate was spot inoculated on Colorex ESBL agar (Media Mage) and incubated in normal air overnight at 37ºC. Growth and a pink colour indicated ESBL production.

Screening of ESBL producing isolates for the TEM gene was done by Inqaba Biotech™, South Africa. Inqaba Biotechnology is a company that does molecular analyses. The forward primers GCGGAACCCCTATTTG, reverse primer ACCAATGCTTAATCAGTGAG for the universal blaTEM gene were used for detection (S. Ashiboe-Mensah, *et al.* 2016, Cheddie, *et al.*2017). Sequencing was not requested for financial reasons.

Statistical Analysis

Data was captured on an Excel spreadsheet and imported into R (R software version 3.6.1) for analysis. Prevalence was calculated as the number samples that tested positive over total number of samples tested both phenotypically and genotypically. The Pearson Chi-square test was done to test the differences in proportions between the numbers of positives as well as phenotypic versus genotypic.

Chapter 3

Results

All the isolates were extra-intestinal *E. coli*. The samples were from the infected organs, two from pyoderma, 23 from cystitis, three from cholecystitis, three from otitis and six from septicaemia (Table 1).

Table 1: Frequency table illustrating different organs where samples were collected.

Isolates	Pyoderma	Cystitis	Cholecystitis	Otitis	Septicaemia
37	2	23	3	3	6
%	5.4	62.2	8.1	8.1	16.2

Antimicrobial Susceptibility Testing

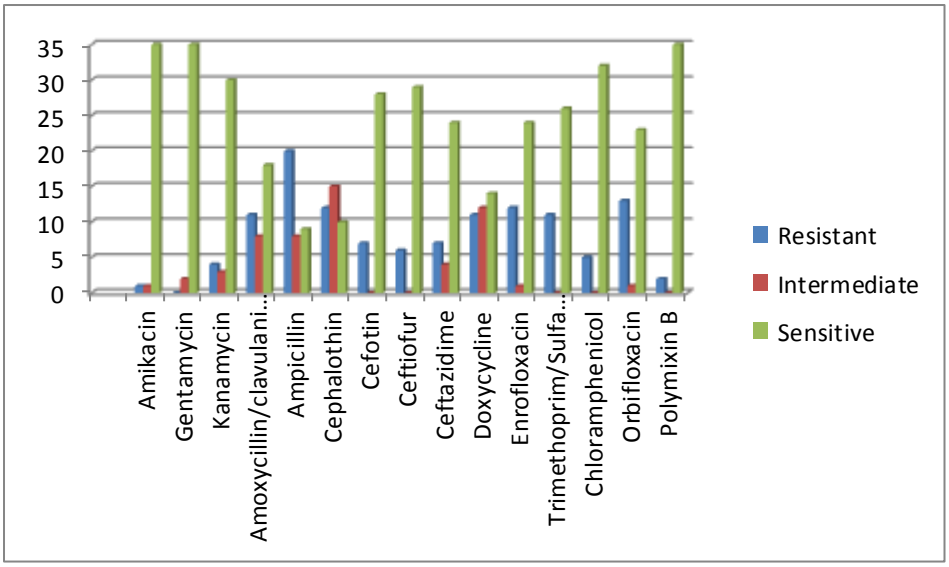
Existing antibiogram data of 37 *E. coli* isolates, obtained by Kirby-Bauer testing, sourced from the laboratory data management system (UVIS) was analysed. The interpretation of results was based on disc diffusion zone diameter and MIC interpretation criteria published by Clinical and Laboratory Standards Institute (CLSI) (2015). High level of resistance prevalence was observed across all isolates.

Resistance was observed in all isolates to ampicillin, amoxicillin/clavulanic acid (Synulox), cephalothin (first generation cephalosporin) and cefoxitin, ceftazidime, ceftiofur (third generation cephalosporin). Resistance against other antimicrobials, aminoglycoside (amikacin, gentamicin, and kanamycin), fluoroquinolone (enrofloxacin, orbifloxacin), doxycycline, chloramphenicol, and polymixin B was observed in all strains. The resistance phenotype observed on the *E. coli* against penicillins and cephalosporins indicate ESBL production by the isolates. Results of disc diffusion result analysis are summarised on table 2.Multidrug resistance (MDR), resistance against three or more antimicrobial classes, was observed in 45.9% of the isolates: 17 *E. coli* isolates classified as MDR. Table 3 illustrate the frequency of the resistance pattern phenotype observed. Eight isolates had resistance pattern against three, 5 isolates against 4, one isolate against five, 2 isolates against 6, and one isolate had resistance pattern phenotype against seven antimicrobial classes.

Table 2: Prevalence among E. coli isolates from dogs treated at the small animal hospital, Faculty of Veterinary Science, University of Pretoria (retrospective analysis of existingKirby-Bauer test results).

Antimicrobial		Resistant		Sensitive	
			Prevalence		
Class	Antibiotic	No	%	No	Prevalence %
	Amikacin	2	5.4	35	94.6
	Gentamycin	2	5.4	35	94.6
Aminoglycosides	Kanamycin	7	18.9	30	81.1
	Amoxycillin/clavulanic acid	19	51.4	18	48.6
Pencillin	Ampicillin	28	75.7	9	24.3
	Cephalothin	27	73	10	27
	Cefotin	7	20	28	80
	Ceftiofur	6	17.1	29	82.9
Cephalosporin	Ceftazidime	11	29.7	24	68.6
Tetracycline	Doxycycline	23	62.2	14	37.8
	Enrofloxacin	13	35.1	24	64.9
Fluoroquinolone	Orbifloxacin	14	37.8	23	62.2
Salfonamides	Trimethoprim/Sulfamethoxazole	11	29.7	26	70.3
Chloramohenicol	Chloramphenicol	5	13.5	32	86.5
Polymixin B	Polymixin B	2	5.4	35	94.6

Graph 1: A frequency bar graph illustrating antibiotic susceptibility of *E. coli* isolates when tested by means of the Kirby-Bauer disk diffusion test.



Y-axis is the number of *E. coli* isolates

Table 3: Multidrug table showing the resistance pattern phenotype status after Kirby-Bauer disk diffusion test.

Number of resistant pattern per isolate	Phenotype of resistance pattern	Number of isolates
7	CN,AMP,CTZ/CP,DXY,ENR,SXT,PLB	1
	AMP,CTZ/CP,DXY,ENR,SXT,CHM,	1
6	CN,AMP,CTZ/CP,DXY,ENR,SXT	1
5	AMP,CTZ/CP,DXY,ENR,SXT	1
	CN,AMP,CTZ/CP,ENR	1
	AMP,CTZ,/CP,DXY,SXT	1
	AMP,DXY,ENR,SXT	2
4	AMP,CTZ/CP,ENR,CHM	1
	AMP,DXY,ENR	3
	CTZ/CP,ENR,PLB	1
	AMP,CTZ/CP,ENR	2
	AMP,SXT,CHM	1
3	AMP,DXY,SXT	1
	AMP,CTZ/CP	3
	AMP,SXT	3
2	AMP,CHM	1
1	AMP	13
MDR		17
NONMDR		20

AMK, Amikacin; KN, Kanamycin; GEN, Gentamycin; AMP, Ampicillin; ACA, Amoxycillin/clavulanic acid; KF, Cephalothin; CF, Cefoxitin; CFT, Ceftiofur; CTZ, Ceftazidime; DXY, Doxycycline; ENR, Enrofloxacin; ORB, Orbifloxacin; CHL, Chloramphenicol; PLB, Polymixin B.

The epsilometer (E-test) method

All the *E. coli* isolates were further investigated and quantified with the epsilometer (E-test) method. Results of the M.I.C.E test are summarized in table 4.Resistance was observed in 29 isolates against ampicillin, amoxicillin/clavulanic acid, ceftazidime, and cefotaxime. Ampicillin resistance was most prevalent78.4%, amoxicillin/clavulanic acid54.1, cefotaxime 24.3% and ceftazidime 21.6% (Table5). All the *E.coli* isolates were sensitive to imipenem. MDR was not observed in all *E. Coli* isolates. ESBL production by *E. coli* isolates was further indicated by the Colorex agar test where 36 isolates were positive and one was negative.

Table 4: Antimicrobial susceptibility of E. coli strains isolated from dogs treated at the small animal hospital, Faculty of Veterinary Science, University of Pretoria (using M.I.C.E test).

Isolates	Cefotaxime		Ceftazidime		Amoxicillin/Clavulanic acid		Ampicillin		Imipenem	
Number	Interpretation	MIC (µg/ml)	Interpretation	MIC (µg/ml)	Interpretation	MIC (µg/ml)	Interpretation	MIC (µg/ml)	Interpretation	MIC (µg/ml)
1	R	0	R	32	R	0	R	0	S	0.25
2	R	0	S	1	R	0	R	32	S	0.25
3	S	0.12	S	0.25	S	8	R	16	S	0.25
4	R	0	R	32	R	0	R	0	S	0.25
5	S	0.06	S	0.25	R	0	R	32	S	0.25
6	R	0	R	4	R	0	R	16	S	0.25
7	S	0.06	S	0.12	S	2	R	32	S	0.25
8	S	0,12	S	0,12	R	0	R	32	S	0.25
9	R	0	R	4	R	0	R	16	S	0.25
10	S	0.12	S	0.25	S	8	R	16	S	0,25
11	R	0	R	64	R	0	R	0	S	0,25
12	S	0.12	S	0.25	R	0	R	32	S	0.25
13	S	0.06	S	0.12	S	4	S	8	S	0.25
14	S	0.06	S	0.12	R	0	R	16	S	0.12
15	S	0.12	S	0.25	R	0	R	64	S	0.25
16	S	0.12	S	0.5	S	8	R	16	S	0.25
17	R	0	R	32	R	0	R	0	S	0.5
18	S	0.12	S	0.25	S	8	R	16	S	0.25
19	S	0.06	S	0.12	S	4	S	8	S	0.25
20	S	0.12	S	0.25	R	0	R	64	S	0.25
21	S	0.25	S	0.5	S	8	R	16	S	0.25
22	S	0.12	S	0.5	S	8	R	16	S	0.25
23	S	0.12	S	0.5	S	8	S	8	S	0.25
24	S	0.12	S	0.25	S	4	S	8	S	0.25

25	R	2	R	4	R	0	R	0	S	0.12
26	S	0.12	S	0.25	R	0	R	32	S	0,25
27	S	0.12	S	0.25	S	4	S	8	S	0.25
28	S	0.12	S	0.25	R	256	R	32	S	0.25
29	S	0.12	S	0.25	S	4	R	16	S	0.25
30	R	0	R	8	R	0	R	32	S	0.25
31	S	0.25	S	0.5	R	0	R	0	S	0.25
32	S	0.25	S	0.5	S	4	S	8	S	0.25
33	S	0.25	S	0.5	R	0	R	64	S	0.25
34	S	0.25	S	0.5	R	0	R	32	S	0.25
35	S	0.12	S	0.25	S	8	S	8	S	0.25
36	S	0.12	S	0.25	S	8	R	16	S	0.25
37	S	0.12	S	0.12	S	4	S	8	S	0.25
CONTROL	S	0.03	S	0.12	S	0.25	S	1	S	0.25

Table 5: Prevalence among E. coli isolates from dogs treated at the small animal hospital, Faculty of Veterinary Science, University of Pretoria (M.I.C.E test)

Antimicrobial	Phenotype Susceptible		Phenotype Resistant	
	No of isolate	%	No of isolates	%
Ampicillin	8	21.6	29	78.4
Amoxicillin/clavulanic acid	17	45.9	20	54.1
Ceftazidime	29	78.4	8	21.6
Cefotaxime	28	75.7	9	24.3
Imipenem	37	100	0	0

B-Lactamase TEM gene screening.

ESBL producing *E. coli* DNA of the 37 *E. coli* isolates in this study were screened by PCR for presence of the TEM gene. Ten *E. coli* isolates out of 37 were positive for TEM. According to this study the prevalence of the TEM gene is 27%. Table 6 shows the distribution of the isolates with and without the TEM gene relative to the antibiotic susceptibility status of the same isolate. Ampicillin resistant isolates had the highest percentage 78.4% TEM gene presence. Twoβ-lactam resistance phenotypes were observed on the ten isolates that carried the TEM gene as follows: ampicillin and amoxicillin/clavulanic acid resistant (n=8) and cefotaxime, ceftazidime, ampicillin, amoxicillin/clavulanic acid resistant (n=2) pattern.

Table 6: Distribution of TEM gene in all isolates according to susceptibility phenotype status.

Drug	Status	TEM Gene		
		Absent	Present	%
Ampicillin	R	19	10	34.5
	S	8	0	0
Amoxicillin/Clavulanic acid	R	10	10	50
	S	17	0	0
Cefotaxime	R	7	2	22.2
	S	20	8	28.5
Ceftazidime	R	6	2	25
	S	21	6	22.2
Imipenem	R	0	0	0
	S	27	10	27
Overall	R	44	24	35.3
	S	93	24	20.5

Statistical Analysis

Analysis output of the Chi-square test done to test the differences in proportions between the numbers of positives as well as phenotypic versus genotypic report, Appendix 1. Mantel-Haenszel chi-squared test with continuity correction in R demonstrated significant statistical association between proportion of the resistance observed after the M.I.C.E test for all five antibiotics against the TEM gene with a p-value close to 0 (p-value = 3.614e-12). The p-value = 1.754e-10 observed after further test demonstrate significant statistical association between phenotypic resistance observed in isolates against three antimicrobials and the TEM gene.

A statistical analysis of the phenotypic status, resistant and susceptible, with or without the TEM gene was done separately for the overall status and for the individual antimicrobials. A measure of chi-square contribution showed that the TEM gene contributed by a factor of 2.129toward the phenotypic resistance observed in

36.4% of the *E. coli* isolates. Fourteen isolates with the TEM gene and 19 without the TEM gene were resistant. A much lower chi-square contribution factor of 0.788was observed in the isolates were phenotypic resistance was identified, but the TEM gene was absent. The chi-square contribution was much lower in sensitive isolates with TEM gene by factor 0.729 and by of factor 0.249 insensitive isolates without the TEM gene.

Chapter 4

Discussion

The 37 isolates in this study were from routine clinical diagnostic samples taken to aid veterinary clinicians incoming to informed decisions in managing clinical cases. The samples were collected from animals with pyoderma, cystitis, cholecystitis, otitis and septicaemia. The observation that 62.2% of the clinical isolates where from cystitis cases is consistent with other studies where *E. coli* was observed to be among the most prevalent cause of UTI in dogs and cats (Lother & Beutin, 1999) and an important cause of UTI in humans (Vila, *et al*. 2016) although it also causes infection in other extra-intestinal organs.

In this study, antibiotic susceptibility test results indicated varying levels of resistance among the 37 isolates ranging from 5.4 to 75.7% against the 15 antimicrobials. MDR was observed in 17 (45.6) isolates during the retrospective analysis of Kirby-Bauer susceptibility test results. Analysis for MDR was not possible because the MICE tested only 2 classes of antimicrobials. MDR has been observed in the studies of companion animals (Shaheen, *et al.* 2011;Katherine, *et al*. 2008), poultry, pigs, bovine and human studies (Ewers, *et al*. 2012).45.6% MDR observed in this study is higher than 18% observed by Tuerena, *et al*. 2016. The general phenotypic resistance pattern observed is similar to studies done in other countries. In our study, similar to reports in other publications, older or antimicrobials that have been over prescribed a longer time had the highest resistance prevalence for example ampicillin 78.4% and amoxicillin/clavulanic acid 54.1%.

The Kirby-Bauer disk diffusion test indicated ESBL producing isolates when resistance was observed to beta lactams, penicillin, and at least one third generation cephalosporin, but antibiotic susceptibility to one of the cephamycins (cefoxitin). The M.I.C.E test result of resistance phenotype seen against cephalosporins (ceftazidime and cefotaxime) and penicillins (ampicillin and amoxicillin/clavulanic acid) further indicated the presence of ESBL producers as well as the degree of resistance. The prevalence of 75.7% resistance to ampicillin in the disk diffusion antibiogram is similar to that observed in the M.I.C.E (78.4%). MICs of resistant isolates were 256µg/ml or more. The prevalence of resistance to amoxicillin/clavulanic acid

(51.4%) in the disk diffusion antibiogram is similar to that seen in the M.I.C.E. test (54.1%). Twenty percent resistance was detected to ceftazidime by disk diffusion compared to a prevalence of 21.6% resistance observed against ceftazidime in the MIC test. MICs of resistant isolates ranged from 16 to 64µg/ml. Disk diffusion and MIC results agreed on six ESBL isolates.

Results of the Colorex ESBL agar did not correlate with the ESBL producing isolates indicated by the disk diffusion and MIC tests. Colorex agar identified 35 isolates as ESBL producers, while disk diffusion identified seven and MIC ten isolates. The better identification of ESBL producers by the MIC, ten isolates compared to seven isolates identified by the disk diffusion, can be attributed to the difference in the antimicrobial susceptibility profile. Aarestrup *et al* (2010) evaluated the ability of different 3GC to detect ESBL and concluded that the most superior cephalosporins in detection of ESBL are cefpodoxime, ceftriaxone and ceftriaxone. The MIC test antimicrobial susceptibility profile had better ESBL indicators, (ceftazidime, and cefotaxime) unlike the disk diffusion which had ceftazidime and ceftiofur.In this study, unlike other studies, no phenotypic resistance was detected against carbapenems (Imipenem). Carvalho,*et al*.(2016) reported resistance of 9.5% against cefepime in his study of resistance patterns in dogs and their owners.

Amoxicillin/clavulanic acid is a common antimicrobial used in clinical case management of companion animals. In this study, a prevalence of 54.1% resistance of clinical isolates against amoxicillin/clavulanic acid is higher than findings in other studies at a prevalence of 14% (Tuerena, *et al*. 2016). However, it is higher than prevalence of 3 to 8%, observed in the studies of community dogs most likely because the isolates in this study were from hospitalized dogs. Cases managed at the small animal hospital of the University of Pretoria are predominantly referral cases meaning most cases have been treated on several cases. Treatment on several cases results in exposure to high antimicrobial selection pressure (Katherine, *et al*.2008). All the isolates which tested positive for TEM gene had phenotypic resistance against amoxicillin/clavulanic acid. Clavulanic acid is an inhibitor of the TEM gene enzymes inclusive of other ESBL. Other studies have demonstrated the presence of inhibitor-resistant enzyme variant that is linked to the presence of the

TEM gene (Karczmarczyk, *et al*. 2011; Tuerena, *et al*, 2016). Karczmarczyk, *et al.*(2011), in their study demonstrated transfer of amoxicillin/clavulanic acid and ampicillin resistance on some isolates through conjugation. The high resistance phenotype pattern observed against amoxicillin/clavulanic acid may be due to presence of other ESBL. According to Shaheen, *et al*.(2011),the OXA-1 type β-lactamase hydrolyze β-lactamase inhibitors, like clavulanic acid, and in their research analysis demonstrated that nearly a third (28%) of the *E-coli* isolates were carrying the variant.

This finding suggests that there are other ESBL genes in the isolates causing resistance. In the study by Tuerera, *et al*. 2016, the majority of resistant *E. coli* isolates tested positive for the AmpC gene with the TEM gene second most common.

In this study, resistance to ceftazidime, a 3GC, was 21.6%.Of these *E. coli* isolates, 78.4% tested negative for the TEM gene. The prevalence in this study is relatively lower than prevalence detected by Tuerera, *et al*. 2016, of 27% in *E. coli* isolates from hospitalized dogs.

In this cross sectional study, the TEM gene was detected by PCR in 10 isolates with a prevalence of 27% (Table 6). The forward primer GCGGAACCCCTATTTG and the reverse primer ACCAATGCTTAATCAGTGAG used in this study, like in other studies, are for the general detection and amplification of the *bla*TEM gene but not the subtype of the blaTEM gene (S. Ashiboe-Mensah, *et al.*2016, Cheddie *et al.*2017). The prevalence in this study is low compared to prevalence of ESBL production of 43-63% (Baede, *et al*.2015). Most cross sectional studies carried out in other countries has prevalence range ESBL-producing clinical Enterobacterales between 3.1% and 54.4%. The prevalence was calculated from isolates derived from cats and dogs (Baede, *et al*.2015). According to Baede, *et al* (2015) most studies found a prevalence of 16-22% ESBL producing Enterobacterales in healthy companion animals. In contrast to this study, other studies included other ESBL genes besides TEM. In this study TEM gene, beside financial resource limitation, was selected because TEM-gene is documented as most commonly found ESBL in

E. coli isolated from young children in South Africa (DeFrancesco et al. 2017). Huber, *et al.* 2013, detected the TEM gene in some of the clinical isolates from dogs and cats with urinary infection admitted at the University of Zurich, small animal clinic. The observation that all isolates detected with TEM gene tested phenotypically positive for resistance is consistent with other studies. A study in Brazil detected ESBL genes in 26.6% of all isolates that were resistant on the antibiotic susceptibility test. According to Carvalho, *et al.* 2016, 66.6% of the isolates from dogs that tested positive for ESBL genes had gene TEM which is higher than in our result of 42.4%. The TEM gene was the most prevalent, detected in 66 out of 74 isolates (89%) (Karczmarczyk, *et al.* 2011).

The first TEM gene, TEM-1, was reported in 1965 was isolated from *E. coli* with the capacity to hydrolyse penicillins and first generation cephalosporins. Variants with increased activity against cephalosporins have since evolved, TEM-2, TEM-3. In England, Liverpool in 1982, the first plasmid corded gene encoding reduced ceftazidime susceptibility was isolated, TEM-12 (Shaik, *et al*. 2014). According to Shaik, *et al*. 2014, this was a classic case of resistance developing due to selective pressure induced by use of third generation cephalosporins. The prevalence of the TEM gene observed in this study, 27%, is lower than the susceptibility resistance. This is consistence with findings in other studies. In China Sun, *et al.* 2010, demonstrated that 41.3% of the screened healthy and sick dogs and cats were positive ESBL producers. 54.5% of isolates from sick animals were positive for ESBL production which is higher than 24.5% observed as ESBL producers on health animals. The high number of ESBL producers observed was attributed to the fact the 50% of the dogs had been treated with a cephalosporin within the past 12months (Sun, *et al.*2010).

The major drivers of development of antimicrobial resistance are antimicrobial usage of β-lactam antibiotic and third generation cephalosporins resulting in direct selection and spread of ESBL-producing *E. coli* in livestock, wildlife and human. (Cavaco, *et al*, 2008; Lautenbach, *et al.* 2001). In addition, it is thought that the introduction of ceftiofur for treatment of mastitis in cattle and septicaemia in poultry is one of the drivers of selection of ESBL-producing *E. coli*. Hence, the rise in prevalence of

resistance in dairy and poultry (Report by the Joint Working Group of DARC and ARHAI).The use of ceftiofur in dairy herds in the Netherlands was associated with reduced susceptibility to ceftiofur in bacterial isolates from the herds (Santman-Berends, *et al*. 2017)**.**

This study demonstrates the need for multidisciplinary collaboration between health, animal and environmental professional in order to come up with mitigatory strategies that result in early detection and monitoring of antimicrobial resistance.

According to the World Health Organisation (WHO 2014), there are profound gaps in surveillance and international collaboration. Particularly with respect to data of emerging antimicrobial resistance in pets, livestock, wildlife bacteria and their impact on environmental, human and animal health. Pets pose a high risk of transmission between human and animals because they live in close proximity. The WHO 2001 report identified that antimicrobial use in food animals pose a great risk for resistance development. Containment of antimicrobial resistance can be mitigated through strict obligatory prescription and cease usage of growth promoters if concurrently used for treatment in food animals. Development of surveillance system that result in early detection of resistance assist with containment of antimicrobial resistance. The government has a major role in mitigation of antimicrobial resistance through formulating policy for prudent use of antimicrobial. The government must also establish regulations for registrations, dispensing, manufacture of drugs treatment guidelines for both animal and human use (WHO 2001).

CONCLUSION AND RECOMMENDATIONS

The alternative hypothesis was confirmed as antibiotic resistance as well as ESBL producers were detected. Variable prevalence of resistance was observed against all isolates after analysis of existing Kirby-Bauer antibiogram data. The M.I.C.E test further indicated the presence of ESBL producing isolates. The high general prevalence of resistance across the isolates compared to the prevalence of the TEM gene indicates existence of other ESBL genes contributing to the observed phenotype of resistance. Inclusion of more sensitive cephalosporin disks could improve detection of ESBLs by the microbiology laboratory.More research is necessary to investigate trends of antimicrobial resistance over time as well as the presence of other ESBL genes.

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Appendix1

Analysis output of the Chi-square test done to test the differences in proportions between the numbers of positives as well as phenotypic versus genotypic.

Antimicrobial resistance patterns and prevalence of ESBL producers among Escherichia coli isolates from dogs

Manatsa Samson 17388855

09/12/2019

```
library(readr)
## Warning: package 'readr' was built under R version 3.5.3
library(readxl)
## Warning: package 'readxl' was built under R version 3.5.3
MICE_1 <- read_excel("~/MSC UP/Research/Thesis/results/Data analysis/MICE 1.xlsx")
#View(MICE_1)
attach(MICE_1)
library(gmodels)
## Warning: package 'gmodels' was built under R version 3.5.3
library(Rmisc)
## Warning: package 'Rmisc' was built under R version 3.5.3
## Loading required package: lattice
## Loading required package: plyr
## Warning: package 'plyr' was built under R version 3.5.3
drug=as.factor(drug)
status=as.factor(status)
tem_gene=as.factor(tem_gene)
mytable<- table(status,tem_gene)#A will be rows, B will be columns
print(table)
## function (..., exclude = if (useNA == "no") c(NA, NaN), useNA = c("no",
##     "ifany", "always"), dnn = list.names(...), deparse.level = 1)
## {
```

```
##     list.names <- function(...) {
##         l <- as.list(substitute(list(...)))[-1L]
##         nm <- names(l)
##         fixup <- if (is.null(nm))
##             seq_along(l)
##         else nm == ""
##         dep <- vapply(l[fixup], function(x) switch(deparse.level +
##             1, "", if (is.symbol(x)) as.character(x) else "",
##             deparse(x, nlines = 1)[1L]), "")
##         if (is.null(nm))
##             dep
##         else {
##             nm[fixup] <- dep
##             nm
##         }
##     }
##     miss.use <- missing(useNA)
##     miss.exc <- missing(exclude)
##     useNA <- if (miss.use && !miss.exc && !match(NA, exclude,
##         nomatch = 0L))
##         "ifany"
##     else match.arg(useNA)
##     doNA <- useNA != "no"
##     if (!miss.use && !miss.exc && doNA && match(NA, exclude,
##         nomatch = 0L))
##         warning("'exclude' containing NA and 'useNA' != \"no\"' are a bit contradicting
")
##     args <- list(...)
##     if (!length(args))
##         stop("nothing to tabulate")
##     if (length(args) == 1L && is.list(args[[1L]])) {
##         args <- args[[1L]]
```

```
##         if (length(dnn)  != length(args))
##             dnn <- if (!is.null(argn  <- names(args)))
##                 argn
##             else paste(dnn[1L],  seq_along(args),  sep = ".")
##     }
##     bin <- 0L
##     lens <- NULL
##     dims <- integer()
##     pd <- 1L
##     dn <- NULL
##     for (a in args) {
##         if (is.null(lens))
##             lens <- length(a)
##         else if (length(a)  != lens)
##             stop("all arguments  must  have  the same length")
##         fact.a <- is.factor(a)
##         if (doNA)
##             aNA <- anyNA(a)
##         if (!fact.a) {
##             a0 <- a
##             a <- factor(a, exclude  = exclude)
##         }
##         add.na <- doNA
##         if (add.na) {
##             ifany <- (useNA  == "ifany")
##             anNAc <- anyNA(a)
##             add.na <- if (!ifany || anNAc) {
##                 ll <- levels(a)
##                 if (add.ll <- !anyNA(ll))  {
##                   ll <- c(ll, NA)
##                   TRUE
##                 }
```

```
##             else if (!ifany && !anNAc)
##               FALSE
##             else TRUE
##         }
##         else FALSE
##     }
##     if (add.na)
##         a <- factor(a, levels = ll, exclude = NULL)
##     else ll <- levels(a)
##     a <- as.integer(a)
##     if (fact.a && !miss.exc) {
##         ll <- ll[keep <- which(match(ll, exclude, nomatch = 0L) ==
##             0L)]
##         a <- match(a, keep)
##     }
##     else if (!fact.a && add.na) {
##         if (ifany && !aNA && add.ll) {
##             ll <- ll[!is.na(ll)]
##             is.na(a) <- match(a0, c(exclude, NA), nomatch = 0L) >
##               0L
##         }
##         else {
##             is.na(a) <- match(a0, exclude, nomatch = 0L) >
##               0L
##         }
##     }
##     nl <- length(ll)
##     dims <- c(dims, nl)
##     if (prod(dims) > .Machine$integer.max)
##         stop("attempt to make a table with >= 2^31 elements")
##     dn <- c(dn, list(ll))
##     bin <- bin + pd * (a - 1L)
```

```
##         pd <- pd * nl
##     }
##     names(dn) <- dnn
##     bin <- bin[!is.na(bin)]
##     if (length(bin))
##         bin <- bin + 1L
##     y <- array(tabulate(bin,  pd), dims, dimnames = dn)
##     class(y) <- "table"
##     y
## }
## <bytecode: 0x00000000090a8230>
## <environment:  namespace:base>
mytable <- table(drug,tem_gene)  # A will be rows,  B will be columns
mytable  # print table
##                          tem_gene
## drug                        negative positive
##  Ampicillin                    27       10
##   Amoxicillin/Clavulanic  acid  27       10
##   Cefotaxime                    27       10
##   Ceftazidime.                  27       10
##   Imipenem                      27       10
margin.table(mytable,  1) # A  frequencies  (summed over B)
## drug
##                Ampicillin                Amoxicillin/Clavulanic  acid
##                        37                           37
##                Cefotaxime                Ceftazidime.
##                        37                           37
##                  Imipenem
##                        37
margin.table(mytable,  2) # B  frequencies  (summed over A)
## tem_gene
## negative positive
```

```
##     135      50
prop.table(mytable) # cell percentages
##                          tem_gene
## drug                        negative    positive
##   Ampicillin                0.14594595 0.05405405
##   Amoxicillin/Clavulanic    0.14594595 0.05405405
##   Cefotaxime                0.14594595 0.05405405
##   Ceftazidime.              0.14594595 0.05405405
##   Imipenem                  0.14594595 0.05405405
prop.table(mytable, 1) # row percentages
##                          tem_gene
## drug                        negative   positive
##   Ampicillin                0.7297297 0.2702703
##   Amoxicillin/Clavulanic  acid  0.7297297 0.2702703
##   Cefotaxime                0.7297297 0.2702703
##   Ceftazidime.              0.7297297 0.2702703
##   Imipenem                  0.7297297 0.2702703
prop.table(mytable, 2) # column percentages
##                          tem_gene
## drug                        negative positive
##   Ampicillin                    0.2      0.2
##   Amoxicillin/Clavulanic  acid  0.2      0.2
##   Cefotaxime                    0.2      0.2
##   Ceftazidime.                  0.2      0.2
##   Imipenem                      0.2      0.2
# 2-Way Frequency Table
mytable <- table(status,tem_gene) # A will be rows, B will be columns
mytable # print table
##             tem_gene
## status       negative positive
##   resistance       42       24
##   sensitive        93       26
```

```
margin.table(mytable, 1) # A frequencies (summed over B)
## status
## resistance sensitive
##         66        119
margin.table(mytable, 2) # B frequencies (summed over A)
## tem_gene
## negative positive
##      135       50
prop.table(mytable) # cell percentages
##             tem_gene
## status         negative  positive
##   resistance 0.2270270 0.1297297
##   sensitive  0.5027027 0.1405405
prop.table(mytable, 1) # row percentages
##             tem_gene
## status         negative  positive
##   resistance 0.6363636 0.3636364
##   sensitive  0.7815126 0.2184874
prop.table(mytable, 2) # column percentages
##             tem_gene
## status         negative  positive
##   resistance 0.3111111 0.4800000
##   sensitive  0.6888889 0.5200000
#Mantel-Haenszel chi-squared test with continuity correction


mantelhaen.test(drug,status,tem_gene)
##
##  Cochran-Mantel-Haenszel test
##
## data:  drug and status and tem_gene
## Cochran-Mantel-Haenszel M^2 = 59.545, df = 4, p-value = 3.614e-12
# 3-Way Frequency Table
```

```
mytable <- xtabs(~drug+status+tem_gene, data=MICE_1)
ftable(mytable) # print table
##                                  tem_gene negative positive
## drug                    status
## Ampicillin                 resistance              18       10
##                         sensitive                9        0
## Amoxicillin/Clavulanic acid resistance          11       10
##                         sensitive               16        0
## Cefotaxime                 resistance              7        2
##                         sensitive               20        8
## Ceftazidime.               resistance              6        2
##                         sensitive               21        8
## Imipenem                   resistance              0        0
##                         sensitive               27       10
summary(mytable) # chi-square test of indepedence
## Call: xtabs(formula = ~drug + status + tem_gene, data = MICE_1)
## Number of cases in table: 185
## Number of factors: 3
## Test for independence of all factors:
##  Chisq = 73.57, df = 13, p-value = 1.754e-10
##  Chi-squared approximation may be incorrect
## cross tabultaions using gmodel package
## antimicrobial reasistance AMR
CrossTable(status,tem_gene)
##
##
##    Cell Contents
## |-------------------------|
## |                       N |
## | Chi-square contribution |
## |           N / Row Total |
## |           N / Col Total |
```

```
## |         N / Table Total |
## |-------------------------|
##
##
## Total Observations in Table:  185
##
##
##              | tem_gene
##       status |  negative |  positive | Row Total |
## -------------|-----------|-----------|-----------|
##   resistance |        42 |        24 |        66 |
##              |     0.788 |     2.129 |           |
##              |     0.636 |     0.364 |     0.357 |
##              |     0.311 |     0.480 |           |
##              |     0.227 |     0.130 |           |
## -------------|-----------|-----------|-----------|
##    sensitive |        93 |        26 |       119 |
##              |     0.437 |     1.181 |           |
##              |     0.782 |     0.218 |     0.643 |
##              |     0.689 |     0.520 |           |
##              |     0.503 |     0.141 |           |
## -------------|-----------|-----------|-----------|
## Column Total |       135 |        50 |       185 |
##              |     0.730 |     0.270 |           |
## -------------|-----------|-----------|-----------|
##
##
CrossTable(drug,tem_gene)
##
##
##    Cell Contents
## |-------------------------|
```

```
## |                     N |
## | Chi-square contribution |
## |           N / Row Total |
## |           N / Col Total |
## |         N / Table Total |
## |-------------------------|
##
##
## Total Observations in Table:  185
##
##
##                          | tem_gene
##                     drug |  negative |  positive | Row Total |
## -------------------------|-----------|-----------|-----------|
##               Ampicillin |       27 |       10 |       37 |
##                          |    0.000 |    0.000 |          |
##                          |    0.730 |    0.270 |    0.200 |
##                          |    0.200 |    0.200 |          |
##                          |    0.146 |    0.054 |          |
## -------------------------|-----------|-----------|-----------|
##  Amoxicillin/Clavulanic  acid |       27 |       10 |       37 |
##                          |    0.000 |    0.000 |          |
##                          |    0.730 |    0.270 |    0.200 |
##                          |    0.200 |    0.200 |          |
##                          |    0.146 |    0.054 |          |
## -------------------------|-----------|-----------|-----------|
##                Cefotaxime |       27 |       10 |       37 |
##                          |    0.000 |    0.000 |          |
##                          |    0.730 |    0.270 |    0.200 |
##                          |    0.200 |    0.200 |          |
##                          |    0.146 |    0.054 |          |
## -------------------------|-----------|-----------|-----------|
```

```
##              Ceftazidime. |       27 |       10 |       37 |
##                           |    0.000 |    0.000 |          |
##                           |    0.730 |    0.270 |    0.200 |
##                           |    0.200 |    0.200 |          |
##                           |    0.146 |    0.054 |          |
## --------------------------|----------|----------|----------|
##                  Imipenem |       27 |       10 |       37 |
##                           |    0.000 |    0.000 |          |
##                           |    0.730 |    0.270 |    0.200 |
##                           |    0.200 |    0.200 |          |
##                           |    0.146 |    0.054 |          |
## --------------------------|----------|----------|----------|
##              Column Total |      135 |       50 |      185 |
##                           |    0.730 |    0.270 |          |
## --------------------------|----------|----------|----------|
##
##
CrossTable(drug,status)
##
##
##   Cell Contents
## |-------------------------|
## |                       N |
## | Chi-square contribution |
## |           N / Row Total |
## |           N / Col Total |
## |         N / Table Total |
## |-------------------------|
##
##
## Total Observations in Table:  185
##
```

```
##
##                          | status
##                     drug | resistance |  sensitive |  Row Total |
## --------------------------|------------|------------|------------|
##                Ampicillin |         28 |          9 |         37 |
##                           |     16.594 |      9.203 |            |
##                           |      0.757 |      0.243 |      0.200 |
##                           |      0.424 |      0.076 |            |
##                           |      0.151 |      0.049 |            |
## --------------------------|------------|------------|------------|
## Amoxicillin/Clavulanic  acid |         21 |         16 |         37 |
##                           |      4.609 |      2.556 |            |
##                           |      0.568 |      0.432 |      0.200 |
##                           |      0.318 |      0.134 |            |
##                           |      0.114 |      0.086 |            |
## --------------------------|------------|------------|------------|
##                Cefotaxime |          9 |         28 |         37 |
##                           |      1.336 |      0.741 |            |
##                           |      0.243 |      0.757 |      0.200 |
##                           |      0.136 |      0.235 |            |
##                           |      0.049 |      0.151 |            |
## --------------------------|------------|------------|------------|
##               Ceftazidime. |          8 |         29 |         37 |
##                           |      2.048 |      1.136 |            |
##                           |      0.216 |      0.784 |      0.200 |
##                           |      0.121 |      0.244 |            |
##                           |      0.043 |      0.157 |            |
## --------------------------|------------|------------|------------|
##                  Imipenem |          0 |         37 |         37 |
##                           |     13.200 |      7.321 |            |
##                           |      0.000 |      1.000 |      0.200 |
##                           |      0.000 |      0.311 |            |
```

```
##                          |      0.000 |      0.200 |            |
## -------------------------|------------|------------|------------|
##               Column Total |         66 |        119 |        185 |
##                          |      0.357 |      0.643 |            |
## -------------------------|------------|------------|------------|
##
```

Animal Ethics Certificate

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Research Ethics Committee

PROJECT TITLE	**Antimicrobial resistance patterns and prevalence of ESBL producing *E coli* in clinical isolates from dogs and cats admitted at the small animal hospital of the Faculty of Veterinary Science, University of Pretoria**
PROJECT NUMBER	**REC003-18**
RESEARCHER/PRINCIPAL INVESTIGATOR	**Samson Manatsa**

STUDENT NUMBER (where applicable)	
DISSERTATION/THESIS SUBMITTED FOR	**MSc**

SUPERVISOR	**Dr Annelize Jonker**

APPROVED	Date **15 June 2018**
CHAIRMAN: UP Research Ethics Committee	Signature