Characterization and resistance profiles of selected enteric bacteria isolated from non-human primates at a wildlife-human interface

BY

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13404394

A research report submitted in partial fulfilment of the requirements for the degree of Magister Scientiae (Veterinary Science)

November, 2014

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Preface

Declaration by Student

I, **BARBARA AKORFA GLOVER** declare that this dissertation is my own work, carried out originally under the supervision of Prof Moritz van Vuuren and co-supervision of Dr Akinbowale Jenkins of the University of Pretoria and is in accordance with the requirements of the University for the degree of Magister Scientiae (Veterinary Science).

24/02/15

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Date Signature

Acknowledgements

My sincerest gratitude to the following:

- My supervisor, Prof Moritz van Vuuren and co-supervisor Dr Akinbowale Jenkins, for their assistance during the study
- To GOD almighty for the strength and grace to finish this research
- All the laboratories that provided training and isolates for testing: Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria; Institut de Génétique et Microbiologie (France) and the Pasteur Institute, France
- Prof Christophe Sola, Institut de Génétique et Microbiologie, France
- Laboratory training in France:

  Ms Lizânia Spinassé
  Ms Nayanne Dantas
  Ms Bernice Klotoe

- Laboratory training in South Africa

  Anna-Mari Bosman
  Ayesha Hassim
  Choopa Chimvwele
  Jeanette Wentzel

- Funders:

  1. National Research Foundation of South Africa (NRF)
  2. South Africa National Parks-University of Pretoria (SanParks-UP) bursary

3. The University of Pretoria (UP) Postgraduate International Study Abroad bursary
4. The Department of Veterinary Tropical Diseases (DVTD)

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CHAPTER 1

Conventional and molecular detection of antimicrobial resistance in selected bacteria isolated from non-human primates at a wildlife-human interface

Abstract

The direct and repeated exposure to antimicrobials in domestic animals and humans has been shown to produce strong selective pressures for the maintenance of antimicrobial resistance in their enteric bacteria. Though wild animal populations are not directly exposed to antimicrobials, antimicrobial resistance has been reported in some wild animal populations. Proximity to humans has been reported to be associated with a higher prevalence of antimicrobial resistance in wild animal populations.

In this study faecal samples were screened for indicator and zoonotic bacterial organisms from primate populations (baboons and vervet monkeys) located at two primate rehabilitation centres close to the Kruger National Park, South Africa. For comparison purposes, samples were obtained from non-human primates with 3 distinct levels of exposure to humans. The 1st group had regular human contact and consisted of mostly orphaned juveniles that were fed, bathed and cuddled regularly. The 2nd group had been weaned from frequent contact with humans, but had occasional contact. The 3rd group lived in enclosures similar to their natural habitats with almost no human contact. Antimicrobial susceptibility testing was carried out on all isolates using the Sensititre GPALL1F and GNX2F MIC plates (Trekds, UK). The Gram- positive isolates were tested against 22 antimicrobial drugs while the other Gram-negative bacteria were tested against 21 antimicrobial drugs all in dosages used by humans.

In all, 300 fresh faecal samples were collected. Sixty enterococci, 64 *Klebsiella* spp., and 64 *Escherichia coli*/*E. fergusonii* strains were isolated from the baboon samples. From the vervet faecal samples 40 enterococci, 64 *Klebsiella* spp. and 64 *E. coli*/*E. fergusonii* strains were isolated. Over eighty percent of the Gram-negative isolates were resistant to polymyxin B and E (colistin) as well as the carbapenems with occasional resistance recorded against aminoglycosides. Eighty percent of the Gram- positives (all enterococci) were resistant to aminoglycosides, carbapenems and fluoroquinolones. More than 90% of all the Gram-negative isolates were susceptible to penicillins and β-lactamase inhibitor combinations with the exception of the *Salmonella* spp. in which about 50% of the isolates were resistant.

The highest level of antimicrobial drug resistance in the non-human primates was observed in group 1. Comparatively speaking, group 1 had 20% and 23% higher levels of resistance than group 2 and group 3 respectively. This was observed for all the antimicrobials tested, especially polymyxin B and colistin. It was also observed that antimicrobial resistance levels decreased with age; with isolates from older animals being less resistant. This resistance profile was similar in all 3 groups in both rehabilitation centers.

Establishing the presence of microbial exchange between human populations and wildlife (especially primates), even in the absence of immediate disease concerns, may identify points of contact where increased surveillance may be justified because of higher risks of novel disease emergence.

Introduction

Written records of the transmission of diseases between species dates back to early studies on animal health. (Wolfe *et al.,* 1998). There are examples of disease transmission between species for diseases such as anthrax and tuberculosis either directly or via the environment. There have also been reports of the role of multiple wild and domestic hosts in the epidemiology of diseases such as influenza (birds-mammals), rabies (mammal – mammal) and foot-and-mouth disease (buffalo – impala – kudu - cattle) (Kock, 2004).

Population explosions of both human and domestic animals has resulted in increased interactions with nature, thereby resulting in an apparent increase in so-called emerging or novel diseases. With the majority of emerging infectious diseases in humans traced to wildlife reservoirs (Jones & Patel, 2008), investigations into the zoonotic pathogens are increasingly becoming key to understanding the disease transmission dynamics of emerging pathogens.

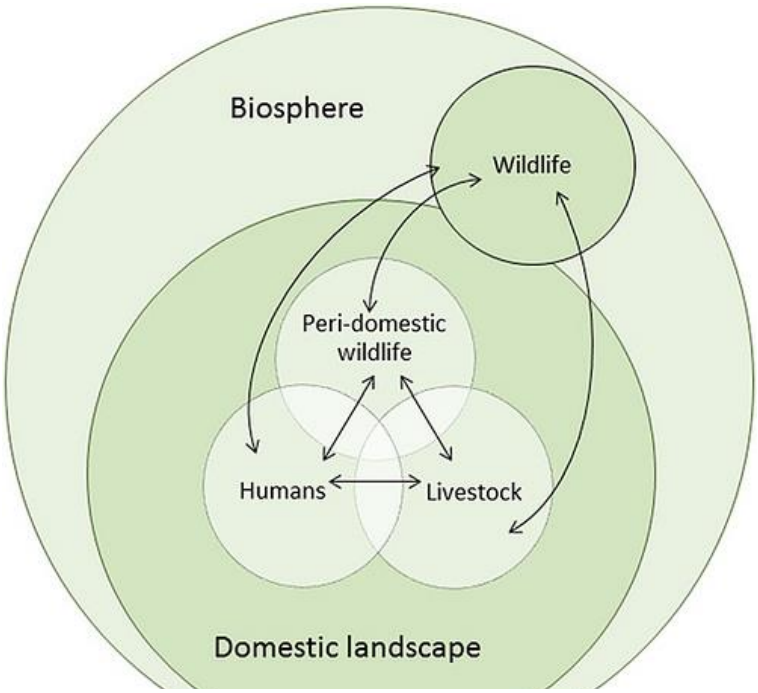


Figure 1: The Wildlife – Livestock – Human Interface (Grace et al., 2013)

Disease pandemics such as swine flu (Neumann *et al*. 2009), severe acute respiratory syndrome (SARS) (Dye and Gay 2003) and the recently re-emerging Ebola virus infection call more attention to the obvious – zoonotic diseases are an increasing threat to humans worldwide, crossing cultural and socioeconomic boundaries both in the developed and developing worlds (Mayer, 2000).

However, detecting inter-organism contacts that pose considerable risks for the transmission of pathogens requires the identification of dependable measures of microbial exchange. Infectious

diseases mostly have no geographical and species boundaries and similarities in pathogen susceptibility between primates in infections like HIV and babesiosis make non-human primates ideal laboratory models for infectious disease research.

In "recognizing the continued emergence of new bacterial pathogens in animals that are resistant to antimicrobials considered critically important for human therapy, there is good reason to further strengthen global efforts to prevent and control the emergence and spread of resistance from animals to humans" and vice versa (Radhouani *et al.*, 2014).

This research project focused on the 'proximity to humans' as drivers of antimicrobial drug resistance (ADR) transfer from human bacteria to those of non-human primates. This information may be important for the management of the wildlife-human interface, human health and the environment in which we live.

Results from this research may identify points of contact between wildlife and human populations, meriting increased surveillance, because of higher risks of disease emergence. This knowledge could also buttress the fact that wildlife are becoming major reservoirs of ADR genes - a threat to the fight against ADR bacteria found in humans and livestock.

Monitoring the incidence of resistance of indicator bacteria such as *E. coli* and *Enterococcus* spp. in wild animals makes it possible to show that wildlife have the potential to serve as an environmental reservoir and 'melting pot 'of bacterial resistance (Radhouani *et al.*, 2014).

Literature review

Enteric bacteria

The term "enteric bacteria" is generally used in reference to organisms of the Family *Enterobacteriaceae*, many members of which occur in the intestinal tract of humans and animals in health and disease. Examples of enteric bacteria relevant to this research are reviewed below.

Escherichia coli is a facultatively anaerobic organism in the large intestine, and its presence in water, food or anywhere else indicates faecal contamination and the possibility of associated

intestinal pathogens (an indicator bacterium). *E. coli* may cause diseases of the urinary tract, and certain strains cause severe intestinal disease (Mahon *et al.*, 2007).

Enterococcus faecium is a Gram-positive, alpha-haemolytic or non-haemolytic bacterium in the genus Enterococcus. It can be commensal (innocuous, coexisting organism) in the human intestine, but it may also be pathogenic, causing diseases such as neonatal meningitis (Ryan & Ray (Eds), 2004).

Enterococcus faecalis is a Gram-positive commensal bacterium inhabiting the gastrointestinal tracts of humans and other mammals. It can cause life-threatening infections in humans, especially in the nosocomial (hospital) environment, where the naturally high levels of ADR found in *E. faecalis* contributes to its pathogenicity (Ryan & Ray (Eds), 2004).

Klebsiella, on the other hand is a non-motile, Gram-negative, oxidase-negative, rod-shaped bacterium with a prominent polysaccharide-based capsule (Ryan & Ray (Eds), 2004). They are known to be ubiquitous in nature (Bagley, 1985). *Klebsiella* species are characteristically found in the gastrointestinal tract as normal flora; however, they can also behave as opportunistic human and animal pathogens (Ristuccia & Cunha, 1984).

Salmonella spp. are facultative, intracellular parasites that invade the mucous membranes of the human gut, and are transmitted to humans mainly through water, meat and poultry products (D'Aoust, 1991; Hunter, 1997). *Salmonella* infection is the most frequent food-borne gastrointestinal disease transmitted from animals to humans. The genus *Salmonella* is comprised of two species *S. enterica* and *S. bongori*. Salmonellae are Gram-negative, motile (with a few exceptions), facultatively anaerobic bacteria (D'Aoust, 1997). With the exception of a limited number of human-host-adapted serotypes (also referred to as the typhoidal salmonellae), the members of the genus *Salmonella* are regarded as zoonotic or potentially zoonotic (Acha and Szyfres, 2001).

Antimicrobial Drug Resistance (ADR)

The unwanted effects of bacterial (including enteric bacteria) growth have been controlled using antimicrobials. Antimicrobials are mostly chemicals produced by microbes, which are capable of destroying or inhibiting the growth of another microbe (Taylor *et al.*, 2004). The microbes that produce antimicrobials are mostly bacteria, but a few fungi also produce them. There are two types of antimicrobials: bacteriostatic and bactericidal (Taylor *et al.*, 2004). Bacteriostatic agents inhibit the growth and multiplication of susceptible microbes whilst bactericidal agents kill microbes.

Resistance is a description of the relative insusceptibility of a microorganism to a particular treatment under a particular set of conditions (Gilbert & McBain, 2003). A major problem that is increasing is that many bacteria have been found to show resistance to antimicrobials. It is even recorded, that not long after he discovered penicillin, Nobel laureate Alexander Flemming identified staphylococci that were resistant to this first 'wonder drug' (Okeke & Sosa, 2003). There are two general categories of ADR traits displayed by microorganisms:

(i) Those that allow microorganisms to withstand relatively high levels of a specific antimicrobial agent, which are conferred by mutations in genes responsible for antimicrobial uptake or binding sites, as well as those gained by acquisition of genes on mobile elements (Davies, 1994; Davies, 1996; Mazel, 2000); and

(ii) Those provided by genes conferring non-specific low-level resistance to multiple antimicrobials, such as the multiple antibiotic resistance (*mar*) locus (Aleksun and Levy, 1983; George and Levy, 1983; Hächler, *et al* 1991).Bacterial strains that are resistant to an antimicrobial drug can produce enzymes that inactivate the drug. Okeke & Sosa, (2003) reported that bacteria acquire the ability to be resistant to antimicrobial drugs through gene mutation or horizontally acquired resistance genes often carried on transmissible plasmids from one cell to another by conjugation. Some ADR genes are held within mobile elements called transposons or integrons and these elements not only capture and organize the expression of resistance genes but are also capable of moving from plasmids to the chromosome, a feature that stabilizes their inheritance (Okeke & Sosa, 2003; Mazel, 2000).

Antimicrobial drug resistance (ADR) develops principally because of exposure to antimicrobials in the enteric bacteria of humans and domestic animals, (Sayah *et al*., 2005; Skurnik *et al*., 2006; Witte, 1998). Colonization or infection by a resistant bacterial strain can indirectly occur as a result of:

1. The treatment of bacterial infections with antimicrobials,
2. The uncontrolled consumption of antimicrobials or antimicrobial residues,
3. The transfer of resistance genes through direct contact with resistant bacteria or
4. The consumption of food and water contaminated with a resistant bacterial strain (Blanco *et al*., 2009; Sayah *et al*., 2005).

There is evidence of the existence of antimicrobial resistant bacteria before the worldwide use of antimicrobials though it was reportedly in low levels (Caldwell & Lindberg, eds. 2011). However, the spread of resistant strains is partly attributed to evolutionary pressures from the use of antimicrobials (Nelson, 2009). Naturally occurring antimicrobial resistance is common and the genes that confer this resistance are known as the environmental resistome (Nelson, 2009). Unfortunately, these resistomes may be transferred from non-pathogenic bacterial strains to pathogenic bacterial strains leading to clinically significant antimicrobial resistance (Wright, 2010)

Antimicrobial drug resistance in humans

Bacteria cause a significant proportion of infections in humans in Africa (Okeke & Sosa, 2003) and other parts of the world and the development of antimicrobial drugs provided relief to human healthcare systems. Current data however suggests that, in a remarkably short time, resistance to antimicrobial drugs has weakened the ardent hope that bacterial infections would cease to be an important cause of death and disease in humans. The WHO 2012 fact sheet on ADR reported that:

- About 440 000 new cases of multidrug-resistant tuberculosis (MDR-TB) emerge annually, causing at least 150 000 deaths.
- Resistance to earlier generation antimalarial medicines such as chloroquine and sulphadoxine-pyrimethamine is widespread in most malaria endemic countries.
- Resistance is an emerging concern for treatment of HIV infection, following the rapid expansion in access to antiretroviral medicines in recent years
- A high percentage of hospital-acquired infections are caused by highly resistant bacteria such as methicillin-resistant *Staphylococcus aureus* (MRSA).

  and

- Inappropriate and irrational use of antimicrobial medicines provides favourable conditions for resistant microorganisms to emerge, spread and persist (WHO, 2012).

Current resistance data also points to major risks in healthcare from resistant bacteria by increasingly compromising the outcome of many infections that were, until recently, treatable. It has been documented that:

1. ADR has become a serious problem for treatment of gonorrhoea (caused by *Neisseria gonorrhoeae*), involving even "last-line" oral cephalosporins (WHO, 2012) due to the widespread distribution of penicillinase-producing *Neisseria gonorrhea*, (van Dyck *et al*., 2001).
2. The prevalence of gonococcal resistance to affordable alternatives - such as tetracyclines, thiamphenicol and spectinomycin - continues to rise and resistance to fluoroquinolones has emerged (Van Dyck *et al*., 2001).

3. Resistance to earlier generation antimalarial medicines such as chloroquine and sulphadoxine-pyrimethamine is widespread in most malaria- endemic countries. Falciparum malaria parasites resistant to artemisinins are emerging in South-East Asia and infections show delayed clearance after the start of treatment – indicating resistance (Okeke & Sosa, 2003; WHO, 2012).

Antimicrobial drug resistance in animals

Antimicrobial drugs are used as growth promoters in animals that serve as human food via animal feeds. Traces of these drugs, however, can be found in the meat, milk, and eggs produced from those animals (Schneider & Garrett, 2009) and these could in turn build up resistance in the enteric bacteria of humans when consumed regularly. It has also been reported that, persistent use of these antimicrobial drug-laced feeds gives rise to multi-drug resistant bacteria often labelled as superbugs with reports of possible transmission of these superbugs to humans via the food chain. The World Health Organization recommends that antimicrobial drugs as growth promoters in animal feeds should be prohibited, in the absence of risk assessments.

Some commercial farmers use sub-therapeutic levels of antimicrobial drugs in feed as growth promoters. Some farmers further abuse these drugs by applying them in therapeutic levels as means of eradicating disease in farm animals. The Union of Concerned Scientists (UCS), in a research report, estimated that more than 70% of the antimicrobial drugs used in the US are given to food animals (for example, chickens, pigs and cattle), in the absence of disease. This places the levels of antimicrobial drugs present in the ‘treated’ animals at "sub-therapeutic" levels – insufficient to combat disease in the absence of no demonstrable disease (Union of Concerned Scientists, 2001). The sub-therapeutic levels however have “been shown to select for ADR in both commensal and pathogenic bacteria in

a) the animals themselves;

b) subsequent animal-based food products; and

c) Water, air, and soil samples collected around large-scale animal feeding operations.” (Sapkota *et al.*, 2007)

A study designed to assess the extent of antimicrobial drug use in small and large commercial poultry producers in Ghana, West Africa, evaluated responses from 483 poultry farmers in the Greater Accra, Ashanti and Central regions of Ghana (Turkson, 2008). The tetracyclines formed the largest class of antimicrobial drugs used by the poultry producers on their birds (35.7%, n=831), followed by the nitrofurans (23.1%), penicillin-streptomycin combinations (18%), and sulphonamides and sulphonamide combinations (8.3%). Interviews with the farmers however indicated that the animals were fed with feed laced with antimicrobial drugs in the absence of sickness and some farmers were ignorant of the use of these antimicrobial drugs – other than to promote growth.

Wild animals are not expected to be exposed directly to antimicrobials, and the source of ADR in the bacteria of wild animals is not clear. However, resistant bacteria have been found at high prevalence in the intestinal bacteria of wild rodents living in proximity to livestock with Literak *et al*., (2009) reporting 20% and Kozak *et al*., (2009) reporting 54% resistance. Antimicrobial resistant *Escherichia coli* and Enterococcus spp. isolates obtained from wildlife were first reported in Japanese wild birds (Sato *et al*., 1978).

Among *Escherichia coli* isolates recovered from the faeces of wild Canadian geese (*Branta canadensis*) and choughs (*Pyrrhocorax pyrrhocorax*) that were feeding and/or living in close proximity to livestock waste, 72% (Blanco *et al*., 2009) and 21%(Cole *et al*., 2005) respectively were resistant to amoxicillin-clavulanic acid.

The origins of ADR in wildlife is important to human health because of the increasing importance of zoonotic diseases as well as the need for predicting emerging resistant pathogens.

ADR transfer between organisms at the wildlife-human-livestock interface

The possible transfer of multiple drug-resistant *Salmonella enterica* serovar Typhimurium bacterial strains from calves to humans was reported by Anderson & Lewis (1965) after earlier similar descriptions in 1961 (Watanabe and Fukasawa, 1961; Anderson & Datta, 1965) – heightening concerns on the dangers and implications of drug resistance development in farm animals. Chee-Sanford *et al.*, 2001 reported that up to 75% of tetracycline administered to swine was excreted unaltered. The expelled drugs can persist in the environment, creating an

opportunity for resistance selection within exposed bacterial populations (Rosenblatt-Farrell, 2009).

Furthermore, the possibilities of ADR transfer between animals was also studied by Graham *et al.*, (2009) on flies collected from the areas surrounding a poultry production facility. Antimicrobial drug resistant bacteria isolated from the flies had resistance patterns consistent with antimicrobial drugs used in the poultry production units; thereby indicating that these flies probably acquired these resistant bacteria through the faecal samples of the poultry in the facility. Furthermore, it is suggested that migratory birds are responsible for the unexpectedly high presence of drug-resistant *Escherichia coli* in Arctic wildlife in studies by Sjöland *et al*., (2008).

High genetic correlations were observed in repetitive sequence-based polymerase chain reaction (rep-PCR) fingerprinting and multilocus sequence typing polymerase chain reaction (MLST-PCR) profiles of antimicrobial drug resistant *E. coli* populations in the gut flora of the banded mongoose (*Mungos mungo*) and humans in northern Botswana recently (Pesapane *et al.*, 2013). These banded mongooses feed on human faeces in the study area and indiscriminate human waste disposal was identified as a contributing factor to the transfer of the resistance genes.

Proximity to humans has also been associated with higher prevalence of ADR in wildlife. For example, resistant bacteria were detected more frequently in baboons feeding on human refuse than in animals living in more remote areas with no human contact (Rolland *et al*., 1985).

On the Galápagos islands, resistant enteric bacteria (*E. coli* and *Salmonella*) in Galápagos wildlife were investigated and reported in 2012 by Wheeler *et al.* Reptile faeces from tourism sites had antimicrobial resistant enteric bacteria whilst enteric bacteria of reptiles living on protected beaches on more isolated islands did not show resistance (Wheeler *et al.*, 2012).

Measuring drug resistance using phenotypic characteristics

The conventional method used in detecting ADR or susceptibility in bacteria is the Kirby-Bauer disc diffusion method. It is a relatively flexible and inexpensive technique used to test rapidly growing aerobic bacteria. Specified amounts of antimicrobial agents are infused into filter paper discs, which are then placed on agar plates uniformly inoculated with test bacteria. The diameters of the zones of inhibition (in millimetres) are then measured and compared with standards for zone size interpretations.

Also, the determination of the minimum inhibitory concentration (MIC) of antimicrobials for bacterial strains are carried out using broth microdilution tests. In this test, 96-well microtitre plates are used and the antimicrobial drugs are serially diluted to the lowest concentration of antimicrobial drug to be tested. Equal volumes of test organisms in broth are then inoculated into the wells and incubated under standard growth conditions – usually for 18-24 hours. The lowest drug concentration that inhibits bacterial growth is the MIC. Comparisons of the recorded MIC with standard MIC breakpoints for a particular organism aids in interpreting the antimicrobial susceptibility or resistance of the test bacteria

Measuring drug resistance using genotypic characteristics

Integrons and antimicrobial drug resistance genes

With advancements in technology newer methods to analyze drug resistance have been developed. The detection of ADR by molecular methods requires the identification of genes that code for ADR in microbiomes.

Integrons are described as highly efficient tools used by bacteria for ADR acquisition and expression (Skurnik *et al*.,2006). Mazel (2006) reported that multi-antimicrobial drug resistance was known to be associated with transmissible plasmids by the 1970s and the role of integrons in the acquisition of multi-antimicrobial drug resistance in bacteria came to the fore in the late 1980s. Mazel further explained that integrons are assembly platforms that incorporate exogenous

open reading frames (ORFs) by site-specific recombination and convert them to functional genes by ensuring their correct expression.

There are two main types of integrons namely the Mobile integrons (MI) and the Chromosomal integrons (CI). MIs correspond to functional platforms that are physically associated with transposons that can be carried by conjugative plasmids whilst CIs possess a specific integrase similar to integrases in MI but is not mobile (Cambrey *et al*., 2010) For the purposes of this research, much focus would be on the mobile integrons.

The sequences of the encoded integrases of the MIs groups them into 5 classes of integrons and all five classes have been associated with antibiotic-resistance determinants (Cambrey *et al*., 2010). This research focuses on the first three classes due to their historical involvement in the spread of multiresistance phenotypes.

Class 1 integrons are detected in 22%-59% of Gram-negative clinical isolates (Labbate *et al.*, 2009) with occasional detections in Gram-positive isolates (Martin *et al.*, 1990; Nandi *et al.*, 2004) – making them, comparatively, the most widely spread and clinically important of all the classes of integrons. Class 2 (Biskri & Mazel *et al.*, 2003) and 3 (Arakawa *et al.*, 1995) integrons are located in transposoons (Collis *et al.*, 2002) with the latter being less prevalent and the former being mostly associated with Tn7 derivatives.

A) The integron/gene cassette site-specific recombination system (Chowdhury *et al.*, 2013).

The integron platform consists of the *intI* gene, attI site and Pc promoter (Figure 2). The gene cassette usually consists of a single promoterless ORF and a recombination site called attC. In its free form, the gene cassette is circularized and is recombined into the integron by an integrase-mediated reaction between attI and attC (or attC and attC in lower frequencies).

B) The genetic structure of a typical clinically derived class 1 integron.

The 5′-conserved segment (CS) contains the core integron consisting of IRi, *intI*1, attI and Pc. The variable region (VR) is where gene cassettes are inserted. The 3′-CS minimally consists of qacED and sulI. However, sometimes ORF5 and ORF6 make up part of it. Remnants of the Tn402 transposition module (tni) and IRt can sometimes be found downstream of the 3′-CS. (Chowdhury *et al.*, 2013)

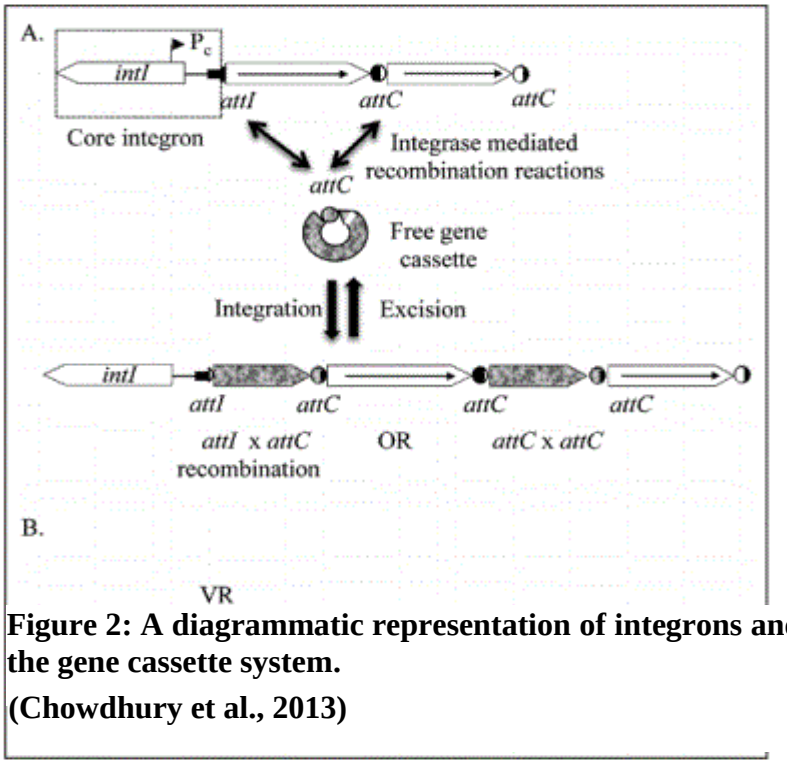


Figure 2: A diagrammatic representation of integrons and the gene cassette system.
(Chowdhury et al., 2013)

There has been extensive studies particularly on multidrug resistant *E. coli* isolates from human faeces and water and most have been identified to carry class 1 integrons and conjugative plasmids (Asem *et al.*, 2011; Al-Dweik & Shehabi, 2009; Shehabi *et al.*, 2006). Almost all of the integrons characterised to date contain gene cassettes coding for resistance to antimicrobial agents (Maguire *et al.*, 2000) and thus are useful tools in determining ADR in bacteria. There have been various research reports on the use of PCR assays in detecting integrons in bacteria,

including conventional PCR techniques (Seward *et al.*, 1999) and real-time PCR assays (Maguire *et al*., 2000).

There is the increasing problem of resistance to a wide spectrum of antimicrobial agents in an increasing number of bacterial isolates that still needs to be addressed (Maguire *et al.*, 2000), both in the hospital and in community settings. Studies on integrons have shed more light on the dissemination of ADR genes between organisms and patients and it is consequently important to screen for integrons in bacterial isolates.

Sulphonamides are structural analogues and competitive antagonists of *para*-aminobenzoic acid (PABA). They inhibit normal bacterial utilization of PABA for the synthesis of folic acid, an important metabolite in DNA synthesis (Lipman, 2008). This inhibition ability gives them a biostatic feature that is utilized in the production of antimicrobial drugs. Bacterial resistance to sulphamethoxazole (a type of sulphonamide) is caused by mutations in the enzymes involved in folic acid synthesis that prevent the drug from binding to it. Bacterial resistance to sulphonamides occurs through mutations in the chromosomal dihydropteroate synthase (DHPS) gene (folP) or through acquisition of an alternative DHPS gene (sul), whose product has a low affinity for sulphonamides (Petersen and Dalsgaard, 2003). Of the two pathways, sul genes are the most prevalent mechanism of sulphonamide resistance (Enne *et al*., 2002; Petersen and Dalsgaard, 2003).

Tetracyclines are defined as “a subclass of polyketides having an octahydrotetracene-2-carboxamide skeleton" (McNaught & Wilkinson, 2006). They are used in the treatment of infections of the urinary tract, respiratory tract, and the intestines and are also used for the treatment of *Chlamydophila* infections, especially in patients allergic to β-lactams and macrolides. Resistance to the tetracyclines results from changes in permeability of the microbial cell wall of target bacteria. In susceptible organisms, the drug is concentrated in the cell and does not readily leave the cells. In resistant organisms, the drug is not actively transported into the cells or leaves it so rapidly that inhibitory concentrations are not maintained. This is often plasmid-controlled (Connel *et al*, 2003).

The tetracycline resistance determinant in transposon Tn10 consists of two genes, the *tetA* resistance gene and the *tetR* repressor gene, that are transcribed from divergent overlapping

promoters (Daniels *et al.*, 1985). Thus, detecting the presence of these genes in bacteria aids in determining possible resistance to tetracycline and minocycline in the bacteria.

Tetracycline resistance genes: *tetA* and *tetB* as well as sulphonamide resistance genes *sul1* and *sul2* all aid in determining resistance to the above-mentioned classes of drugs and identifying the presence of these genes by molecular methods aids in the rapid detection of drug resistance.

Aims and objectives of this study

Aims

1. To detect resistance to antimicrobial drugs in bacteria isolated from non-human primates in close association with humans.
2. To identify points of wildlife-human contact that contribute to ADR

Objectives

1. To isolate and identify strains of *Escherichia coli*, *Salmonella* serovars, *Klebsiella sp.*, *Enterococcus faecalis* and *Enterococcus faecium*.
2. To test these isolates for resistance to selected antimicrobial drugs.
3. To identify ADR plasmids in bacteria isolated from non-human primates by targeting integrons whose gene cassettes may encode genes for ADR

Materials and methods

Study location

Baboon and vervet monkey populations of two wildlife rehabilitation centres located within 10 km of each other and approximately 30 km from the Kruger National Park were used for this study. These centres were established to accommodate and provide temporary sanctuary for wildlife casualties, rehabilitate them to a point of self-support and the ultimate release and introduction into nature where they naturally belong. They rehabilitate and provide sanctuary to over 500 orphaned, injured, abused, ex-laboratory or unwanted pets (usually primates) and rehabilitation could take up to 3 years. In both centres, the non-human primates were classified into three groups and this determined the sampling strategies. The first group had regular human contact and were mostly orphaned juveniles that were fed, bathed and cuddled regularly (Figure 3). The second group of non-human primates had been weaned off frequent contact with humans with occasional contact when it was time to feed them. There was no cuddling or bathing of the animals in this group and they only came in

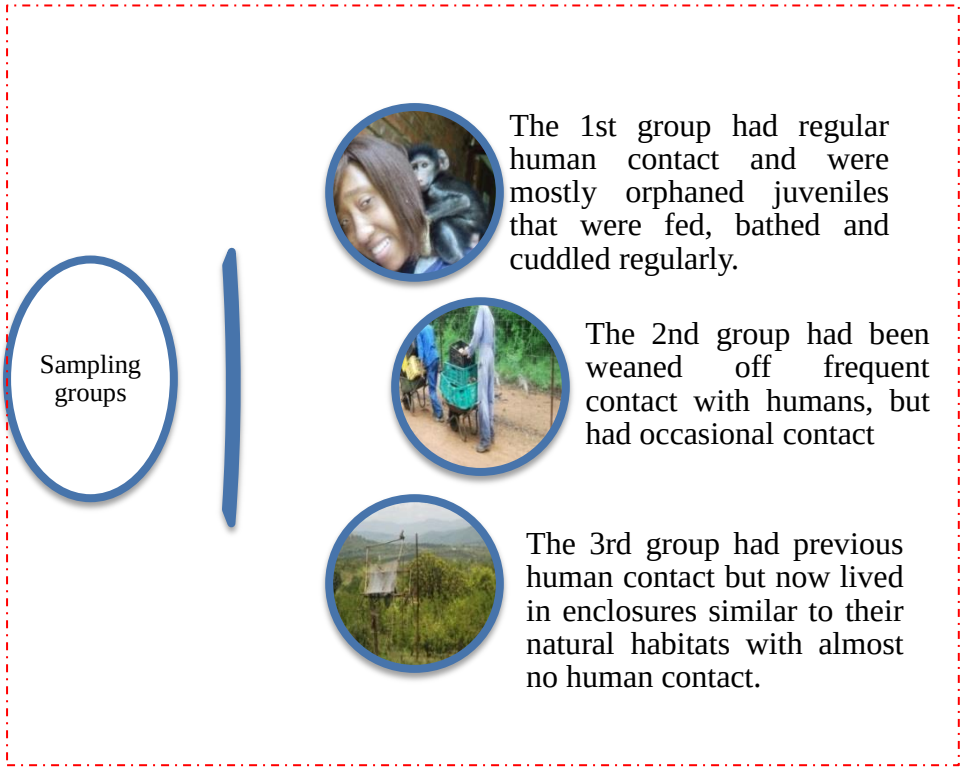


Figure 3: An illustrated view of the sampling groups with descriptions

contact with staff/volunteers during feeding lasting a maximum of 10 minutes, 3 times daily. The third group had previous human contact and lived in enclosures similar to their natural habitats with almost no human contact. They fed off natural shoots in their enclosures and food was only supplemented in harsh weather conditions when plants they fed on were not available

Sample collection

There were three major groups defined per rehabilitation centre for sampling purposes in line with the natural groups in the centres. Three hundred fresh faecal samples were obtained from vervets and baboons in the sampling groups using sterile swabs immersed in Stuart's transport medium (Onderstepoort Veterinary Hospital Pharmacy, South Africa). One-hundred-and-fifty (150) baboon faecal samples and fifty faecal samples from vervets were collected at one wildlife rehabilitation centre whilst another 100 faecal samples from vervets were collected from the second wildlife rehabilitation centre.

Bacterial isolation

For the isolation of Gram-negative bacteria, the 300 faecal samples were inoculated on MacConkey agar (Selecta Media, South Africa) under aseptic conditions and incubated for 24 hours at 37 °C in an O_2 incubator. Pink colonies on the MacConkey agar plates were then Gram-stained and their morphologies and phenotypic characteristics noted. Suspect *E. coli* and *E. coli*-like isolates as well as *Klebsiella isolates* were then inoculated on Eosin Methylene Blue (EMB) (Selecta Media, South Africa) agar. The faecal samples were also inoculated on *Salmonella-Shigella* agar (Selecta Media, South Africa) to isolate *Salmonella* serovars. Finally, the faecal samples were also inoculated on bile esculin agar (Selecta Media, South Africa) for selective isolation of Enterococcus sp.

Biochemical tests

Biochemical characterizations of all the Gram-negative isolates were done using the Remel ® RapID One panel strips (UK). Oxidase tests were done prior to tests on the panel strips to ensure only oxidase negative isolates were tested as per the manufacturer's instructions. This was followed by characterization of the isolates using the Remel RapID ONE kit.

In order to characterise the isolates on the RapID One panel strips, they were transferred into inoculation fluids and then onto wells imbibed with reagents and incubated at 37 ˚C for 4 hours. The biochemical profiles were then determined after incubation based on colour changes in the inoculated wells as indicated by the manufacturer.

Antimicrobial susceptibility tests

Antimicrobial susceptibility tests were carried out using the Sensititre GNX2F MIC and GNX2F (Trekds, UK) broth microdilution plates. Gram-positive isolates were tested against 22 antimicrobial drugs (Table 1) whilst the Gram-negative bacteria were tested against 21 antimicrobial drugs (Table 2) in serial dilutions to determine the minimum inhibitory concentrations (MICs). The plates were read using the UV lamp readers commonly used for reading PCR products on agarose gels instead of the conventional MIC plate readers.

Table 1: List of antimicrobial drugs used in the susceptibility tests, their MICs and Clinical and Laboratory Standards Institute CLSI susceptibility cut-offs for Gram-positive bacteria

Antimicrobials (Inoculum concentration: 1 x 10^5 cfu/ml, reconstitution volume: 50µl)	**Type of isolate tested**	**Abbreviation**	**MIC interpretative criteria (µg/mL)**		
			Susceptible	**Intermediate**	**Resistant**
Chloramphenicol	Gram-positive	CHL	≤ 8	16	≥32
Daptomycin	Gram-positive	DAP	≤4	-	-
Gentamicin	Gram-positive	GEN	≤4	8	≥16
Linezolid	Gram-positive	LZD	≤2	4	≥8
Rifampin	Gram-positive	RIF	≤1	2	≥4
Trimethoprim / sulfamethoxazole	Gram-positive	SXT	≤2/38	-	≥4/76

Quinupristin / dalfopristin	Gram-positive	SYN	≤1	2	≥4
Tetracycline	Gram-positive	TET	≤4	8	≥16
Erythromycin	Gram-positive	ERY	≤ 0.5	1-4	≥8
Oxacillin+2%NaCl	Gram-positive	OXA+	≤0.25	-	4
Ampicillin	Gram-positive	AMP	≤8		≥16
Penicillin	Gram-positive	PEN	≤8		≥16
Vancomycin	Gram-positive	VAN	≤2	4-8	≥16
Levofloxacin	Gram-positive	LEVO	≤2	4	≥8
Tigecycline	Gram-positive	TGC	≤0.03	-	≥0.12
Moxifloxacin	Gram-positive	MXF	≤0.06		≥0.5
Clindamycin	Gram-positive	CLI	≤0.5		>0.5
Streptomycin	Gram-positive	STR	≤8	16	≥32
Ciprofloxacin	Gram-positive	CIP	≤1	2	≥4
Nitrofurantoin	Gram-positive	NIT	≤32	64	≥128
D Test 1	Gram-positive	DT1	-	-	-
D Test 2	Gram-positive	DT2	-	-	-
Cefoxitin screen	Gram-positive	FOXS	≤4	-	≥8

Table 2: List of antimicrobial drugs used in the susceptibility tests, their MICs and CLSI susceptibility cut offs for Gram-negative bacteria

Antimicrobials (Inoculum concentration : 50µl, Reconstitution volume: 50µl)	Type of isolate tested	Abbreviation	MIC Interpretative Criteria (µg/mL)		
			Susceptible	Intermediate	Resistant
Amikacin	Gram-negative	AMI	≤16	32	≥64
Ticarcillin / clavulanic acid	Gram-negative	TIM2	≤16/2	32/2–64/2	≥128/2
Aztreonam	Gram-negative	AZT	≤4	8	≥16
Piperacillin / tazobactam	Gram-negative	P/T4	≤16/4	32/4–64/4	≥128/4
Trimethoprim / sulfamethoxazole	Gram-negative	SXT	≤ 2/38	-	≥4/76
Gentamicin	Gram-negative	GEN	≤4	8	≥16
Cefepime	Gram-negative	FEP	≤8	16	≥32
Tobramycin	Gram-negative	TOB	≤4	8	≥16
Levofloxacin	Gram-negative	LEVO	≤2	4	≥8
Doxycycline	Gram-negative	DOX	≤4	8	≥16
Ciprofloxacin	Gram-negative	CIP	≤1	2	≥4
Minocycline	Gram-negative	MIN	≤4	8	≥16
Meropenem	Gram-negative	MERO	≤1	2	≥4
Cefotaxime	Gram-negative	FOT	≤1	2	≥4
Tigecycline	Gram-negative	TGC	≤2	4	≥8

Ertapenem	Gram-negative	ETP	≤0.5	1	≥2
Imipenem	Gram-negative	IMI	≤1	2	≥4
Doripenem	Gram-negative	DOR	≤1	2	≥4
Colistin	Gram-negative	COL	≤2	2	≥2
Polymyxin B	Gram-negative	POL	≤2		≥4
Ceftazidime	Gram-negative	TAZ	≤		≥

Integron PCR

Detection of class 1, 2 and 3 integrons in multi-drug resistant bacterial isolates were accomplished with PCR and primer pairs targeting Integron 1 (*Int 1)*, Integron 2 (*Int 2)* and Integron 3 (*Int 3*) genes (Burjaq & Shehabi, 2013). Twenty (20) resistant bacterial strains representing all three samplings groups were randomly screened for the presence of integrons. Pure DNA extractions from the selected bacterial strains served as DNA templates. PCR reactions were performed in a final volume of 25µl containing 12.5µl Fermentas Taq (ThermoScientific, USA), 0.5µl of each primer (Table 3) and 2µl of purified DNA. The PCR conditions were: initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 1 min; primer annealing at 59°C for 1 min; extension 72°C for 1 min and final extension at 72°C for 7 min. Tubes were kept at 4°C when the cycles ended. The PCR product was analysed by performing 1.5 % agarose gel electrophoresis for 80 min at 90 V using a horizontal electrophoresis apparatus. The gels were visualized under UV light.

Detection of *tetA & B* and *sul1 & 2* genes by means of PCR

The same 20 bacterial strains screened for integrons were further screened for tetracycline resistance (*tet*) and sulphonamide resistance (*sul*) genes. PCR reactions were performed in a final volume of 25µl containing 2µl of extracted DNA, 12.5µl of Kapa Hotstart Readymix? (Kapa Biosystems, Cape Town, South Africa), 0.5µM of each primer (Table 3), 8.5µl of molecular grade water, and 2µl of bacterial DNA. Cycling conditions were 95^{o}C for 3 min, followed by 35 cycles of 98^{o}C for 20 sec, 65^{o}C for 30 sec, 72^{o}C for 30 sec, 72^{o}C for 60 sec and then a hold at 4^{o}C. The PCR product was analysed by performing agarose gel electrophoresis for 80 min at 90 V using horizontal electrophoresis apparatus. The gels were visualized under UV light.

Table 3: The specific primer sequences used in the detection of class 1, 2 and 3 integrons, *tetA* and *tetB* gene cassettes and *sul1* and *sul2* genes.

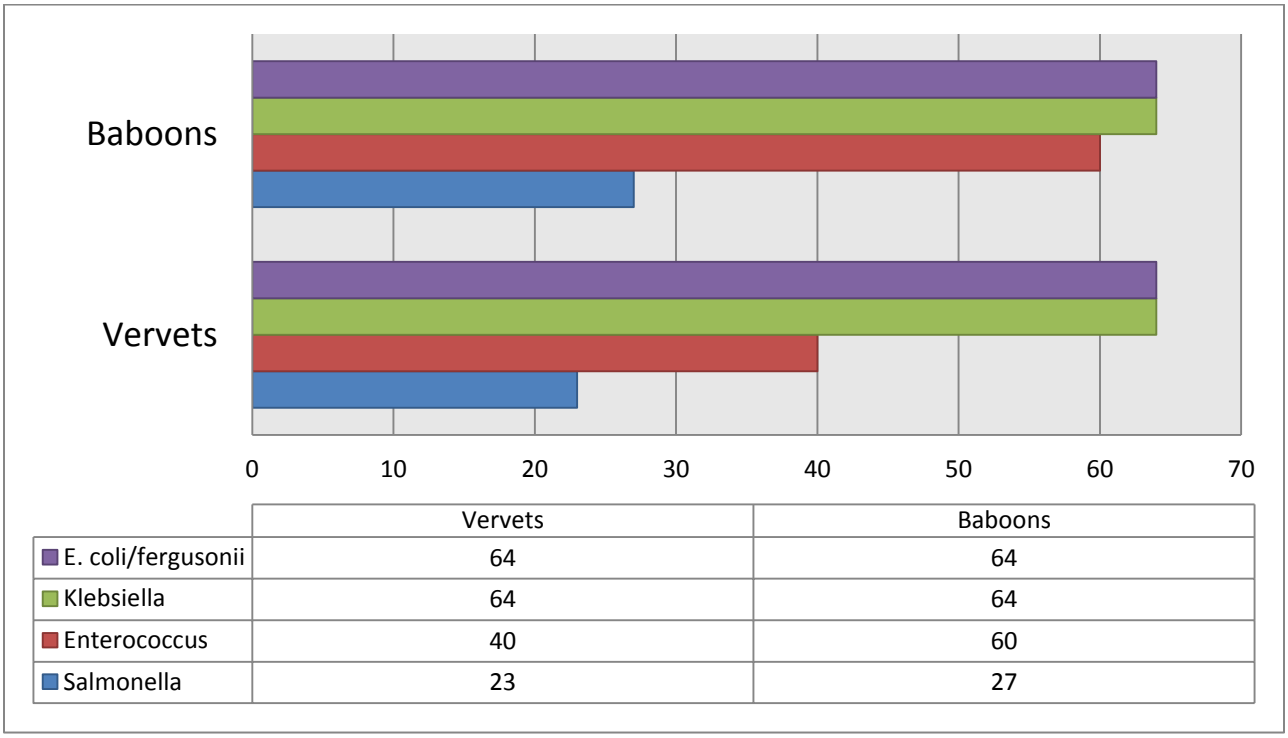
(Dillon *et al.*,, 2005; Sengeløv *et al.*, 2003 ; Leverstein-van Hall *et al.*, 2003)

Amplicon size (bp)	Primer sequence (5'-3')	Gene target
160	F 5' – CAGTGGACATAAGCCTGTTC-3' R 5'-CCCGAGGCATAGACTGTA-3'	Int1
788	F 5'- CACGGATATGCGACAAAAAGGT-3' R 5'- GTAGCAAACGAGTGACGAAATG -3'	Int2
977	F 5'- GCCTCCGGCAGCGACTTTCAG-3' R 5'-ACGGATCTGCCAAACCTGACT-3'	Int3
956	F 5'-GTAATTCTGAGCACTGTCGC-3' R 5'-CTGCCTGGACAACATTGCTT-3'	*tetA*
414	F 5'-CTCAGTATTCCAAGCCTTTG-3' R 5'-ACTCCCCTGAGCTTGAGGGG-3'	*tet*B
660	F 5'- GTGACGGTGTTCGGCATTCT-3' R 5'- TTTACAGGAAGGCCAACGGT-3'	*sul1*
393	F 5'- GGCAGATGTGATCGACCTCG-3' R 5'- ATGCCGGGATCAAGGACAAG-3'	*sul2*

Results

Bacterial Isolation

Escherichia coli isolates showed dark blue-black colonies with metallic green sheens on EMB agar – indicating vigorous fermentation of lactose and acid production, which precipitates the green metallic pigment. *Klebsiella* sp. isolates showed brown, dark-centred, mucoid colonies indicating lactose fermentation and acid production. Hydrogen sulphide positive *Salmonella* serovars produced black-centred colonies on *Salmonella-Shigella* agar for easy detection. Other *Salmonella* isolates were colourless.



	Vervets	Baboons
E. coli/fergusonii	64	64
Klebsiella	64	64
Enterococcus	40	60
Salmonella	23	27

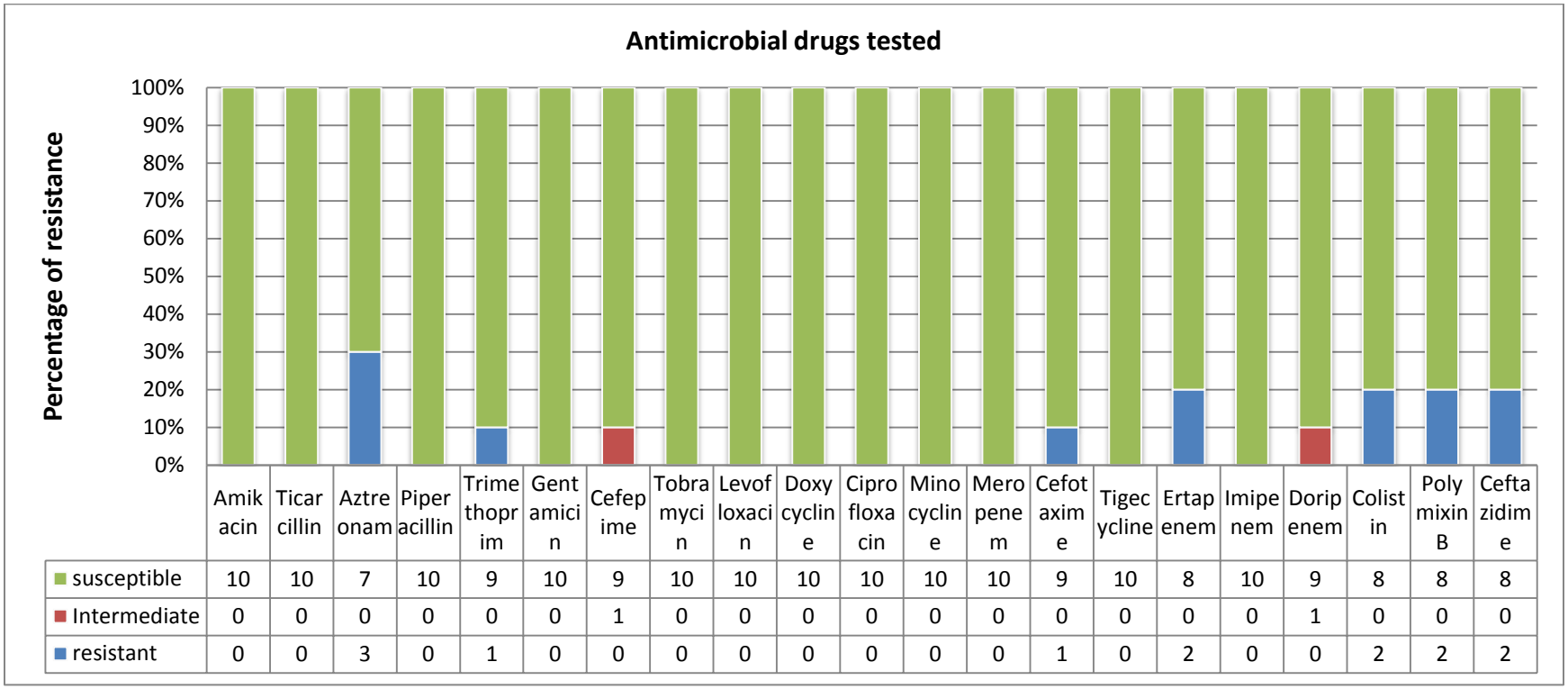
Figure 4: Graphical representation of isolated bacteria from all three sampling groups

In total, 406 bacterial strains were isolated from the 300 fresh faecal samples – belonging to four different genera of bacteria – namely *Escherichia*, Enterococcus, *Salmonella* and *Klebsiella* – and two different non-human primates. All the samples were from vervets and baboons. Fifty *Salmonella* strains were obtained from vervets and baboons with 23 isolated from vervets and 27

from baboons. Sixty-four *Klebsiella* strains were isolated from vervets and baboons whilst 64 *Escherichia* strains were isolated from vervets and baboons.

Antimicrobial susceptibility tests

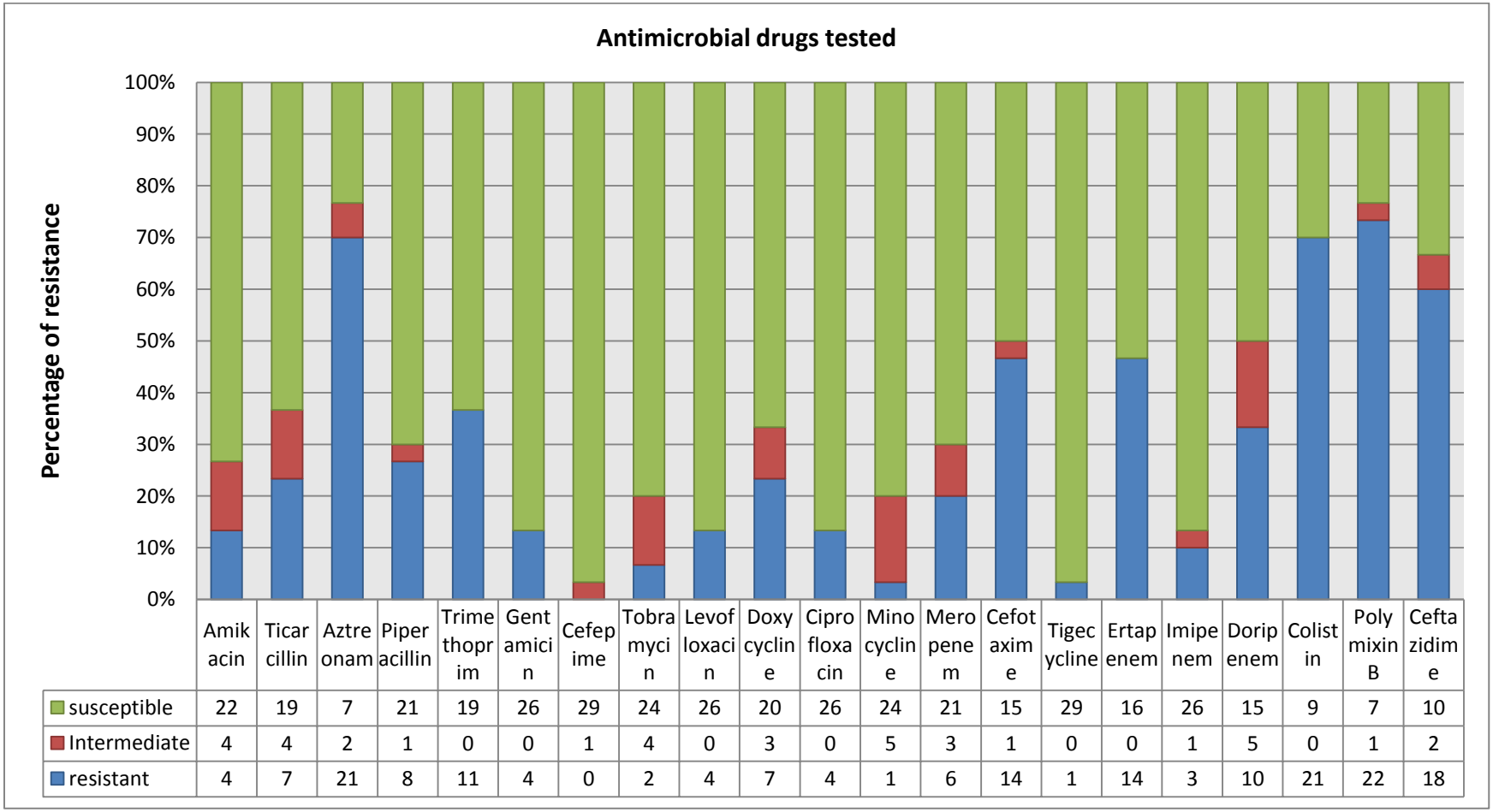
There were low levels of resistance recorded in all the ten randomly selected strains of *Klebsiella* tested (Figure 5). However, ≥20% resistance observed to aztreonam, ertapenem and the polymyxins.



	Amikacin	Ticarcillin	Aztreonam	Piperacillin	Trimethoprim	Gentamicin	Cefepime	Tobramycin	Levofloxacin	Doxycycline	Ciprofloxacin	Minocycline	Meropenem	Cefotaxime	Tigecycline	Ertapenem	Imipenem	Doripenem	Colistin	Polymixin B	Ceftazidime
susceptible	10	10	7	10	9	10	9	10	10	10	10	10	10	9	10	8	10	9	8	8	8
Intermediate	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0
resistant	0	0	3	0	1	0	0	0	0	0	0	0	0	1	0	2	0	0	2	2	2

Figure 5: The results of antimicrobial susceptibility tests of ten *Klebsiella* isolates

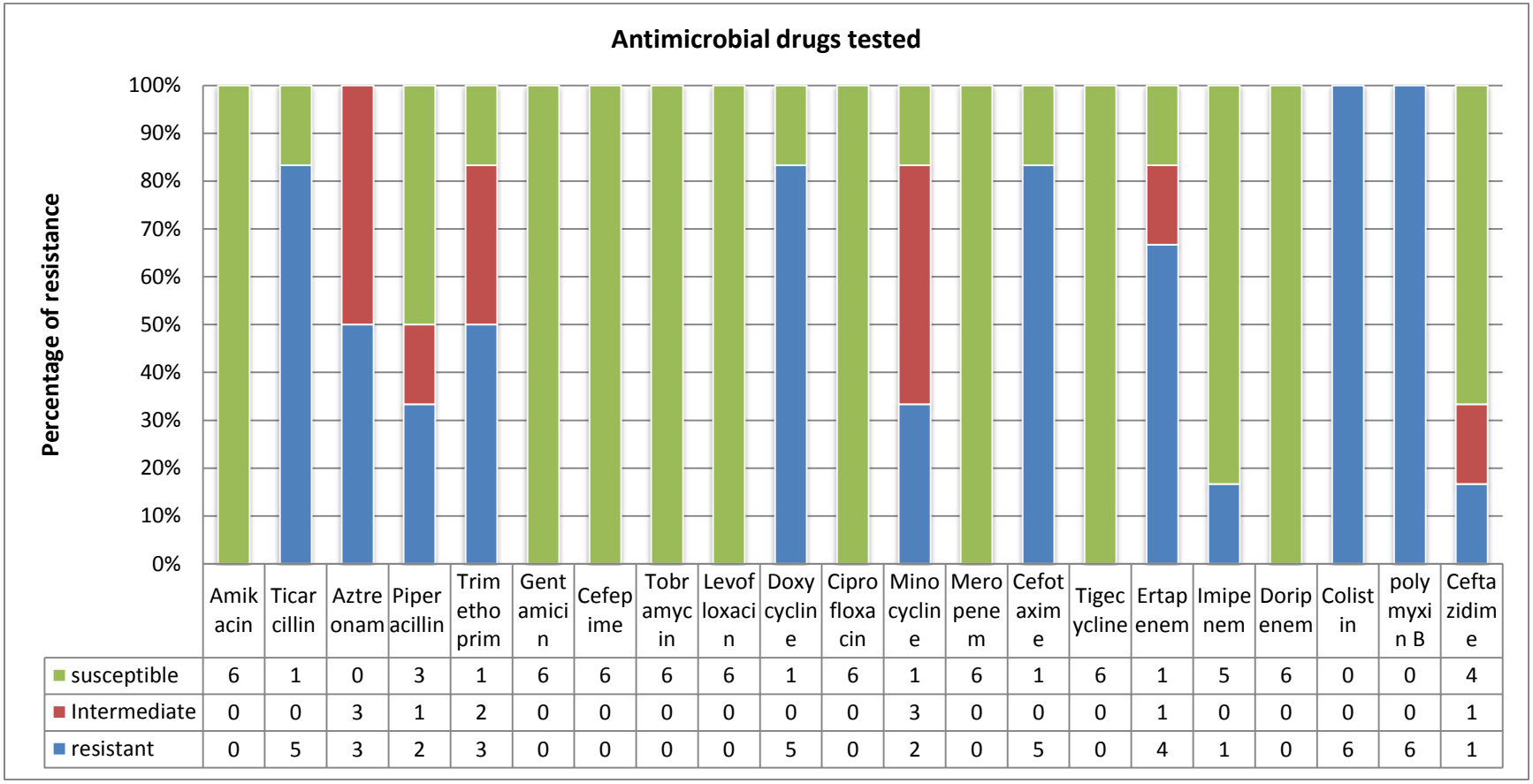
Resistance patterns in the *Escherichia* isolates were similar for most strains analysed (Figure 6). There was ≥70% resistance observed to the polymyxins B and C (colistin), aztreonam, cefatoxime, ertapenem as well as ceftazidime.



	Amikacin	Ticarcillin	Aztreonam	Piperacillin	Trimethoprim	Gentamicin	Cefepime	Tobramycin	Levofloxacin	Doxycycline	Ciprofloxacin	Minocycline	Meropenem	Cefotaxime	Tigecycline	Ertapenem	Imipenem	Doripenem	Colistin	Polymixin B	Ceftazidime
susceptible	22	19	7	21	19	26	29	24	26	20	26	24	21	15	29	16	26	15	9	7	10
Intermediate	4	4	2	1	0	0	1	4	0	3	0	5	3	1	0	0	1	5	0	1	2
resistant	4	7	21	8	11	4	0	2	4	7	4	1	6	14	1	14	3	10	21	22	18

Figure 6: The results of antimicrobial susceptibility tests of thirty *Escherichia* isolates

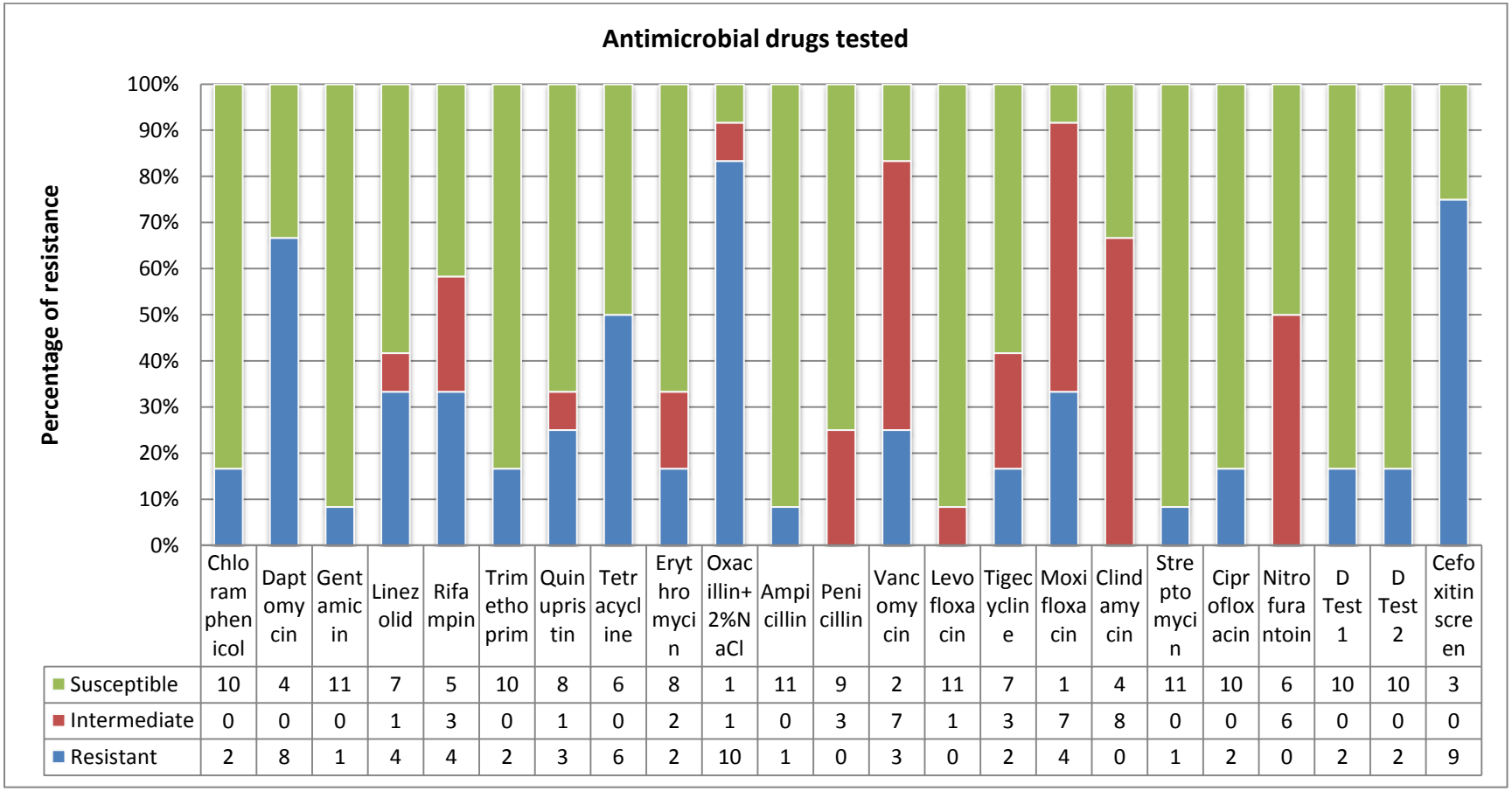
The *Salmonella* isolates showed 100% resistance to polymyxin B and colistin (Figure 7). There was also >60% resistance against ticarcillin, doxycycline, cefotaxime and ertapenem. There was observed susceptibility to gentamicin and tobramycin as well as cefepime, amikacin and levofloxacin among others.



	Amikacin	Ticarcillin	Aztreonam	Piperacillin	Trimethoprim	Gentamicin	Cefepime	Tobramycin	Levofloxacin	Doxycycline	Ciprofloxacin	Minocycline	Meropenem	Cefotaxime	Tigecycline	Ertapenem	Imipenem	Doripenem	Colistin	polymyxin B	Ceftazidime
susceptible	6	1	0	3	1	6	6	6	6	1	6	1	6	1	6	1	5	6	0	0	4
Intermediate	0	0	3	1	2	0	0	0	0	0	0	3	0	0	0	1	0	0	0	0	1
resistant	0	5	3	2	3	0	0	0	0	5	0	2	0	5	0	4	1	0	6	6	1

Figure 7: The results of antimicrobial susceptibility tests of six *Salmonella* isolates

The highest resistance was recorded against Oxacillin+2%NaCl and the lowest were recorded against streptomycin, ampicillin and gentamicin in the Enterococcus isolates (Figure 8).



	Chloramphenicol	Daptomycin	Gentamicin	Linezolid	Rifampin	Trimethoprim	Quinupristin	Tetracycline	Erythromycin	Oxacillin+2%NaCl	Ampicillin	Penicillin	Vancomycin	Levofloxacin	Tigecycline	Moxifloxacin	Clindamycin	Streptomycin	Ciprofloxacin	Nitrofurantoin	D Test 1	D Test 2	Cefoxitin screen
Susceptible	10	4	11	7	5	10	8	6	8	1	11	9	2	11	7	1	4	11	10	6	10	10	3
Intermediate	0	0	0	1	3	0	1	0	2	1	0	3	7	1	3	7	8	0	0	6	0	0	0
Resistant	2	8	1	4	4	2	3	6	2	10	1	0	3	0	2	4	0	1	2	0	2	2	9

Figure 8: The results of antimicrobial susceptibility tests of twelve Enterococcus isolates

Integrons and resistance genes

In Figure 9, the presence of 393 base pair bands indicate the presence of *Sul*2 genes in the isolates screened. Seventeen out of twenty bacterial isolates screened tested positive for *sul*2 genes. Lanes 1 to 12, 14, 16-19 were positive for *sul2* genes whilst lanes 13 and 15 were negative. Lane 20 was water as control.

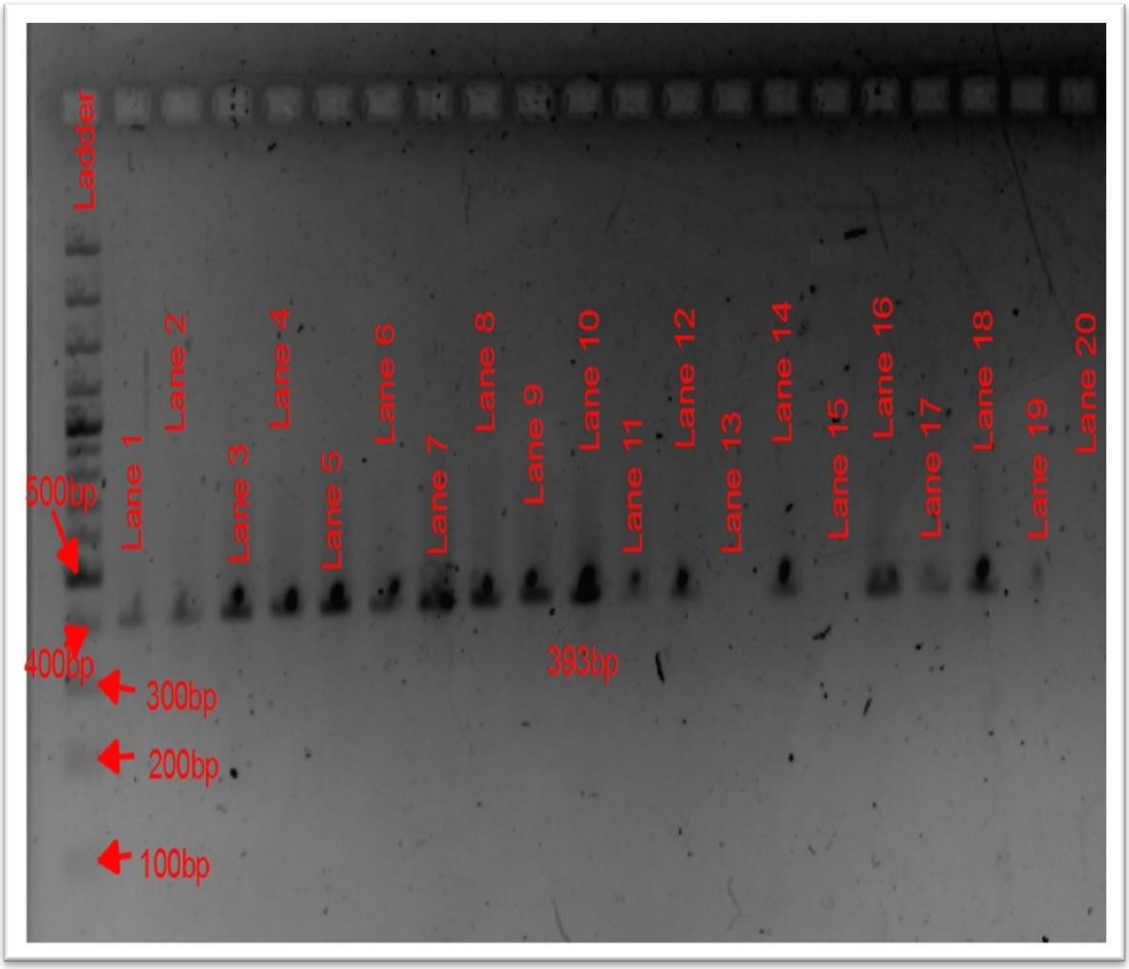


Figure 9: Gel image of PCR for the detection of *sul*2 genes

The presence of 660 base pair bands indicate the presence of *Sul*1 genes in isolates screened. Twelve out of twenty isolates screened tested positive for *sul*1 genes. Lanes 1-12 all tested showed 660bp bands for *sul1* genes whilst lane 13 was a control(water)

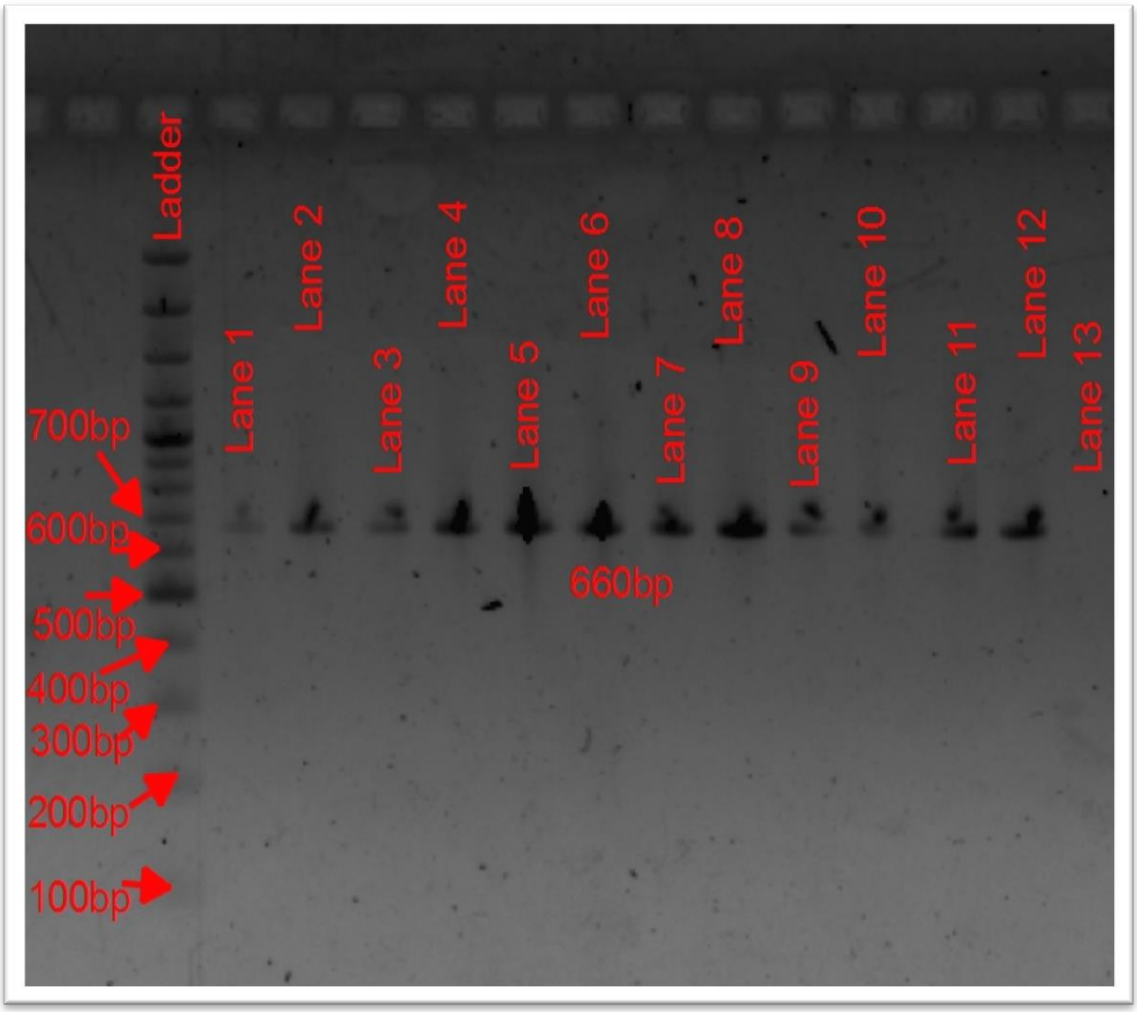


Figure 10: Gel image of PCR for the detection of *sul*1 genes

The presence of 956 base pair bands indicate the presence of *tet*A genes in the isolates screened. Eight out of twenty isolates screened tested positive for *tet*A genes. Lanes 1 to 8 all showed 956bp bands and were thus positive for *tetA* genes.

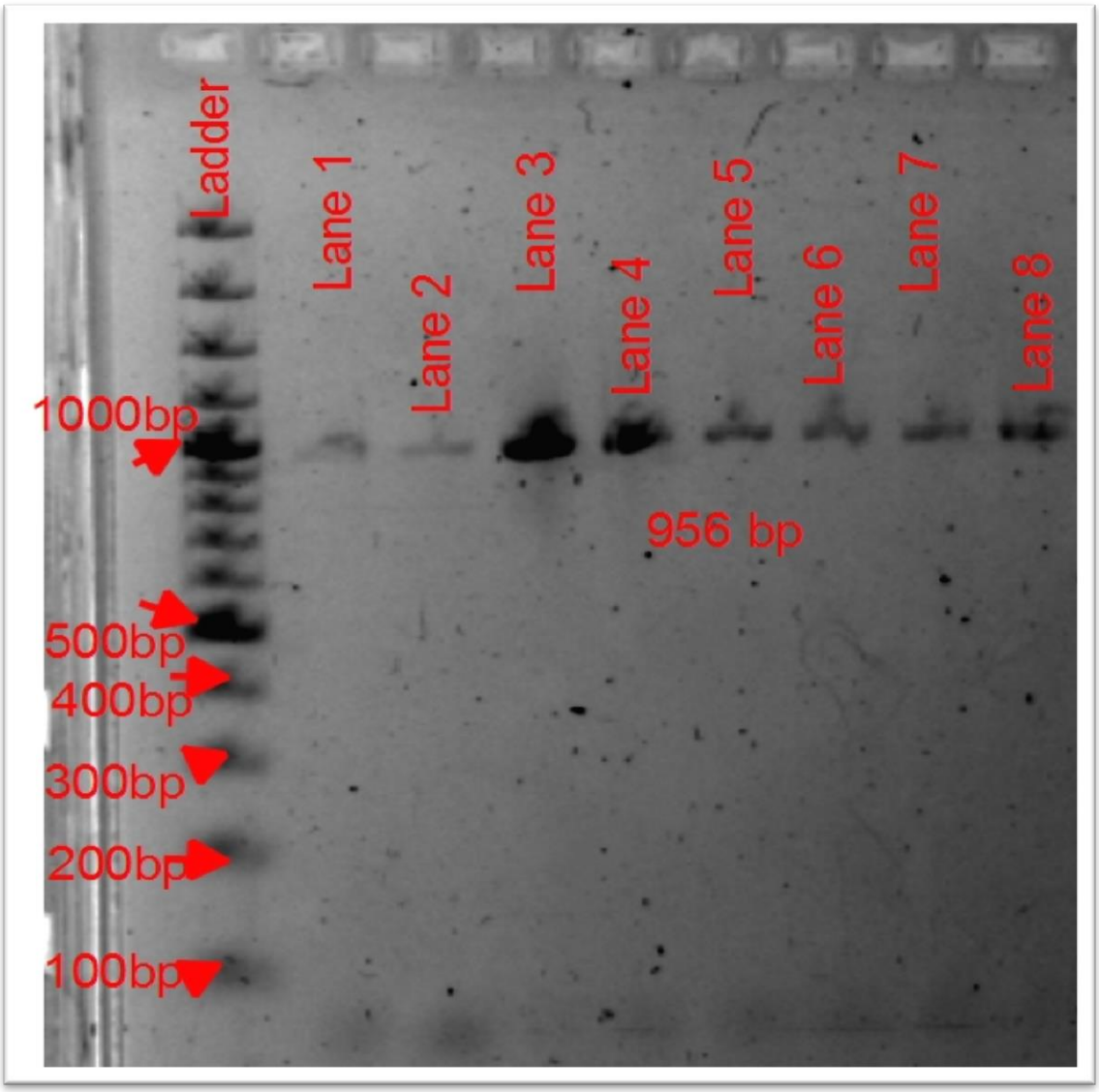


Figure 11: Gel image of the PCR for the detection of tetA genes

The presence of 414 base pair bands indicate the presence of *tet*B genes in isolates screened. Fifteen out of twenty isolates screened tested positive for *tet*B genes. Lanes 1-3 and 5-11 showed bands for *tetB* genes. This image is a section of the final gel image. Lanes 4 and 12 had faint bands in this regions, lanes 12and 13 had 180bp that might be inteprated as false negatives whilst lanes 14 and 15 were non-template controls.

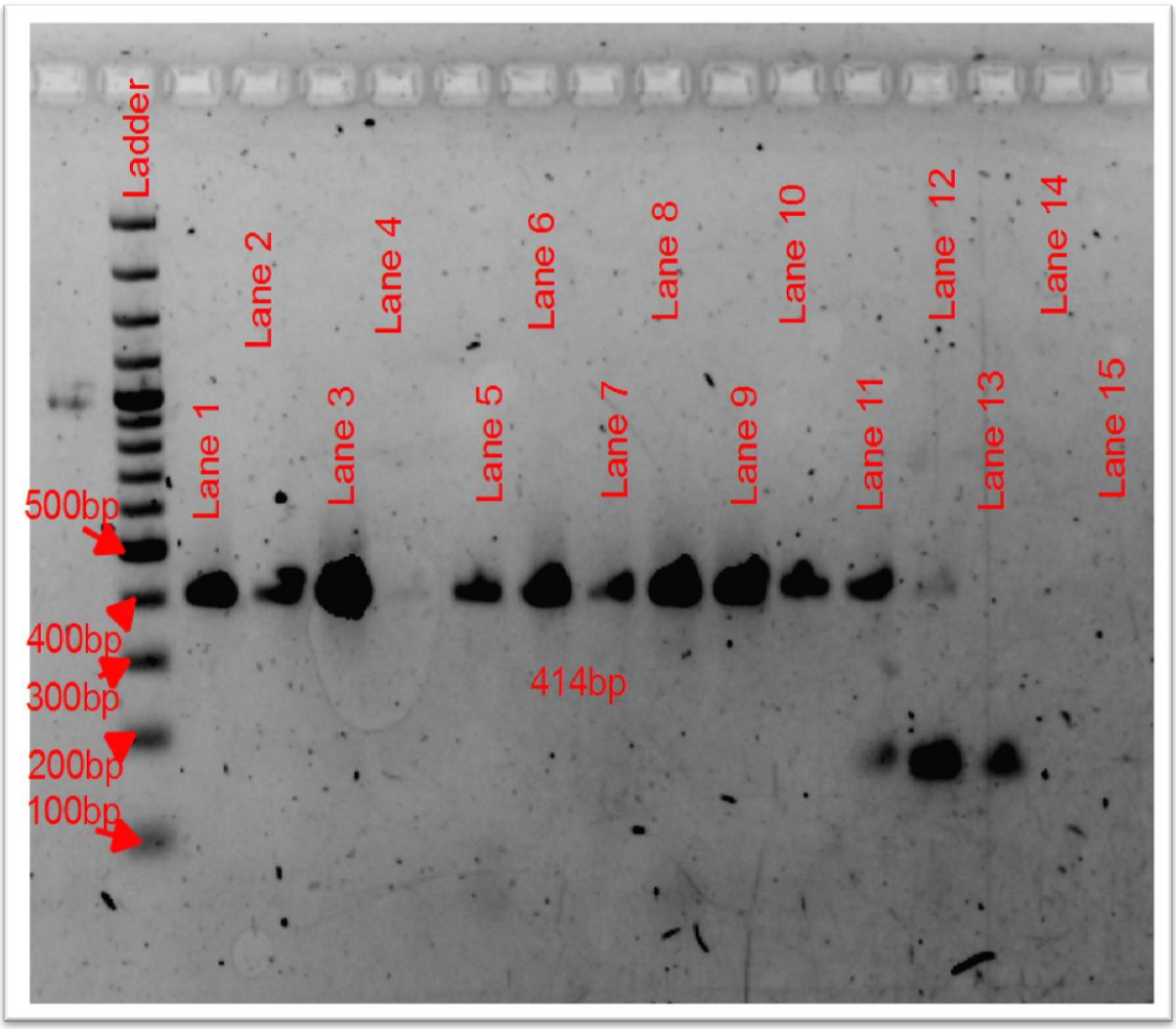


Figure 12: Gel image of the PCR for the detection of tetB genes

The presence of 160 base pair bands indicate the presence of *Int*1 genes in isolates screened. Seventeen out of twenty isolates screened tested positive for Int1 genes. Lanes 1-10 all showed bands for Int1 genes. This image is a section of the final gel image. Lane 11 was a control(water).

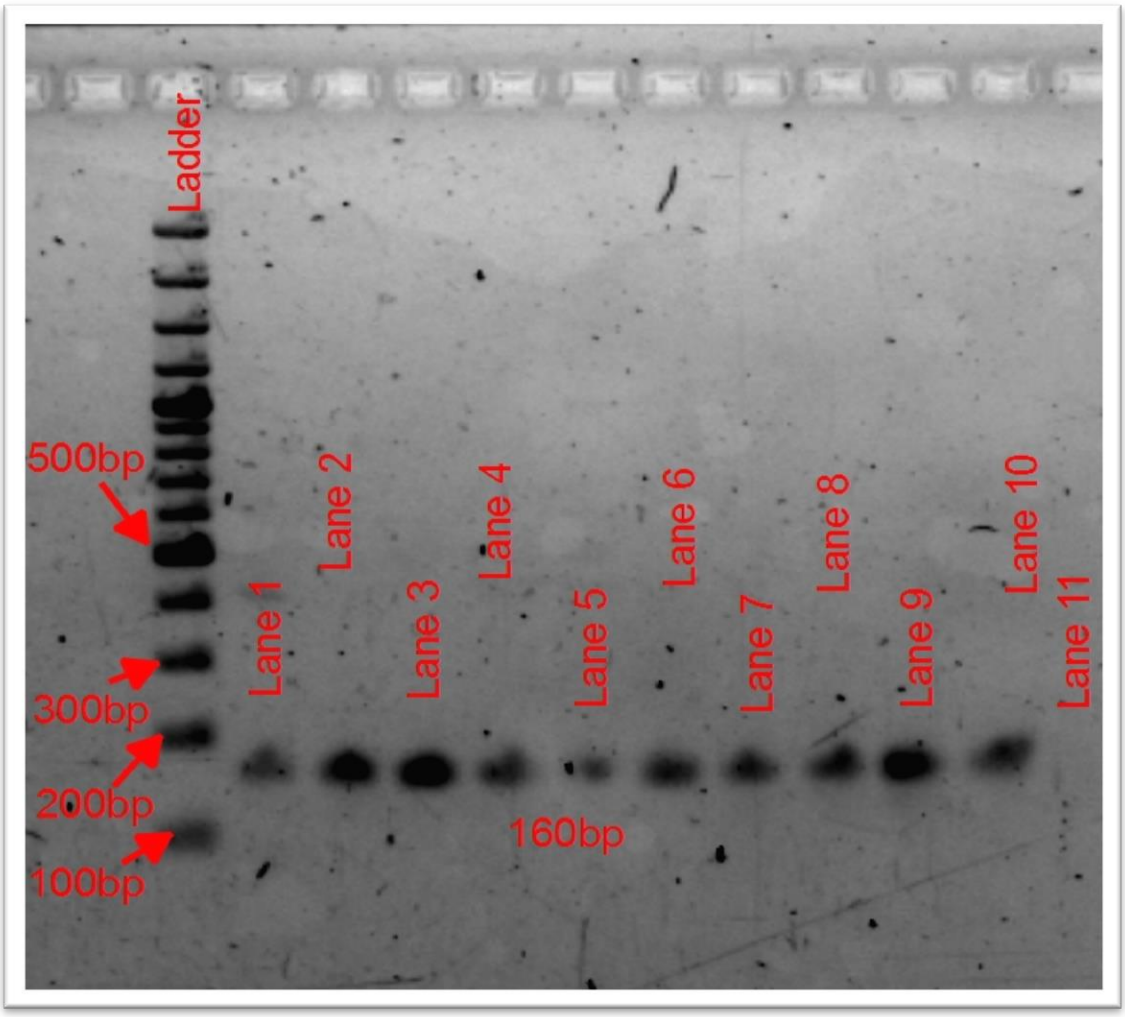


Figure 13: Gel image of the PCR for the detection of Class 1 Integrons

No Class 3 integrons were detected whilst Class 2 integrons detected produced very faint bands

Table 4: The results of PCR for the detection of *tet*A, *tet*B, *sul*1, *sul*2, Class 1 & 2 genes and resistance profiles of isolates tested

Type of bacteria	Resistance profile	Presence of class 1 integrons	Presence of Class 2 integrons	Presence of *tetA* genes	Presence of *tetB* genes	Presence of *sul1* genes	Presence of *sul2* genes
Enterococcus	DAP, LZD, SYN, ERY, OXA+, VAN, TGC, MXF, CLI, DT1, FOXS	+	-	-	-	+	+
Enterococcus	DAP, LZD, TET, OXA+, TGC, MXF, CLI, FOXS	+	-	-	+	+	-
Enterococcus	DAP, OXA+, MXF, CLI, NIT	+	+	-	-	-	+
Enterococcus	TET, OXA+, MXF, FOXS, CLI	+	-	-	-	-	+
Enterococcus	CHL, DAP, LZD, RIF, SXT, SYN, TET, ERY, OXA+, AMP, PEN, VAN, TGC, MXF, CLI, STR, CIP, NIT, DT1, DT2 FOXS	+	+	-	+	+	+
Enterococcus	DAP, RIF, TET, OXA+, VAN, MXF, CIP, DT2, FOXS	+	-	+	+	+	+
Escherichia spp.	AMI, AZT, TOB, MERO, ETP, DOR, COL, POL,TAZ	+	+	-	+	-	+
Escherichia coli	TIM2, AZT, P/T4, SXT, DOX, MIN, FOT, ETP, IMI, DOL, COL, POL, TAZ	+	-	-	+	+	+
Escherichia spp.	TIM2, AZT, P/T4, SXT, GEN, TOB, LEVO, DOX, CIP, MIN, FOT, ETP, COL, POL, TAZ	+	-	+	+	+	+
Escherichia spp.	TIM2, AZT, SXT, MERO, FOT, TGC, ETP, IMI, DOR, COL, POL, TAZ	+	-	+	-	+	+
Escherichia	POL, TAZ	+	-	-	-	-	-

coli							
Escherichia coli	TIM2, AZT, SXT, DOX, MIN, FOT, ETP, COL, POL	+	-	-	+	+	-
Escherichia spp.	TIM2, AZT, P/T4, SXT, MERO, FOT, TGC, ETP, IMI, DOR, COL, POL, TAZ	+	-	+	+	+	+
Klebsiella	AZT, ETP, DOL, COL, POL, TAZ	+	-	+	+	-	+
Salmonella	TIM2, SXT, DOX, FOT, ETP, COL, POL	+	-	-	-	+	+
Salmonella	TIM2, SXT, DOX, MIN, FOT, ETP, COL, POL	+	-	-	+	+	+
Klebsiella	AZT, FEP, FOT, ETP, COL, POL, TAZ	+	-	-	+	-	+
Salmonella	AZT, DOX, MIN, COL, POL, TAZ	+	-	-	-	-	+
Salmonella	TIM2, AZT, SXT, DOX, MIN, FOT, ETP, COL, POL	+	-	+	+	-	+
Klebsiella	AZT, ETP, DOR, COL, POL, TAZ	+	-	+	+	+	+

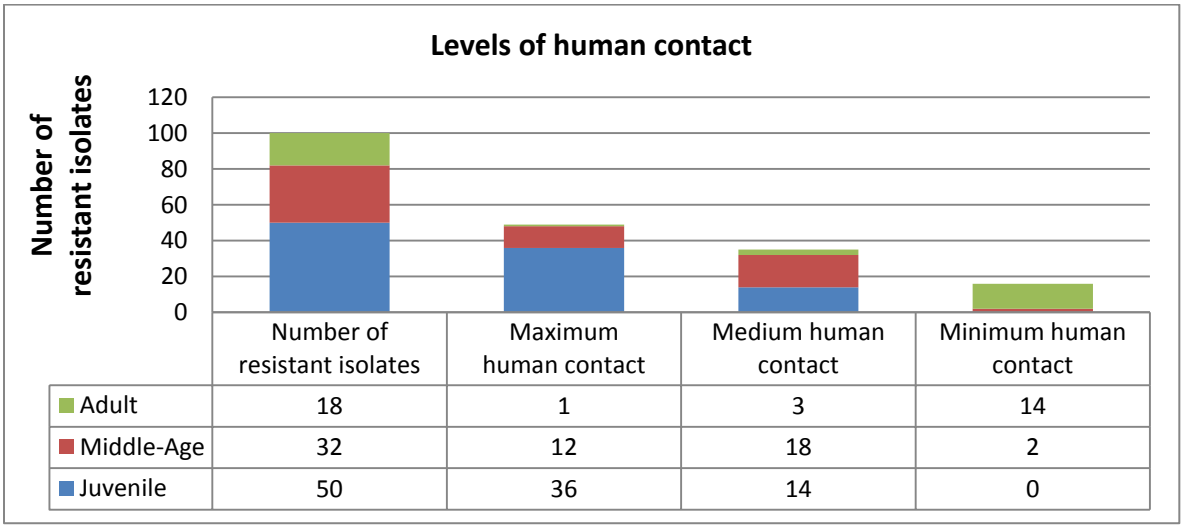
Discussion

Bacterial growth in wells of the microbroth dilution plates for detecting ADR were determined using UV lamp readers to increase reading accuracy. Wells with bacterial growth glowed under the UV lamp reader for easier detection compared to the conventional readers. Analysis of the antimicrobial susceptibility results with selected antimicrobials, revealed over 80% susceptibility to gentamicin. It is active against a wide range of human bacterial infections, mostly Gram-negative bacteria including *Pseudomonas*, *Proteus*, *Serratia*, and the Gram-positive *Staphylococcus*. Gentamicin is also useful against *Yersinia pestis* and *Francisella tularensis* (Golijan, 2011). However, gentamicin has a limited use due to the side effects attributed to its use.

Ciprofloxacin is a 100% synthetic drug and does not occur naturally. The bacterial isolates were almost 100% susceptible (except among the *Escherichia* isolates where a few isolates were resistant to it). Ciprofloxacin is used to treat a number of human infections including infections of bones and joints, endocarditis, gastroenteritis, malignant otitis externa, respiratory tract infections, cellulitis, urinary tract infections, prostatitis, anthrax, chancroid, among others (AHFS, 2011). However, in most of these treatments, it is not recommended as a first-line antibiotic (Goosens *et al*., 2007) due to its adverse effects currently under debate (Heidelbaugh *et al*., 2013; Brown *et al*., 2013; Falagas *et al*., 2006) and this could be the reason for the susceptibility.

Polymyxin B sulphates and colistin had not been used for many years in the 20th century (from the 1970's to early 2000) for the treatment of bacterial infections in humans because less toxic antimicrobials were available (Evans *et al*., 1999; Biswas *et al*., 2012). However, increasing resistance to the carbapenems (such as imipenem and meropenem) – which are often considered as the last resort treatment for nosocomial infections caused by multi-resistant Gram-negative organisms – has resulted in a return of the polymyxins and colistin (Michalopoulos & Karatza, 2010). It's re-use started in the late 2000's (Biswas *et al*., 2012; Dhariwal & Tullu, 2013). Thus, the high rates of resistance shown by all the Gram-negative strains isolated in this research are not strange (since they are not new drugs) but of concern because of the dependence of current antimicrobial treatment programs on these drugs.

Two Enterococcus isolates each 'passed' the D-tests 1 and 2 whilst nine isolates passed the cefoxitin screen tests. The D test is performed for the detection of inducible clindamycin resistance whilst cefoxitin screen tests are to determine resistance to oxacillin.



	Number of resistant isolates	Maximum human contact	Medium human contact	Minimum human contact
Adult	18	1	3	14
Middle-Age	32	12	18	2
Juvenile	50	36	14	0

Figure 14: The number of multi-drug resistant isolates based on the level of human contact of their host

The data showed that non-human primates with maximum human contact in Group 1 and in possible contact with other forms of human detritus had greater levels of antibiotic-resistant gut bacteria than did their nearly wild and wild counterparts in Groups 2 and 3.

Group 1 had 20% and 23% higher resistance prevalence than Groups 2 and 3 respectively due probably to maximum human contact (Literak *et al*, 1985; Rolland *et al*, 1985; Kozak *et al* 2009; Blanco, *et al*, 2009; Cole, *et al*. 2005 and Pesapane *et al.*, 2013).

Group 3 however, contained relatively lower numbers of resistant Gram-negative intestinal bacteria, a finding in accordance with other studies of humans and animals not exposed to modern medicine and human contact (Hughes & Datta, 1983; Mare *et al*, 1968). The activation of their natural, wild instincts in their natural environments makes those enclosures dangerous for volunteers and staff in general and it ensures minimal human contact. This could be the reason for the low levels of resistance.

Various factors could contribute to the relatively high numbers of resistant bacteria present in group 1.

Firstly, on a normal day, monkeys in group 1 are taken out of their cages and individually given warm baths. They are fed via feeding bottles by surrogate human mothers, played with and placed back in the cages. They are fed at least three times in a day via feeding bottles. One centre permitted more access to this group of animals than the other but this group generally required trained personnel with experience on handling juveniles. Thus, these animals have enough human contact to facilitate the transfer of resistance genes from humans to the animals and probably back to other humans. The rehabilitation centre policies regulated who had contact with the primates in this group but one centre enforced these regulations more seriously than the other centre. As expected, resistance patterns in the centre with less strict regulations were higher due to the number of people that had regular contact with the animals.

Since proximity to human populations has been established to contribute to antimicrobial resistance transfer to wildlife, it can be said that this was an avenue through which these animals acquired resistance strains - though it is yet to be proven.

In addition, the two rehabilitation centres studied thrived on donations and services rendered by international volunteers. The daily handling of non-human primates are done by local staff as well as international volunteers from around the world who dedicate a number of weeks to living and working at the centres. Thus, animals in these centres are introduced to a bevy of antibiotic resistant strains from across the world – often on a monthly basis. These volunteers often stay for a maximum of up to 6 months and a minimum of a month. No special gear or prior medical tests are required to volunteer at these centres or to handle the animals concerned thereby creating a conductive atmosphere for the transfer of resistant strains not just to the animals but between the volunteers themselves through the animals. These animals thus act as reservoirs of antimicrobial resistant strains that can be transferred back to other volunteers that establish contact with them.

Lastly, as rehabilitation centres, non-human primates under their care are given regular medical treatment when diseases/infections are suspected. Most of the primates come in injured and are put on antimicrobials until their wounds heal. The caretakers admitted to giving the injured primates penicillin and other antimicrobials and this could also contribute to resistance.

In research on the Cape Peninsula baboon population, a human parasite *Trichuris trichiura* was identified which provides the first evidence of likely anthroponotic infection of baboons (Ravasi,

2009). Anthroponotic infections are the transmission of infectious diseases from humans to animals; in this case the baboons. This finding likely supports assertions that pathogens can be transferred from humans to non-human primates, and these can include antimicrobial resistant pathogens.

All 20 (100%) randomly selected isolates tested were positive for class one integrons by means of PCR with 160 base pair bands when analysed molecularly using primers that target class 1 integrons. Class 1 integrons are found extensively in clinical isolates, and most of the known antibiotic-resistance gene cassettes belong to this class (Mazel 2006). They contain elements that are known to confer resistance to all known β-lactams, all aminoglycosides, chloramphenicol, trimethoprim, streptothricin, rifampin, erythromycin, fosfomycin, lincomycin and antiseptics of the quaternary-ammonium-compound family (Rowe-Magnus & Mazel, 2002; Fluit & Schmitz, 2004). This probably explains why all the isolates with class 1 integrons were multi-drug resistant.

Three of the isolates bore class 2 integrons whilst none bore class 3 integrons. Class 2 integrons are 'historical' classes that are involved in the multi-drug resistance phenotype and exclusively associated with Tn*7* derivatives (Hall 1997; Brown *et al.*, 1996; Sundstrom *et al.*, 1991). Consistent with other research reports on members of the family *Enterobacteriaceae*, it does not frequently occur in gut flora (Burjag & Shehabi, 2013).

TetB genes were present in 15 out of 20 isolates representing 75% of isolates tested whilst 8 samples had *tetA* genes representing 40% of isolates tested. It is believed that the acquisition of resistance to tetracyclines by bacteria is due to the localization of *tet* genes on plasmids, transposons, and integrons. Thus it is not surprising that strains that tested positive for integrons had either *tetA* or *B* genes present – and in some instances, both. The data was also in line with MIC tests done of similar isolates in another project (Leverstein-van Hall, 2003). Four out of 6 Enterococci tested were resistant to tetracyclines.

Twelve *sul1* genes were identified in the tested isolates representing 60% of the isolates while 17 isolates tested positive for *sul2* genes (85%). Co-trimoxazole resistance is often associated with the presence of *sul1* or *sul2* genes or both (Burjag & Shehabi, 2013) and this was reflected in the resistance profiles of the test isolates. The genes present correlated with MIC results obtained

with observed resistance to trimethoprim/sulfamethoxazole. Nine of the twenty isolates tested were resistant to trimethoprim/sulfamethoxazole.

The correlation between class 1 integrons and *sul* genes has been previously studied (Byrne-Bailey *et al*., 2009). It has been determined for example that the *sul1* gene is located on the 3' conserved region of the class 1 integron and is usually identified with integrons in soil environments (Hindi *et al.*, 2013) – drawing attention to a possible mode of transmission of these genes to the non-human primates. Since humans regularly, feed and bath these primates as well as clean their cages, the environment can be implicated as a conduit. The wild non-human primates outside the rehabilitation centre have a 'stealing' habit and often would raid bins in search for food. Faecal samples from some of these wild primates were also tested for *tet* and *sul1* genes and they all tested positive. They could also be implicated in the transfer of these genes to their counterparts in the rehabilitation centres since they are sometimes seen in the cages and enclosures.

Also, the *sul2* genes were the most prevalent of all resistance genes detected – in line with findings by Kerrn *et al.*, (2002), Trobos *et al* ., (2009) and more recently (Hindi *et al.*, 2013). *Sul2* genes are also often related to class 1 integrons (Antunes *et al*., 2005)

Conclusions and recommendations

The results of this project implicate regular human contact as a source of resistant bacteria in the intestines of wildlife in the absence of known antibiotic selective pressure in line with earlier studies by Levy *et al* (1981). Moreover, these data call more attention to an (until recently) unrecognized pathway by which antibiotic resistance plasmids may be transmitted to wild animals and subsequently spread to the natural environment – direct human contact.

The results of this study also provides support to the assertions that integron-bearing bacteria often carry *sul* genes and *sul2* genes are the most prevalent in members of the family *Enterobacteriaceae.* The presence of class 1 integrons as well as the *tet* and *sul* genes further support the data obtained from the antimicrobial susceptibility tests.

It is recommended that further research be done on the human handlers in and around the rehabilitation centres so as to determine the kinds of antibiotics frequently used by the centre staff, the resistance profiles as well as their degree of similarity to the strains from the non-human primates. Susceptibility tests on their enteric bacteria would aid in detecting which drugs these bacteria are resistant to in order to confirm any transmission from the humans to the baboons.

It is recommended that more integron screening tests be carried out on intestinal bacteria of wildlife to determine their potential to be intrinsically resistant to antimicrobial drugs. To achieve this objective, rapid molecular techniques are preferred in comparison to conventional MIC tests.

In addition, screening for the less known Class 2 and 3 integrons and further research on the roles they play in antibiotic resistance should be carried out.

The wildlife-human interface is described as a high-risk interface that continues to increase because of expanded ecotourism and forest encroachment (Wolfe *et al.,* 1998). Non-human primates in South Africa are often caught within this interface and study models on the pathogen susceptibility and transfer between these primates and humans are important in monitoring the interface for emerging infectious diseases.

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CHAPTER 2

The isolation, biochemical and molecular identification of *Escherichia fergusonii* and a review of the literature

Abstract

Farmer et al (1985) proposed *Escherichia fergusonii* as a new species within the genus *Escherichiae* and family *Enterobacteriaceae* in 1985 with a 64% similarity to *Escherichia coli* when analyzed through DNA hybridization. *Escherichia fergusonii* has been isolated from human patients with pancreatic carcinoma, sepsis, enteric disease, wound infection, urinary tract infection, and cystitis. It was first described in animals in cases clinically suggestive of salmonellosis and subsequently identified to be of enteric origin. It has since been isolated from goats, pigs, cattle, and sheep and birds.

Bacterial isolates from fecal samples of non-human primates at two wildlife rehabilitation centres in South Africa were screened for the presence of *Escherichia coli*. Biochemical characterization of *E. coli* and *E. coli*-like bacteria using the Remel ® RapidID One panel strips (UK), showed adonitol positive, sorbitol negative strains. Two tests were carried out to further confirm the *E. coli* and *E. coli*-like samples using the Simmons citrate agar supplemented with 2% adonitol and biochemical tests based on their ability to ferment cellobiose and D-arabitol (Huys et al., 2003). Antimicrobial susceptibility tests were run on the isolates using microbroth-dilution-plates imbibed with 21 different antimicrobial drugs in varying human dosages. A duplex PCR for molecular confirmation was carried out targeting two genes; the yliE gene (cellulose synthase and regulator of cellulose synthase island) and the EFER_1569 (putative transcriptional activator for multiple antibiotic resistance). Positive samples were confirmed by the presence of 233bp for the yliE gene and 432bp for the EFER_1569 gene.

Twenty three *E. coli*-like bacteria were confirmed as *E. fergusonii* based on the confirmatory tests and they were in 100% agreement. Five times as many *E. coli* relative to *E. fergusonii* were isolated. Resistance patterns to the antimicrobial drugs in both *E. coli* and *E. fergusonii* were 95% similar with observed differences to Amikacin. Approximately 86.95% of *E. fergusonii* were resistant to polymixins B and E (Colistin) as well as the carbapenems with occasional resistance to Amikacin. All *E. fergusonii* bacteria isolated from juvenile vervet monkeys in both centers showed positive amplification to EFER_1569 (multiple antimicrobial resistance) whilst in the other groups, variable amplification of EFER_1569 was observed.

This is the first reported isolation and confirmation of *E. fergusonii* in non-human primates. The presence of EFER_1569 genes in all the isolates from young vervets that had consistent human contact is suggestive of the possible routes of transmission into the non-human primate population. The findings support the assertion that *E. fergusonii* is an emerging pathogen of zoonotic importance.

Introduction

In the last four decades, descriptions of *Escherichia blattae* (Burgess *et al.*, 1973), *E. hermanii* (Brenner *et al.*, 1982a) *E. vulneris* (Brenner *et al.*, 1982), *E. fergusonii* (Farmer *et al.*, 1985) and *E. albertii* (Huys *et al.*, 2003) have been published as additions to the genus *Escherichia*. The recent identification of other species in this genus and clades within the genome of *Escherichia coli* have been attributed to the improvement in diagnostic techniques (Gaastra *et al.*, 2014). As a result of the availability of an increasing variety of growth media and the introduction of PCR based assays and whole genome sequencing; phenotypic similarities are not limiting factors anymore in differentiating between species of the genus *Escherichia*.

Escherichia fergusonii was proposed as a new species within the genus *Escherichia* and family *Enterobacteriaceae* in 1985 with a 64% similarity to *Escherichia coli* when analyzed by means of DNA hybridization (Farmer *et al.*, 1985). This bacterial strain was formerly known as Enteric Group 10, due to its biochemically distinct nature compared to other species and biogroups of the genus *Enterobacteriaceae.* DNA-DNA hybridization to determine the relatedness of *E. fergusonii* strains to other species within the genus *Enterobacteriaceae* using ^{32}P-labeled DNA in 60°C hydroxyapatite, showed 90-97% relatedness to the type strain (holotype) *E. fergusonii* ATCC 35469. In relation to other species, the closest was *Escherichia coli* with up to 64% similarity (Farmer *et al.*, 1985).

E. fergusonii was initially isolated from human clinical samples collected from the blood, urine, abdominal wounds and faeces of patients (Farmer *et al.*, 1985). It has been isolated from gall bladder fluids of patients (Funke *et al*., 1993) and from food products such as montasio cheese (Maifreni *et al.*, 2013) and beef (Fegan *et al.*, 2006). It has also been isolated from faecal samples of goats (Hariharan *et al.*, 2007), sheep (Bain and Green, 1999), horses (Weiss *et al.*, 2011), raptors (Bangert *et al.*, 1988), turkeys, ostriches (Herráez *et al.*, 2005), chickens (Oh *et al*., 2012), cattle and pigs (Farmer *et al.*, 1985).

Literature Review

Phenotypic characteristics and growth properties

In common with other *Escherichia* species, *E. fergusonii* is a rod-shaped, Gram-negative member of the family *Enterobacteriaceae*. It is a non-spore-forming, predominantly motile, peritrichous flagellated bacterium. It has a diameter of 0.8–1.5 µm and lengths from 2 to 5 µm. *E. fergusonii* grows optimally on growth media at 37–40 ºC under aerobic conditions with variations between 21-45 º C (Savini *et al.,* 2008; Gaastra *et al.,* 2014).

Citrate adonitol agar has been reported to be an effective selective growth medium for isolating *E. fergusonii* from faecal samples (Wragg *et al.,* 2009. This medium was first described in 1984 as a selective medium for the isolation of certain K99 *E. coli* strains based on their ability to ferment adonitol. Recent studies also employed the use of CHROMAgar Orientation media in isolating *Escherichia* species. *E. coli* colonies appear pink on the growth media while *E. fergusonii* colonies appear pink with brown discolorations around the colonies (Lagace´-Wiens *et al.,* 2010).

The biofilm formation properties of *E. fergusonii* were studied by Ingle *et al.,* (2011) in comparison to similar properties in *E. coli, E. albertii* and cryptic clades of *Escherichia* (clades III, IV, and V). Generally, most strains of the *Escherichia* genus studied had greater biofilm formation at 24 º C than at 37 º C when grown in nutrient broth rather than minimal glucose media. *E. fergusonii* however, revealed biofilm formation the best at 24 º C on minimal glucose medium in comparison with nutrient broth. It was also observed that neither a strain's maximal growth rate nor its optimal temperature for growth varied with respect to the strain's phylogenetic affiliation (Ingle *et al.,* 2011).

Biochemical properties / characterization

Biochemically, *E. fergusonii* is positive for indole, methyl red, lysine decarboxylase, and ornithine decarboxylase (Farmer *et al.,* 1985). It is also oxidase-negative, catalase-positive and is

a facultative anaerobe, which implies that it can obtain energy from the oxidation and reduction of organic materials (Savini *et al.*, 2008). *E. fergusonii* is however, unable to break down lactose; a similarity it shares with species of the genus *Salmonella* (Rimoldi & Moeller, 2013). It is negative for the Voges-Proskauer test, urea hydrolysis, phenylalanine deamination, growth in Potassium Cyanide media KCN, fermentation of lactose, sucrose, *myo*-inositol, D-sorbitol, raffinose, α-methyl-D-glucoside and arginine dihydrolase (Farmer *et al.*, 1985). It ferments L-rhamnose, maltose, D-xylose, trehalose, cellobiose D-glucose with gas production, adonitol, L-arabinose and D-arabitol (Farmer *et al.*, 1985). Citrate utilisation in *E. fergusonii* is generally negative with only 17% of samples tested in one study being positive (Farmer *et al.*, 1985). Its inability to ferment sorbitol and the failure to produce β-glucuronidase is a common/shared feature with *E. coli* O157:H7 (Rice *et al.*, 1991; Gaastra *et al.*, 2014).

Adonitol fermentation by *E. fergusonii* is a characteristic shared with *E. coli* strains with either a cryptic K99 antigen or a glucose dependent K99 antigen (Pohl *et al.*, 1984) and is a feature, which aids in identifying and characterising *E. fergusonii*.

Commonly used commercial kits for biochemical characterisation include the API 20E strip (Analytab Products, BioMeriaux Canada) and the Vitek 2 GN card (bioMe´rieux, Marcy L'e´toile, France).

Molecular identification and characterization

Simmons *et al.*, (2014) described a novel PCR assay for the detection of *E. fergusonii* directly from caeca and cloacal samples of poultry without pre-enrichment. For that study, primers targeting specific genes, including *yli*E (encoding a conserved hypothetical protein of the cellulose synthase and regulator of cellulose synthase island), EFER_1569 (encoding a hypothetical protein, putative transcriptional activator for multiple antibiotic resistance), and EFER_3126 (encoding a putative triphosphoribosyl-dephospho-coenzyme A [CoA]), were designed for the detection of *E. fergusonii* by conventional and real-time PCR methods.

A fluorescent dye-labelled probe for the enumeration of *E. fergusonii* cells was tested by *in situ* hybridization and epifluorescence microscopy and found to be able to stain cells of *E. coli*,

Shigella spp. and E. fergusonii (Regnault *et al*., 2000). The specificity of the probe is limited to the aforementioned species; having being tested on 169 other species.

These molecular techniques improved the sensitivity and speed of diagnosis of *E. fergusonii* infections. As an opportunistic pathogen, usually isolated from infections in which the causative pathogen is initially unknown, molecular detection speeds up detection/confirmation processes considerably (Savini *et al.,* 2008). Sequence-based identification and strain typing, together with the development of tools that can probe for thousands of markers, will allow detailed strain fingerprinting to assist in disease management and control (Gilbert, 2002).

The ability to detect antimicrobial resistance genes as described by Forgetta *et al*, (2012) also aids in treating infections - in instances where first-line antibiotic treatments fail. Availability and access to whole genome sequences of *E. fergusonii* strains would be useful in future in informing health interventions and in managing outbreaks and controlling the spread of multi-drug resistant strains.

Pathogenicity and virulence

E. fergusonii is described as an emerging pathogen that occurs in both humans and animals; though infrequently (Savini *et al*., 2008; Gaastra *et al.,* 2014). They are opportunistic pathogens and have occasionally been associated with wound infections in humans (Savini *et al.,* 2008). The biochemical reactions, antimicrobial susceptibility patterns, susceptibility to polyvalent phage 0-1, and rRNA gene restriction analysis of four *E. fergusonii* strains suggested they were of clonal origins and had great potential to be human pathogens (Funke *et al.,* 1993). Though it's clinical significance was uncertain from initial isolations by Farmer *et al.,* 1985, further research reports of isolations from both humans and animals with pancreatic carcinoma (Funke *et al.,* 1993), septicaemia (Weiss *et al.,* 2011), pneumonia (Regnault *et al*., 2000), endophtalmitis (Gokhale *et al.*, 2014), and other disease conditions cleared up the uncertainties (Mahapatra *et al.*, 2005; Wragg *et al.,* 2009; Gaastra *et al.,* 2014).

In analysing biofilm formation, strains of *E. fergusonii* did not replicate at temperatures below 11 º C (Ingle *et al.*, 2011). This observation is confirmed by the fact that *E. fergusonii* has until now

not been isolated from environmental samples; but has only been isolated from warm-blooded animals. This is suggestive of the facultative pathogenic properties of the organism (Ingel *et al.*, 2011; Gaastra *et al.*, 2014).

Enteropathogenicity testing of *E. fergusonii* strains for diarrhoeagenic potential in the ileal loops of Charles-Foster strains of albino rats, using whole live cells and culture filtrates showed significant fluid accumulation ranging from 0.15 to 0.2 mL/cm. This indicates that *E. fergusonii* strains are potentially diarrhoeagenic (Chaudhury *et al.*,, 1999). Similarly, whole live cells and culture filtrate results indicate that enteropathogenicity is most probably mediated through enterotoxins (Chaudhury *et al.*,, 1999). Twenty one different biochemical types of heat-labile enterotoxin (LT)-producing genes were isolated from *E. fergusonii* strains from healthy chickens in an experiment designed to demonstrate the presence of LT-genes in *E. fergusonii* and its plasmid-mediated LT toxin gene transfer to other members of the family *Enterobacteriaceae* (Oh *et al.*,, 2012).

The PCR screening of *E. fergusonii* strains for the presence of virulence factors involved in intestinal (*elt* [LT-A], *ast*A, *eae*A, and *cdt*A) and extraintestinal virulence (*kps*E, *sfa, iro*N, *aer/iut*A, *pap*C, *pap*G, *hly*D, *cnf*1, *omp*T, *ibe*A, and *fyu*A) as described by Gordon *et al.*, 2005 and Diard *et al.*, 2007, only detected traits *aer/iut*A and *kps*E - and at high frequencies in the latter (Ingel *et al.*, 2011). To assess the role of these virulence factors, a mouse model of systemic infection was used. Mice were challenged subcutaneously around the abdomen with a standardized bacterial inoculum of *E. fergusonii* (with aer/*iut*A and *kps*E virulence factors) (10^9 CFU of log-phase bacteria/ml in 0.2 ml of Ringer's solution). No mortality was recorded 7 days post challenge and none of the 10 mice were killed by the bacterial inoculum during the experiment. The researchers therefore concluded that *E. fergusonii* has a low potential to cause extraintestinal infections. However, isolations have been recorded from blood and tissues outside the gastrointestinal tract of both humans (Funke *et al.*, 1993) and animals (Weiss *et al.*, 2011); questioning the validity of this conclusion.

Infections in humans

The first reported isolation of *E. fergusonii* from humans apart from its description by Farmer *et al.,* (1985) was from the blood of an immunocompromised patient with clinically diagnosed septicaemia in France (Freney *et al.,*, 1987). Although appropriate antibiotic therapy was given, the patient's condition deteriorated and he died some days later of acute respiratory failure due to pulmonary aspergillosis. Blood culture and gall bladder fluid showed the presence of an *E. fergusonii* infection in a 69-year-old urban male that had a history of weight loss, jaundice, and acholic stools with clinical signs of ascending cholangitis. The patient's general condition deteriorated and he died of acute heart failure (Funke *et al*., 1993). Subsequently, A 73-year-old man who had a history of diabetes mellitus and pancreatic cancer, presented with fever, vomiting, and abdominal discomfort in a case study by Lai *et al*., (2011) where blood cultures helped identify an ampicillin-resistant *E. fergusonii* strain. The patient died on the 37th day after admission.

Various case studies over the years have implicated *E fergusonii* as an emerging opportunistic pathogen in humans with some cases leading to death (Farmer *et al.,* 1985; Funke *et al.,* 1993; Mahapatra *et al.,* 2005; Weiss *et al.,* 2011; Wragg *et al.,* 2009; Gokhale *et al.,* 2014).

Infections in animals

E. fergusonii has been found to cause diseases that were suggestive of salmonellosis in sheep and cattle and clinical manifestations included abortion, diarrhoea and mastitis (Bain and Green, 1999). Samples of the intestine, lung, liver, faeces, and kidney from a euthanized 42-month-old goat with a 2-month history of poor weight gain and diarrhoea yielded a moderate growth of *E. fergusonii*. A clinical case of enteritis and septicemia with subsequent death of an adult horse was also reported as the result of *E. fergusonii* infection with the horse reportedly dying after 24 hours of clinical signs that included erosive stomatitis and fever (Weiss *et al.,* 2011). Anorexia, prostration, and severe hemorrhagic diarrhoea were clinical signs observed in two ostriches with *E. fergusonii* infections, and resulted in locally extensive areas of haemorrhage, and fibrino-necrotic typhlitis with a white-yellowish material covering the mucosal surface. Multiple serosal

petechial haemorrhages and fibrinous peritonitis were also observed during post mortem examination (Herráez *et al.*, 2004). *E. fergusonii* has also been isolated from healthy chickens (Oh *et al.*, 2012).

Antimicrobial susceptibility

In a case report of a 52-year old human with acute cystitis, *E. fergusonii* was implicated as the causal agent (Savini *et al.*, 2008). Susceptibility to antimicrobials was tested using Vitek2 AST-N021 and AST-N041 cards and resistance to ampicillin, piperacillin, ciprofloxacin, levofloxacin, gentamicin, netilmicin, tobramicin and cotrimoxazole were observed. The organism was found to be susceptible to amoxicillin-clavulanic acid, ampicillin/sulbactam (MIC, 8 mg/L), piperacillin-tazobactam, cefoxitin, cefixime, cefotaxime, ceftazidime, imipenem (MIC, 2 mg/L), meropenem, nitrofurantoin and tetracycline (Savini *et al.*, 2008). Earlier experiments on *E. fergusonii* strains recorded resistance to ampicillin, tetracycline and co-trimoxazole and susceptibility to cephalosporin and netilimicin (Chaudhury *et al.*, 1999).

Susceptibility was variable for nalidixic acid (77% susceptible), sulfadiazine (62% susceptible), streptomycin (46% susceptible), tetracycline (62% susceptible), ampicillin (77% susceptible), carbenicillin (77% susceptible), and cephalothin (31% susceptible) in selected *E. fergusonii* isolates reported by Farmer *et al.*, (1985) with complete susceptibility to colistin, gentamicin, and chloramphenicol and recorded resistance to penicillin.

Lagace´-Wiens *et al.*, (2010) also reported the presence of large quantities (>108) colony-forming units per liter (CFU/liter) of Extended-spectrum beta-lactamases (ESBL)-producing *E. fergusonii* in a 76-year-old Caucasian woman presenting to the emergency department of a community hospital. Antimicrobial susceptibility test results identified a multi-drug resistant organism with demonstrated high-level resistance to cephalosporins, fluoroquinolones, sulphonamides, monobactams, and aminopenicillins, which suggested the ESBL mechanism of resistance. This was further investigated using the Clinical and Laboratory Standards Institute (CLSI) disk diffusion method using ceftriaxone and ceftazidime disks with and without clavulanate.

Ampicillin-resistant *E. fergusonii* isolates from farm animals were tested for extended-spectrum β-lactamase (ESBL) phenotypes by double disk diffusion tests using three indicator cephalosporins: cefotaxime, ceftazidime and cefoxitin, both alone and in combination with amoxicillin-clavulanic acid (AMC). The disk diffusion test results revealed that all *E. fergusonii* isolates were resistant to more than three antibiotics commonly used on farms. Of the isolates, eight exhibited resistance to ampicillin. One *E. fergusonii* isolate showed reduced sensitivity to the indicator cephalosporins. PCRs with oligonucleotide primer sets targeting TEM, SHV, CTXM, or AmpC β-lactamases were performed on all the β-lactam-resistant isolates as described previously by Rayamajhi, *et al*., (2010). PCR and sequencing results showed that all eight ampicillin-resistant isolates carried the TEM-1 gene, while the single *E. fergusonii* isolate with reduced sensitivity to the indicator cephalosporins carried an additional CTXM-15 β-lactamase gene (Rayamajhi *et al.,* 2011).

PCR and sequencing of SHV, CTX-M, and TEM β-lactamase genes as described by Mulvey *et al*., (2004) revealed the presence of TEM-1 and SHV-12 genes – the presence of the latter conferring the ESBL phenotype (Lagace´-Wiens *et al*, 2010). This was determined based on β-lactamase classifications by Bush & Jacoby, (2010).

In another study on day-old-chick models (Forgetta *et al.,* 2012), it was observed after genome analysis of an isolated *E. fergusonii* strain that the existence of several resistance genes to multiple classes of antibiotics was present in the strain, thus making the treatment of infection caused by such *E. fergusonii* strains difficult when using currently available antimicrobials (Forgetta *et al.,* 2012).

The presence of multiple resistance genes in their genomes, gives rise to multi-drug resistant *E. fergusonii* strains. Most *E. fergusonii* strains that are isolated from both humans and animals – in cases where general first-line drug treatments for bacterial infections fail – are multi-drug resistant (Diarrasouba *et al.,* 2007; Savini *et al.,* 2008; Fricke *et al.,* 2009).

Genome analysis

A phylogenetically divergent and multi-drug resistant *Escherichia* spp. isolate, ECD-227, previously isolated from a broiler chicken and possessing virulent genes was characterised using whole genome sequencing and comparative genome analysis with pathogenic (*E. coli* O157:H7TW14359, APEC O1:K1:H7, and UPEC UTI89) and non-pathogenic species (*E. coli* K-12 MG1655 and *E. fergusonii* ATCC 35469) (Forgetta *et al.,* 2012).

Comparisons were done to determine the presence of known and putative antibiotic resistance and virulence open reading frames. *Escherichia* sp. isolate ECD-227 was identified as *E. fergusonii* through the phylogenetic analysis of 537 open reading frames present in 110 different intestinal bacterial species. The analyses of 537 protein sequences homologous in all test isolates were carried out on 110 bacterial species that included *E. fergusonii* (ATCC 35469), 74 *E. coli*, 29 *Salmonella*, and 6 other enteric bacterial species.

An analysis of the BLAST results of 4,058 protein sequences predicted to be the chromosomal protein sets of ECD-227 against the National Centre for Biotechnology Information (NCBI) non-redundant database showed that 49% of the protein sequences had a higher similarity to *E. fergusonii* (ATCC 35469) than to *E. coli* K-12 MG1655. Twelve percent of the protein sequences were equally similar between *E. fergusonii* ATCC 35469 and *E. coli* K-12 MG1655 and only 14% had greater similarity to *E. coli* K-12 MG1655. It was observed that 23% of the predicted proteins were not present in both *E. fergusonii* ATCC 35469 and *E. coli* K-12 MG1655 and 16% had significant alignment to *E. fergusonii* ATCC 35469 only. Finally, the remaining 6% of predicted proteins were either unique to the ECD-227 strain or similar to *E. coli* K-12 MG1655 only (Forgetta *et al.,* 2012).

Studies on the presence of antibiotic resistant and virulence genes localized in chromosomes and plasmids showed a mutation in *gyr*A (S83L) that is involved in fluoroquinolone resistance. Those plasmids were *Salmonella*-like and harboured antimicrobial resistance genes on a class I integron (*aadA, qacEΔ-sul1, aac3-VI, and sulI*) as well as numerous virulence genes (*iucABCD, sitABCD, cib, traT*) (Forgetta *et al.*, 2012).

Biodegradable properties

The capability of enriched *E. fergusonii* cultures to degrade crude oil was estimated using microcosm studies under saline conditions (Pasumarthi *et al*., 2013). *E. fergusonii* strains were discovered in a crude oil-contaminated sediment sample using diesel oil as the sole carbon source, which led to this analysis. Results showed that the *E. fergusonii* strains isolated degrade crude oil as a sole carbon source and have shown preference for aliphatic hydrocarbons under saline conditions (3% NaCl), which mimics the marine environment. Another study on a specific *E. fergusonii* KLU01 strain isolated from oil-contaminated soil was identified to be hydrocarbon degrading, heavy metal tolerant and a potent producer of biosurfactant using diesel oil as the sole carbon and energy source (Sriram et *al*., 2011). This makes the *E. fergusonii* KLU01 strain a new class of biosurfactant producer with potential environmental and industrial applications, especially in hydrocarbon degradation and heavy metal bioremediation.

Epidemiology

No reports of *E. fergusonii* isolations have been documented from Africa either in humans or in animals to date. Since the majority of reported *E. fergusonii* isolations were case reports (Gaastra *et al*., 2014), data on host species dynamics, reservoirs and transmission are currently unknown.

E. fergusonii prevalence rates in birds in an Australian study were low (4 out of 643) that suggested their rareness in bird populations (Walk *et al*., 2009; Gaastra *et al*., 2014). However, a research project to demonstrate and epidemiologically characterise the presence of heat-labile enterotoxin (LT)-producing *E. fergusonii* in healthy chickens and its plasmid-mediated LT toxin gene transfer to other *Enterobacteriaceae* was conducted in South Korea (Oh *et al*., 2012). Seven provinces were included namely: Gyeonggi, Chungnam, Chungbuk, Jeju, Jeonbuk, Jeonnam, and Kyungbuk. *E. fergusonii* strains carrying LT toxin genes were isolated from the faecal samples of 184 broiler birds in 78 chicken farms in South Korea. The 78 farms sampled represented 2.3% (78/3403) of the total chicken farms in Korea. Most of the *E. fergusonii* strains (28/43, 65.1%) carrying the LT gene were predominantly distributed in the broiler flocks from the Chungnam

and Chungbuk provinces, located in the middle portion of the Korean peninsula. These reports contradict earlier suggestions of the rarity of *E. fergusonii* strains in free flying birds.

A research project on human gastrointestinal tract infections in Latin America also reported that *E. fergusonii* was the third most frequently occurring isolate in the human gastrointestinal tract infections in Latin America (Bours *et al.*, 2010)

Materials and methods

Study location

Two wildlife rehabilitation centres located within 10 km of each other and approximately 30 km from the Kruger National Park were study sites for this study. These centres were established to accommodate and provide temporary sanctuary for wildlife casualties, rehabilitate them to a point of self-support and the ultimate release and introduction into nature where they naturally belong. They rehabilitate and provide sanctuary to over 500 orphaned, injured, abused, ex-laboratory or baboon and vervet monkey populations. In both centres, the non-human primates were classified into three groups and this informed the sampling strategies. The first group had regular human contact and were mostly orphaned juveniles that were fed, bathed and cuddled regularly. The second group of non-human primates had been weaned off frequent contact with humans with occasional contact when it was time to feed them. There was no cuddling or bathing of the animals in this group and they only came in contact with staff/volunteers during feeding lasting a maximum of 10 minutes, 3 times daily. The third group had previous human contact and lived in enclosures similar to their natural habitats with almost no human contact. They fed off natural shoots in their enclosures and food was only supplemented in harsh weather conditions when plants they feed on were not available.

Sample collection and processing

There were three major groups defined per rehabilitation centre for sampling purposes in line with the groups within the centres. Three hundred fresh faecal samples were obtained from

vervets and baboons in sampling groups using sterile swabs immersed in Stuart's transport medium. One-hundred-and-fifty (150) baboon faecal samples and fifty faecal samples from vervets were collected at one wildlife rehabilitation centre whilst another 100 faecal samples from vervets were collected from the second wildlife rehabilitation centre.

Bacterial isolation

To isolate Gram-negative bacteria, the 300 faecal samples were inoculated on MacConkey agar under sterile conditions and incubated for 24 hours at 37 °C. Suspect *E. coli* and *E. coli*-like isolates were then inoculated on Eosin Methylene Blue (EMB) agar.

Biochemical tests

Biochemical characterizations of *E. coli* and *E. coli*-like isolates were carried out using the Remel ® RapidID One panel strips (UK). Oxidase tests were done prior to the panel strip tests to ensure only oxidase-negative isolates were tested as recommended by the manufacturer. Samples were transferred into inoculation fluids and then into wells imbibed with reagents and incubated at 37 ˚C for 4 hours. The biochemical profiles were then determined after incubation based on colour changes in the inoculated wells as indicated by the manufacturer.

Some *E. coli*-like isolates were observed to be adonitol positive and sorbitol negative and were classified as suspected *E. fergusonii* strains. Two tests were carried out for further confirmation of *E. coli* and *E. coli*-like colonies, namely selective isolations on Simmons citrate agar supplemented with 2% adonitol and biochemical tests based on their ability to ferment cellobiose and D-arabitol (Huys et al., 2003).

Antimicrobial susceptibility tests

Antimicrobial susceptibility tests were carried out using the Sensititre GNX2F MIC (Trekds, UK) microdilution plates imbibed with 21 different antimicrobial drugs in serial dilutions in order to determine the minimum inhibitory concentrations. The plates were read using the UV lamp readers commonly used for reading PCR products on agarose gels instead of the conventional MIC plate readers.

Table 5: List of antimicrobial drugs used in the susceptibility tests, their MICs and CLSI susceptibility cut offs for *E. fergusonii*

Antimicrobials (Inoculum concentration: 50µl, Reconstitution volume: 50µl)	Type of isolate tested	Abbreviation	MIC Interpretative Criteria Microgram per milliliter (ug/mL)		
			Susceptible	Intermediate	Resistant
Amikacin	Gram-negative	AMI	≤16	32	≥64
Ticarcillin / clavulanic acid	Gram-negative	TIM2	≤16/2	32/2–64/2	≥128/2
Aztreonam	Gram-negative	AZT	≤4	8	≥16
Piperacillin / tazobactam	Gram-negative	P/T4	≤16/4	32/4–64/4	≥128/4
Trimethoprim / sulfamethoxazole	Gram-negative	SXT	≤ 2/38	-	≥4/76
Gentamicin	Gram-negative	GEN	≤4	8	≥16
Cefepime	Gram-negative	FEP	≤8	16	≥32
Tobramycin	Gram-negative	TOB	≤4	8	≥16
Levofloxacin	Gram-negative	LEVO	≤2	4	≥8
Doxycycline	Gram-negative	DOX	≤4	8	≥16
Ciprofloxacin	Gram-negative	CIP	≤1	2	≥4

Minocycline	Gram-negative	MIN	≤4	8	≥16
Meropenem	Gram-negative	MERO	≤1	2	≥4
Cefotaxime	Gram-negative	FOT	≤1	2	≥4
Tigecycline	Gram-negative	TGC	≤2	4	≥8
Ertapenem	Gram-negative	ETP	≤0.5	1	≥2
Imipenem	Gram-negative	IMI	≤1	2	≥4
Doripenem	Gram-negative	DOR	≤1	2	≥4
Colistin	Gram-negative	COL	≤2	2	≥2
Polymyxin B	Gram-negative	POL	≤2		≥4
Ceftazidime	Gram-negative	TAZ	≤		≥

Duplex PCR

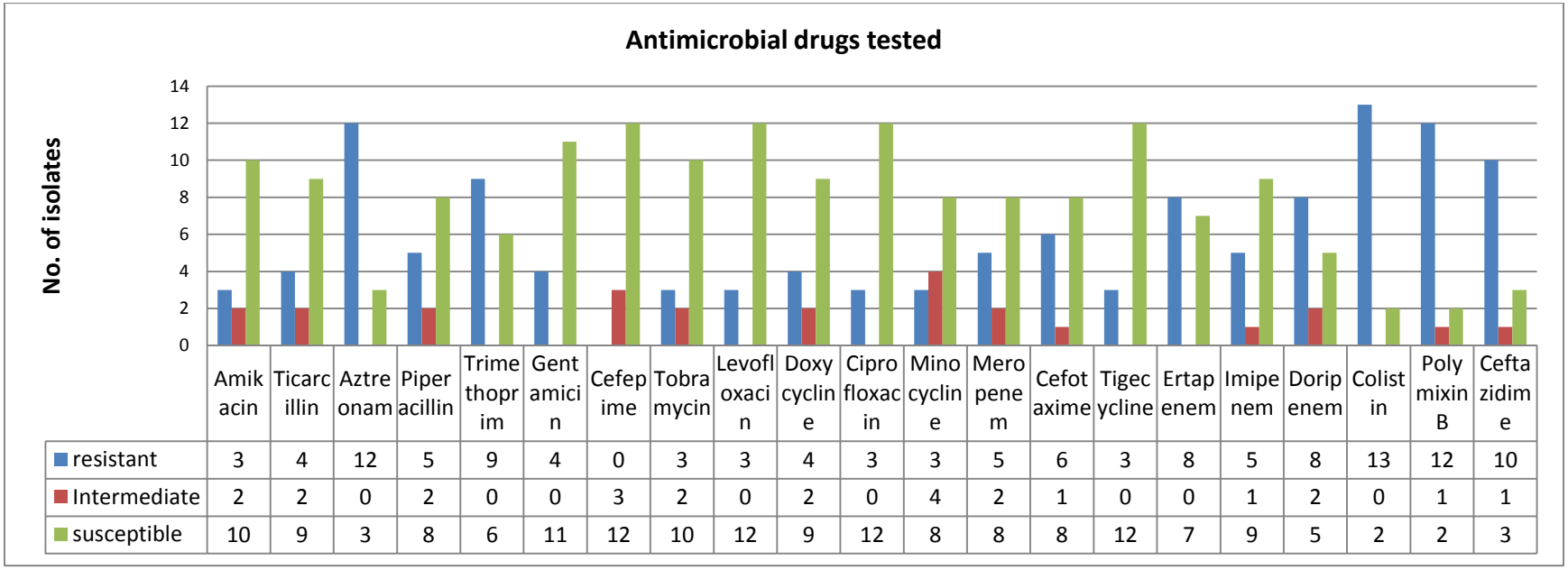
A duplex PCR for molecular confirmation of *E. fergusonii* targeting two genes, namely the *yli*E gene and the EFER_1569 were carried out according to standard protocols as previously published (Simmons *et al.*, 2014). Positive samples were confirmed by the presence of 233bp of the yliE gene and 432bp of the EFER_1569 gene.

The PCR conditions were slightly modified to suit the Taq polymerase used in the reaction as well as to match the concentrations of DNA used. The reported annealing temperature of 56.5^{o}C was adjusted to 60^{o}C and the number of cycles per reaction kept at 30. The 25µl final volume of the PCR mixture contained 12.5µl of Kapa 2G Fast Multiplex PCR kit (Kapa Biosystems, Cape Town, South Africa), 0.5µM of each primer, 8.5µl of molecular grade water, and 2µl of bacterial DNA. The cycling conditions were 94^{o}C for 3 min, followed by 35 cycles of 94^{o}C for 30 sec, 56.5^{o}C for 30 sec, 72^{o}C for 30 sec, and then a hold at 4^{o}C. The PCR products were separated on a 2% Tris–acetate-EDTA buffer agarose electrophoresis gel stained with ethidium bromide (1µl/10 ml) and the bands were referenced to a GeneRuler 100 bp DNA ladder (Fermentas, Ottawa, Ontario) to size the amplicons (Simmons. *et al.*, 2014).

Results

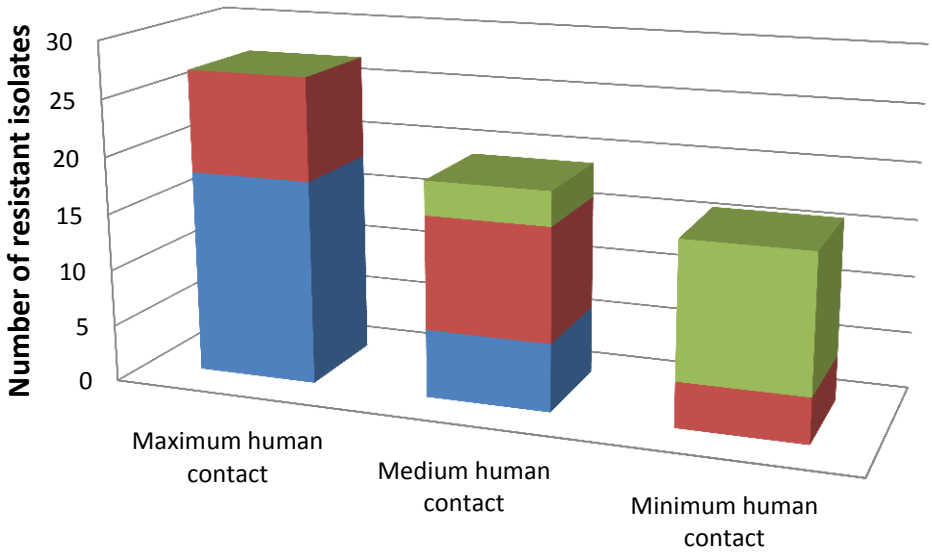
Antimicrobial susceptibility tests

Thirty *E. fergusonii* strains were isolated – fifteen each from vervet monkeys and baboons. The *E. coli*-like bacteria were confirmed as *E. fergusonii* based on the confirmatory tests and they were in 100% agreement. The drugs most bacteria were resistant to included polymyxin B and colistin. Seventy percent of the *E. fergusonii* isolates were resistant to polymyxin B and colistin as well as to the carbapenems with occasional resistance recorded against aminoglycosides.



	Amikacin	Ticarcillin	Aztreonam	Piperacillin	Trimethoprim	Gentamicin	Cefepime	Tobramycin	Levofloxacin	Doxycycline	Ciprofloxacin	Minocycline	Meropenem	Cefotaxime	Tigecycline	Ertapenem	Imipenem	Doripenem	Colistin	Polymixin B	Ceftazidime
resistant	3	4	12	5	9	4	0	3	3	4	3	3	5	6	3	8	5	8	13	12	10
Intermediate	2	2	0	2	0	0	3	2	0	2	0	4	2	1	0	0	1	2	0	1	1
susceptible	10	9	3	8	6	11	12	10	12	9	12	8	8	8	12	7	9	5	2	2	3

Figure 15: Antimicrobial susceptibility test results of the *Escherichia fergusonii* isolates



	Maximum human contact	Medium human contact	Minimum human contact
Adult	0	3	12
Middle-Age	9	10	4
Juvenile	18	6	0

Figure 16: A representation of the number of multi-drug resistant *E. fergusonii isolates* in the sampling groups

The highest level of resistance was observed in group 1 primates. Comparatively, group 1 had 20% and 23% higher numbers of resistant strains than group 2 and group 3 respectively. This was observed across all the antimicrobials tested, especially polymyxin B and colistin.

Table 6: The number of multi-drug resistant *E. fergusonii* strains isolated and the source of the isolates

		***E. fergusonii* isolates from vervets**	***E. fergusonii* isolates from baboons**
Total number of Isolates		8	7
Number of antimicrobial drugs *E. fergusonii* isolates were resistant to	1	1	0
	2	0	2
	3	2	1
	4	1	0
	5	0	2
	6	0	0
	7	1	0
	8	0	1
	9	1	1
	10	0	0
	≥10	2	0
% Multi resistant (≥3)		87.5	71.4

Screening for *yli*E and EFER_1569 genes

All 23 randomly selected *E. fergusonii* isolates screened were positive for *yli*E genes whilst 12 were positive for EFER_1569

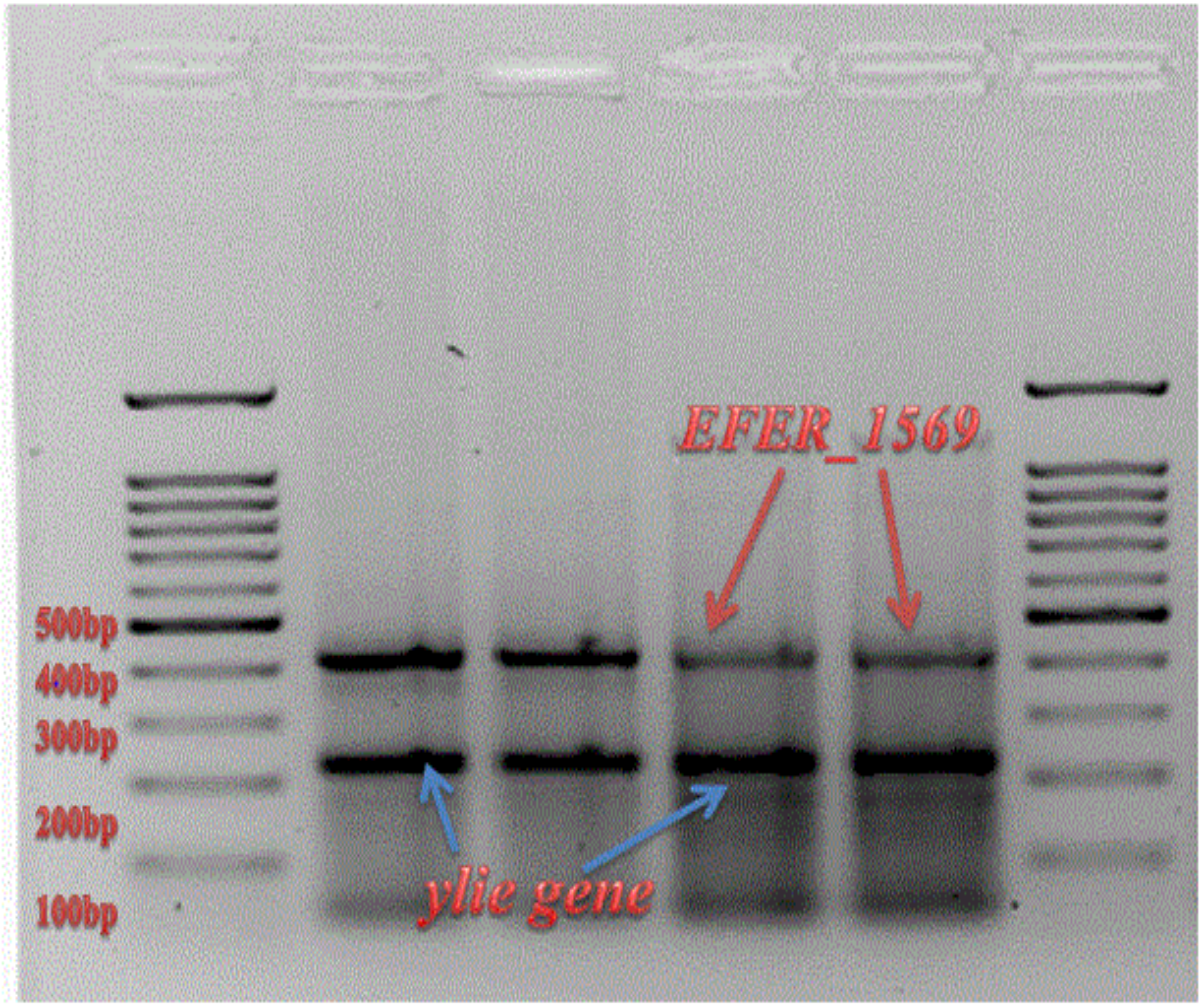
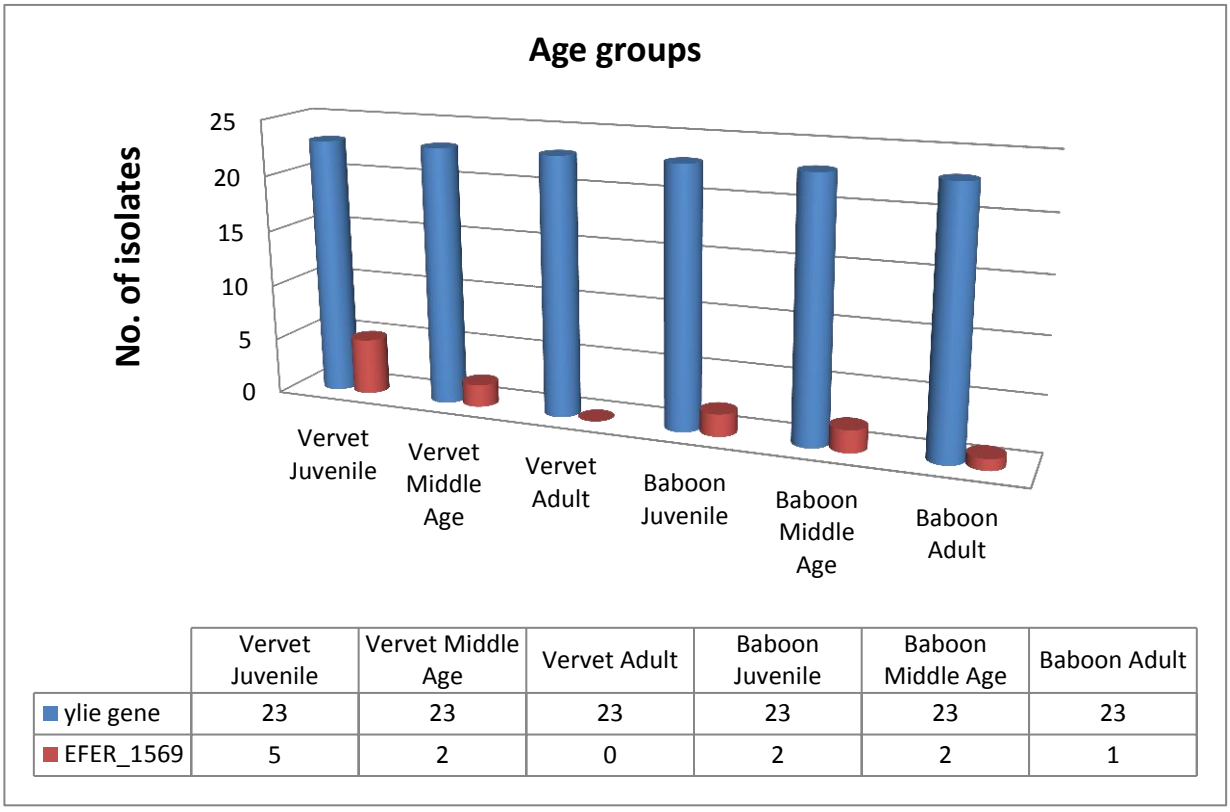


Figure 17: The results of the PCR assay to detect the presence of *yli*E (233bp) and EFER_1569 (432bp) genes in selected *E. fergusonii* isolates

Discussion

The protein encoded by the *yli*E gene synthesises biofilms for colonization resistance in commensal *Escherichia* spp. (233bp) whilst the EFER_1569 gene encodes a transcriptional activator for multiple antibiotic resistances (432bp) solely in *E. fergusonii* (Simmons. *et al.*, 2014)

All *E. fergusonii* bacteria isolated from juvenile vervet monkeys in both facilities yielded positive results with the molecular detection of EFER_1569 (multiple antimicrobial resistance) whilst in the other groups, variable amplification of EFER_1569 was observed.



	Vervet Juvenile	Vervet Middle Age	Vervet Adult	Baboon Juvenile	Baboon Middle Age	Baboon Adult
ylie gene	23	23	23	23	23	23
EFER_1569	5	2	0	2	2	1

Figure 18: The results of the PCR assay to detect the presence of ylie and EFER_1569 genes in the *E. fergusonii* isolates

The highest level of resistance was observed in group 1. Comparatively, group 1 had 20% and 23% higher levels of resistance than group 2 and group 3 respectively. This was observed for all the antimicrobials tested, and especially polymyxin B and colistin. Decreasing levels of antimicrobial resistance was observed with age, with isolates from older animals being less resistant. This resistance profile was similar in both camps. An 87.5% multi-drug resistance was

recorded for isolates from vervets tested against antimicrobial drugs while those from baboons totalled 71.4%.

This is the first documented isolation and confirmation of *E. fergusonii* in Africa, whether in humans or animals. It is also the first report of successful isolation from non-human primates globally. The presence of EFER_1569 genes in all the isolates from young vervets that had consistent human contact is suggestive of the possible routes of transmission into the non-human primate population. However, this cannot be confirmed without molecular fingerprinting analyses which would infer insights into transmission

Consistent with published literature, primate groups with frequent human contact had higher levels of bacteria resistant to antimicrobial drugs than those with less contact (Literak *et al*, 1985; Rolland *et al*, 1985; Cole, *et al*. 2005; Kozak *et al* 2009; Blanco, *et al*, 2009). Resistant bacterial numbers were also observed to diminish with the age of the animals.

Polymyxin B sulphates and colistin had not been used for many years in the 20th century (from the 1970's to early 2000) for the treatment of bacterial infection in humans because less toxic antimicrobials were available (Evans *et al*., 1999; Biswas *et al*., 2012). However, increasing resistance to the carbapenems (such as imipenem and meropenem) – which are often considered as the last resort treatment for nosocomial infections caused by multi-resistant Gram-negative organisms – has resulted in a return of the polymyxins and colistin (Michalopoulos & Karatza, 2010). It's re-use started in the late 2000's. (Biswas *et al*., 2012; Dhariwal & Tullu, 2013). Thus, the high rates of resistance shown by all the Gram-negative strains isolated in this research are not strange (since they are not new drugs) but of concern because of the dependence of current antimicrobial treatment programs on these drugs.

The presence of multi-drug resistant *E. fergusonii* in the non-human primates may be attributed to two factors:

Firstly, *E. fergusonii* strains may have been present in Africa long enough to acquire drug resistance but have not been identified. Since reported isolations outside of Africa have mostly been coincidental, it can be hypothesized that it is not a novel strain in Africa but has eluded

detection. Its similarity to *E. coli*, *Shigella* and even *Salmonella* strains phenotypically have probably contributed to its lack of detection until recently.

Alternatively, international volunteers may be responsible for the introduction of the strains into the non-human primate populations. The rehabilitation centres are both run by international volunteers who come into South Africa to work with these primates for a few weeks before returning to their countries. It is possible that these strains were transferred from some volunteers to these animals during handling. *E. fergusonii* has been identified as an emerging pathogen on every other continent except Africa. Thus, it may have been transferred from international visitors to local primates. Since the human handlers were not sampled, these assertions remain speculative.

Conclusions and recommendations

More evidence is accumulating that suggests that *E. fergusonii* is an emerging multi-drug resistant pathogen that requires further investigation. Its zoonotic potential must be substantiated in order to implement control measures for possible future outbreaks. The absence of data from Africa must also be addressed and further research must be undertaken to investigate *E. fergusonii* populations both in humans and in animals. As an opportunistic pathogen, its role in infections of immunocompromised patients must also be studied especially in regions with high tuberculosis and HIV rates. Further research on the relatedness of *E. coli* K99 and *E. fergusonii* however, must be done to determine if they are indeed two different species or the same.

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CHAPTER 3

Application of the CRISPOL technique for genotyping of *Salmonella enterica* serovar Typhimurium strains

Abstract

Clustered regularly interspaced short palindromic repeats (CRISPR) polymorphisms are prominent in *Salmonella* and strongly correlate with both serotype and MultiLocus Sequence Typing (MLST) which helps discriminate between subtypes within prevalent serotypes. A high-throughput subtyping assay was used in analyzing *Salmonella enterica* serovar Typhimurium strains of both domestic animal and wildlife sources.

Confirmed *Salmonella enterica* serovar Typhimurium strains isolated from horses and bovines were genotyped alongside serologically unconfirmed *Salmonella enterica* serovar Typhimurium strains from non-human primates using the Luminex XMap system. Some challenges were met and results were almost uninterpretable. Deductions from literature are discussed as to why these challenges were experienced.

Introduction

Faecal samples from vervets and baboons in two wildlife rehabilitation centres in South Africa were screened for the presence of *Salmonella* spp. In all, 50 suspect salmonellae were isolated using selective media. Twenty-three salmonellae were isolated from vervet monkeys whilst the remaining twenty-seven were isolated from baboons. These strains were not serotyped.

As a preventive measure against *Salmonella* spp. outbreaks in the academic hospital of the Faculty of Veterinary Science, University of Pretoria, all animals admitted to the hospital for treatment are screened for *Salmonella* spp. infections. Reference strains of bacteria identified to be salmonellae are kept in the bacteriology laboratory of the Department of Veterinary Tropical Diseases (DVTD), University of Pretoria. Over two years (July, 2012 – May, 2014), over 150 salmonella isolates have been stored as reference strains. Of these, 45 are serologically confirmed *Salmonella enterica* serovar Typhimurium strains isolated from bovines and equines.

Serological analysis of salmonella strains have been the gold standard in genotyping salmonella. They are time consuming and require technical expertise to perform. However, newer assays and technologies have been developed that can genotype salmonella strains faster as well as aid in strain tracking for epidemiological purposes especially for *Salmonella enterica* serovar Typhimurium strains. One of such technologies was tested on the 50 suspect *Salmonella enterica* serovar Typhimurium strains isolated from non-human primates (as described above) and from select reference *Salmonella enterica* serovar Typhimurium strains available at DVTD to determine its efficiency and applicability in South African laboratory settings.

Literature review

Salmonellosis is one of the most common causes of food-borne diarrheal disease worldwide. Most infections are zoonotic and are transmitted from food animals to humans through the ingestion of contaminated food. It was reported in 2004 that 1.2 million non-typhoidal *Salmonella* infections occur annually in humans in the United States and these result in approximately 15,000 hospitalizations and 400 deaths (Voetsch *et al.,* 2004). Centers for Disease Control and Prevention (CDC) reports place infection figures of non-typhoidal *Salmonella* infections in the United States at 1.2 million people in 2013 with 100,000 being drug-resistant *Salmonella* infections (CDC,

2013). They also report that there were 21,700,000 *Salmonella typhi* infections worldwide in 2013. Thus, an efficient surveillance system for salmonellosis is essential. Sentinel surveillance, periodic population-based surveys, and laboratory-based surveillance are all strategies aimed at monitoring outbreaks (Fabre *et al.*, 2012).

Laboratory-based approaches often require a network of clinical laboratories and the basic information currently provided by laboratories is the serotype of the isolates. These data contribute to effective monitoring of *Salmonella* outbreaks and aid in the epidemiological characterisation of *Salmonella* strains. Over 200,000 human isolates of *Salmonella* are reportedly serotyped in the United States and Europe annually (Fitzgerald *et al.,* 2007; Fisher, 2004) based on the laboratory-based approach alone. *Salmonella* serotyping requires the determination of two surface antigens– O-polysaccharide and flagellin proteins – by agglutination with a large set of polyclonal rabbit antisera. These surface antigens have so far aided in identifying more than 2,500 serotypes (Grimont & Weill, 2007) of *Salmonella*. The limitations of this technique include its discriminatory capacity due to the highly prevalent feature of two serotypes – Typhimurium and Enteritidis – that account for most outbreaks. "The sensitivity of serotyping for the detection of outbreaks involving these common serotypes, even with the use of cluster-detection algorithms, is therefore unsatisfactory" (Fabre *et al.,* 2012; Bender *et al.,* 2001).

Phage typing has also been identified to be useful in differentiation between isolates within the same serotype by determining the sensitivity of certain *Salmonella* serotypes to several bacteriophage suspensions as reported by Anderson *et al.,* (1977).

With improvements in molecular diagnostics came the use of DNA-based subtyping methods like the pulsed-field gel electrophoresis (PFGE) (Olson *et al.,* 1994). This is a technique used for the separation of large deoxyribonucleic acid (DNA) molecules by applying to a gel matrix an electric field that periodically changes direction. It has over the years, earned the reputation of the gold standard method for this purpose using alternating voltage gradient to improve the resolution of larger molecules (Schwartz & Cantor, 1984). PFGE's limitations include the fact that it is technically demanding and a non-automated method and these limitations have affected the reporting of some disease outbreaks in the past (Hedberg *et al.,* 2008).

In order to obtain more rapid results, the multilocus variable number of tandem repeats (VNTR) analysis (MLVA), which is based on the number of contiguous DNA repeats present at several loci was developed (Fabre *et al.*, 2012). This technique takes advantage of the polymorphism of tandemly repeated DNA sequences in the genetic analysis of pathogenic bacteria. Well-characterised target loci are amplified by polymerase chain reactions (PCR) to determine loci sizes, which are in turn used in determining the number of repeat units on each locus. For *Salmonella* serovar Typhimurium, MLVA assays on five well-characterised loci (with repeat units of 6 to 33 bp) have been standardised for serotyping (Lindstedt *et al.*, 2003). It's advantage over PFGE is that it is rapid, technically simple and suitable for the processing of large numbers of isolates and can distinguish between clonal isolates indistinguishable by PFGE (Fabre *et al.*, 2012). Its limitations however, include that the current assays available are standardised for only two *Salmonella* serotypes, Typhimurium and Enteritidis (van Belkum *et al*., 2007). The repetitive DNA sequences that current assays are based on may also evolve too rapidly, leading to changes in repeat numbers during the course of an outbreak (Hopkins *et al.*, 2007; Hopkins *et al.*, 2011). It is thus advisable to use MLVA assays in conjunction with PFGE or phage typing particularly when monitoring outbreaks (Fabre *et al.*, 2012). Having explored various techniques and their limitations, scientists are now exploring the use of the CRISPR locus of *Salmonella* in managing outbreaks.

The CRISPR locus (Clustered Regularly Interspaced Short Palindromic Repeats) is a genetic marker that allows the identification and genotyping of many pathogenic bacteria. It was characterised at the Netherlands National Institute for Public Health and the Environment (Dutch: *Rijksinstituut voor Volksgezondheid en Milieu* - RIVM) in Netherlands under the name of Direct Repeat locus in *Mycobacterium tuberculosis*, the agent of Tuberculosis. It was used to invent the spoligotyping technique and the Tuberculosis-Spoligo-Rifampin-Isoniazid Typing (TB-SPRINT) assay (IGM, 2014).

Jansen *et al*. (2002) identified a new family of repeated DNA sequences, named CRISPR (clustered regularly interspaced short palindromic repeats) in many prokaryotes (Jansen *et al.*, 2002). This family is characterised by 24–47 bp DNA direct repeats (DRs), separated by variable 21–72 bp sequences called "spacers"(Grissa *et al.*, 2007; Horvath & Barrangou, 2010). A leader

sequence and *cas* (CRISPR-associated sequence) genes are often identified adjacent to the CRISPR locus.

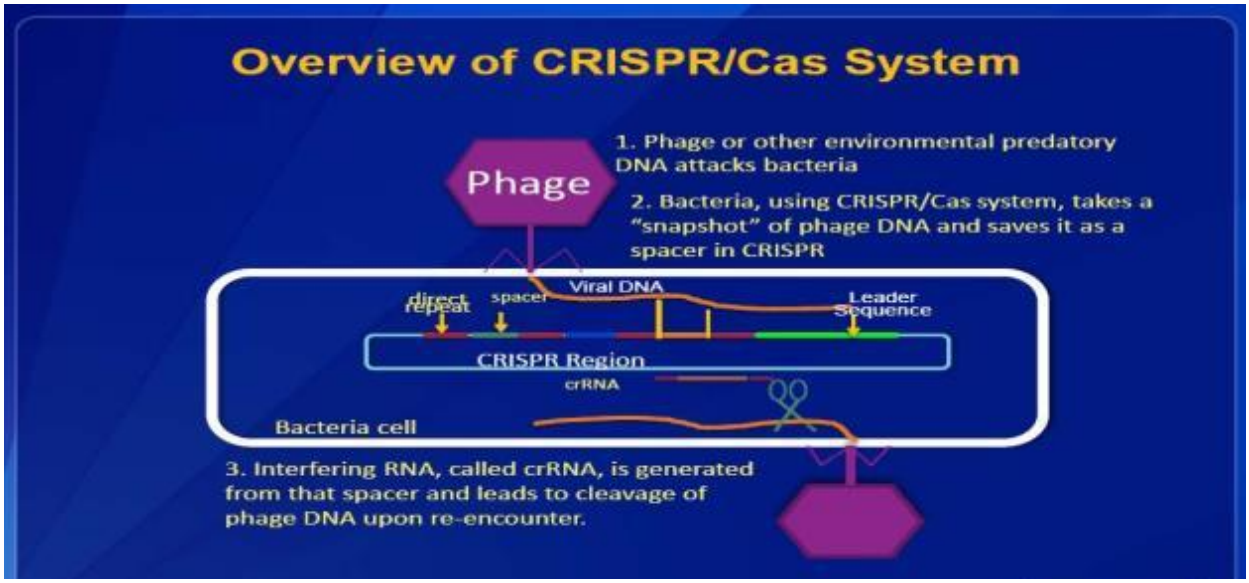

Figure 19: An overview of the CRISPR/*cas* system (Carleton, 2013)

CRISPR loci are present in 100% of Archaea and 60% of bacteria and are believed to encode a sequence-specific defence mechanism against bacteriophages and plasmids (Wiedenheft *et al.*, 2012). CRISPR seem to confer resistance to foreign DNA, such as plasmids and phages, and the newly integrated spacers are derived from the invading DNA (Barrangou *et al.*, 2007; Garneau *et al.*, 2010).

Subtyping methods based on analyses of the spacers of CRISPR loci have since been developed for bacteria of medical interest, such as *Yersinia pestis* (Pourcel *et al.*, 2005), *Corynebacterium diphtheria* (Mokrousov *et al.*, 2007) and *Campylobacter* (Schouls *et al.*, 2003). CRISPR typing aids in "taxonomic characterization of bacterial species, both intra-specific and bio-geographic for molecular epidemiology or populations structure studies" (Fabre *et al.*, 2012). *Salmonella* has 2 CRISPR regions each with 36 analysed spacer regions totalling 72 Plex for *Salmonella enterica* serovar Typhimurium analysis and this is well-adapted to outbreak tracking and inter-laboratory comparisons (Liu F, *et al*. 2011).

Research Aims

1. To analyse the spacer content of the two CRISPR loci in a collection of reference *Salmonella enterica* serovar Typhimurium strains in the Faculty of Veterinary Science, University of Pretoria, South Africa as well as serologically unconfirmed strains from non-human primates in South Africa.
2. To analyse the distribution of the unique spacers identified using BioNumerics and other statistical software and in turn use them in genotyping the strains.
3. To update the open web-accessible database providing a serotype/spacer dictionary and an international tool for strain tracking.

Material and Methods

Sampling

Stored bacterial strains from the bacteriology laboratory of the Faculty of Veterinary Science were inoculated on Columbia blood agar and incubated at 37^{o}C for 24 hours. The DNA of the isolates was then extracted using QIAamp DNA Mini Kit (QIAGEN, South Africa).

Faecal samples from baboons and vervets in South Africa were similarly inoculated on Columbia blood agar and incubated at 37^{o}C for 24 hours. Suspect *Salmonella* spp. Colonies were then transferred unto *Salmonella-Shigella* agar and incubated at 37^{o}C for 24 hours. DNA extraction was then done on pure colonies using QIAamp DNA Mini Kit (QIAGEN, South Africa) The DNA of the strains were then packaged on ice and sent to the Institut de Génétique et Microbiologie in Orsay, France to be analysed by me.

Analysis

Ninety-five *Salmonella* isolates were analysed using the CRISPR polymorphism (CRISPOL) technique of which 45 were confirmed *Salmonella enterica* serovar Typhimurium isolated from horses and cattle in South Africa. The remaining 50 suspect *Salmonella* were from unserotyped non-human primates described in chapter one. This CRISPOL technique has been standardised for *Salmonella enterica* serovar Typhimurium strains involving 72 spacer regions to be amplified. A 25 to 32 bp capture probe was designed for each of 72 of the 79 spacers identified by Fabre *et al.*,(2010) in *Salmonella enterica* serovar Typhimurium strains.

Bead Coupling: The probes to be used for the CRISPR analysis first had to be coupled to microbeads (xMAP beads) before analysis starts. 72 microbead sets are each coupled to probes and each capture probe was coupled to a defined xMAP bead. The probes were grafted on the microbeads in series. Each probe is covalently bound to the microbeads by performing a covalent bond with 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) – a water soluble carbodiimide usually used as a carboxyl activating agent for the coupling of primary amines to yield amide bonds.

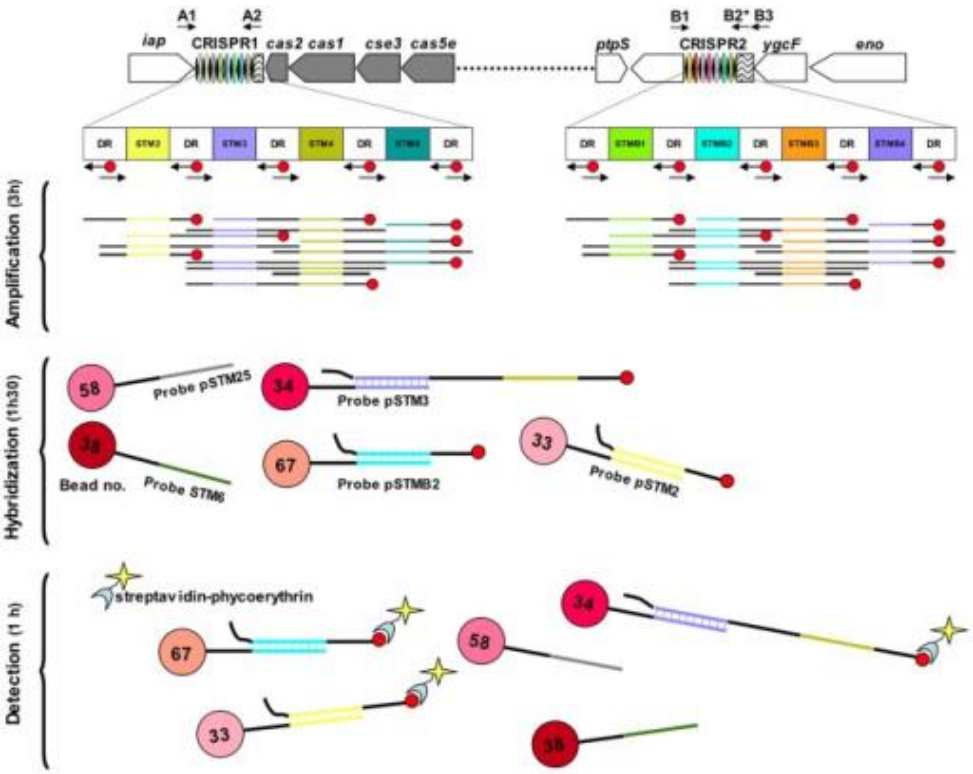

Figure 20: An overview of the bead-based CRISPOL assay for *S. enterica* serovar Typhimurium

CRISPOL PCR: After coupling, beads must be tested using standard controls (confirmed DNA samples) to be sure the bead concentrations and couplings are of standard. A conventional PCR was run with 2 control DNA samples and 2 blanks (water controls) using primers specific for *Salmonella*.

Direct Multiplexed Hybridization: The beads and PCR products were then hybridized by mixing and incubating standard amounts of each according to a standard protocol. The PCR mixture was hybridized with the 72 probe-coupled beads and incubated with streptavidin-phycoerythrin for detection.

The mixture was then analysed using the Luminex technology CRISPOL program that is programmed to take 45 seconds per well/sample to analyse. The technology measures the fluorescence associated with each bead (corresponding to a unique probe/spacer)

Results

Bead readings

The luminex system read mean fluorescence signals of 200 to 2,100 in the presence of the spacer, and of 12 to 150 in the absence of the spacer.

Table 7: A comparison of probe responses in the standard CRISPOL assay against the responses obtained

Probe name (bead no.)	Spacer absent Median (MFI)±SD1 – Original Research – Fabre *et al.*,	Spacer absent Median (MFI)±SD1 – Our Research	Spacer present Median (MFI)±SD – Original research – Fabre *et al.*,	Spacer present Median (MFI)±SD – Our research
pBraB14(29)	65+/-10	65+/-10	3866+/-570	1000+/-800
pSTMB35(30)	70+/-14	70+/-14	3540+/-445	1000+/-800
pHadB20(31)	62+/-8	62+/-8	1544+/-200	1000+/-800
pSTM01(32)	53+/-9	53+/-9	2547+/-332	1000+/-800
pSTM02(33)	81+/-12	81+/-12	2586+/-308	1000+/-800
pSTM03(34)	56+/-4	56+/-4	3808+/-5394	1000+/-800
pSTM03var1(35)	52+/-3	52+/-3	2409+/-5324	1000+/-800
pSTM04(36)	57+/-8	57+/-8	4187+/-390	1000+/-800
pSTM05(37)	173+/-13	173+/-13	5216+/-605	1000+/-800
pSTM06(38)	64+/-4	64+/-4	3019+/-337	1000+/-800
pSTM07(39)	66+/-11	66+/-11	4580+/-4944	1000+/-800

pSTM07var2(40)	65+/-13	65+/-13	3283+/-4714	1000+/-800
pSTM08(41)	55+/-7	55+/-7	709+/-154	1000+/-800
pSTM09(42)	68+/-11	68+/-11	4065+/-397	1000+/-800
pSTM10(43)	94+/-17	94+/-17	4036+/-402	1000+/-800
pSTM11(44)	62+/-9	62+/-9	4564+/-518	1000+/-800
pSTM12(45)	69+/-11	69+/-11	3343+/-5154	1000+/-800
pSTM12var1(46)	61+/-8	61+/-8	1173+/-2804	1000+/-800
pSTM13(47)	58+/-7	58+/-7	3812+/-539	1000+/-800
pSTM14(48)	59+/-8	59+/-8	4183+/-381	1000+/-800
pSTM15(49)	72+/-14	72+/-14	3458+/-452	1000+/-800
pSTM16(50)	71+/-13	71+/-13	4990+/-1061	1000+/-800
pSTM17(51)	89+/-15	89+/-15	2092+/-333	1000+/-800
pSTM18(52)	79+/-14	79+/-14	3863+/-610	1000+/-800
pSTM19(53)	68+/-15	68+/-15	3359+/-289	1000+/-800
pSTM20(54)	65+/-9	65+/-9	3113+/-375	1000+/-800
pSTM21(55)	74+/-14	74+/-14	4100+/-573	1000+/-800
pSTM22(56)	71+/-9	71+/-9	2158+/-304	1000+/-800
pSTM24(57)	50+/-6	50+/-6	4603+/-440	1000+/-800
pSTM25(58)	60+/-11	60+/-11	4178+/-382	1000+/-800
pSTM26(59)	62+/-7	62+/-7	4772+/-521	1000+/-800
pSTM27(60)	59+/-10	59+/-10	4061+/-364	1000+/-800
pSTM28(61)	61+/-9	61+/-9	4186+/-328	1000+/-800

pSTM29(62)	61+/-9	61+/-9	3508+/-418	1000+/-800
pSTM30(63)	73+/-11	73+/-11	4532+/-670	1000+/-800
pSTM31(64)	68+/-10	68+/-10	3209+/-409	1000+/-800
pSTMB0(65)	55+/-12	55+/-12	3952+/-398	1000+/-800
pSTMB01(66)	64+/-16	64+/-16	5409+/-674	1000+/-800
pSTMB02(67)	65+/-11	65+/-11	3987+/-500	1000+/-800
pSTMB03(68)	58+/-9	58+/-9	2508+/-713	1000+/-800
pSTMB04(69)	68+/-13	68+/-13	4913+/-558	1000+/-800
pSTMB05(70)	60+/-8	60+/-8	5196+/-574	1000+/-800
pSTMB06(71)	61+/-8	61+/-8	1802+/-343	1000+/-800
pSTMB07(72)	62+/-10	62+/-10	4447+/-477	1000+/-800
pSTMB08(73)	69+/-10	69+/-10	1210+/-4535	1000+/-800
pSTMB08var1(74)	69+/-10	69+/-10	1266+/-2515	1000+/-800
pSTMB09(75)	6+/-69	6+/-69	1266+/-251	1000+/-800
pSTMB10(76)	64+/-7	64+/-7	2629+/-480	1000+/-800
pSTMB11(77)	59+/-9	59+/-9	2036+/-279	1000+/-800
pSTMB12(78)	73+/-11	73+/-11	2147+/-276	1000+/-800
pSTMB13(79)	69+/-14	69+/-14	5142+/-805	1000+/-800
pSTMB14(80)	64+/-8	64+/-8	2034+/-289	1000+/-800
pSTMB15(81)	67+/-10	67+/-10	3524+/-466	1000+/-800
pSTMB16(82)	78+/-12	78+/-12	5062+/-547	1000+/-800
pSTMB17(83)	94+/-16	94+/-16	2947+/-437	1000+/-800

pSTMB18(84)	80+/-11	80+/-11	3745+/-532	1000+/-800
pSTMB19(85)	101+/-13	101+/-13	4570+/-733	1000+/-800
pSTMB20(86)	75+/-14	75+/-14	4573+/-483	1000+/-800
pSTMB21(87)	73+/-12	73+/-12	3556+/-319	1000+/-800
pSTMB22(88)	84+/-16	84+/-16	5707+/-636	1000+/-800
pSTMB23(89)	69+/-9	69+/-9	5434+/-642	1000+/-800
pSTMB24(90)	71+/-8	71+/-8	3525+/-450	1000+/-800
pSTMB25(91)	72+/-10	72+/-10	2039+/-395	1000+/-800
pSTMB26(92)	193+/-2443	193+/-2443	3599+/-664	1000+/-800
pSTMB27(93)	101+/-15	101+/-15	3464+/-505	1000+/-800
pSTMB28(94)	87+/-12	87+/-12	3218+/-391	1000+/-800
pSTMB29(95)	87+/-13	87+/-13	3542+/-519	1000+/-800
pSTMB30(96)	79+/-10	79+/-10	3805+/-442	1000+/-800
pSTMB31(97)	83+/-15	83+/-15	2195+/-304	1000+/-800
pSTMB32(98)	83+/-12	83+/-12	4832+/-551	1000+/-800
pSTMB33(99)	70+/-8	70+/-8	2143+/-249	1000+/-800
pSTMB34(100)	78+/-11	78+/-11	3109+/-513	1000+/-800

Excel macro patterns

Numeric data outputs of the two CRISPR loci analyses were stored in Excel files in an already designed macro that had cut-off points determined in order to give a pattern as seen in the image below:

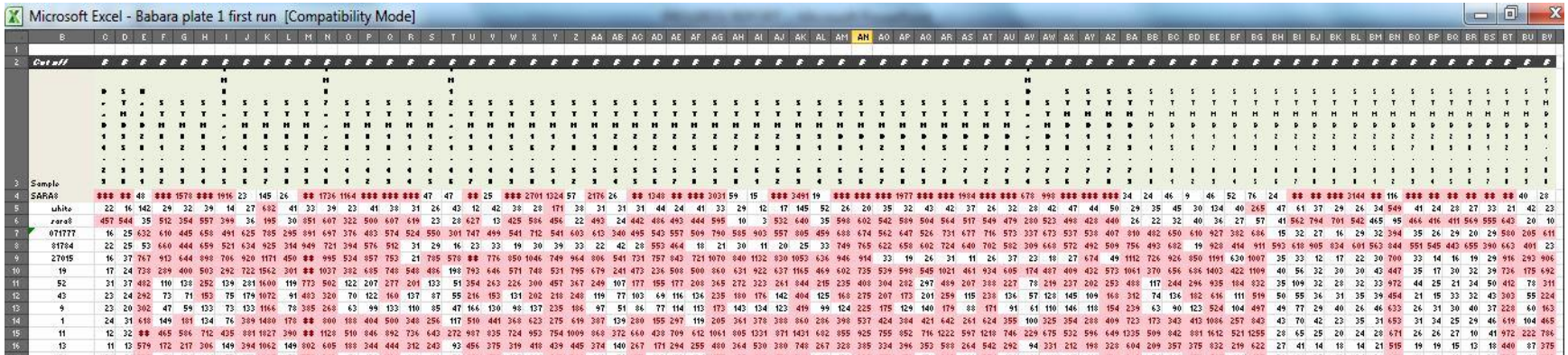

Figure 21: The beads reading analysed using Microsoft Excel

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Bionumerics Analysis

The data were then and exported into the Bionumerics software for data analysis, genetic relationship analysis between isolates and the construction of a phylogenetic tree for epidemiological analysis. The data exported to the Bionumerics software were first converted into categorical data and data were plotted with DICE and WARD statistical tools. Black squares indicate presence of the spacer, as detected by the corresponding probe, whereas white indicates an absence of the spacer. Each of the spacers were treated as a numerical character indicating absence (0) or presence (1)

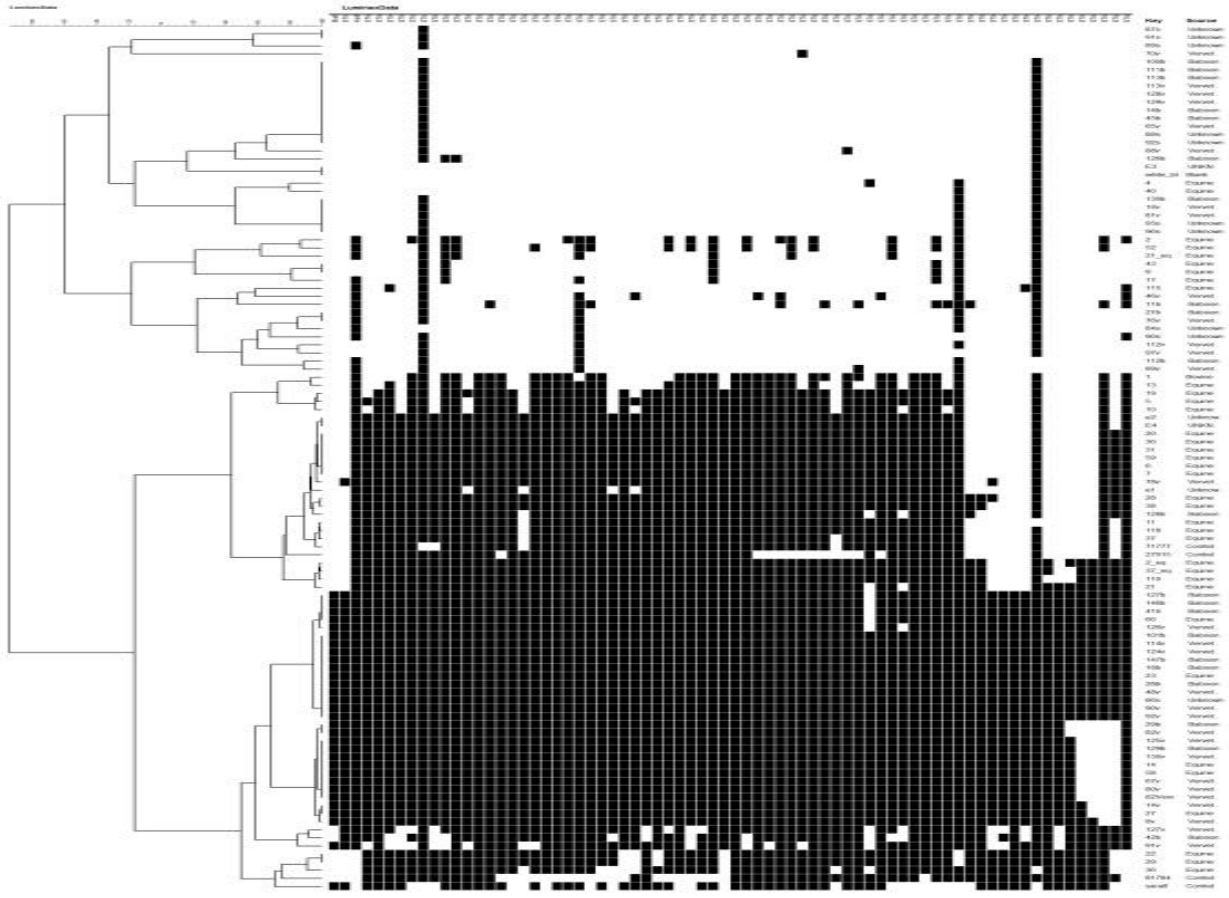
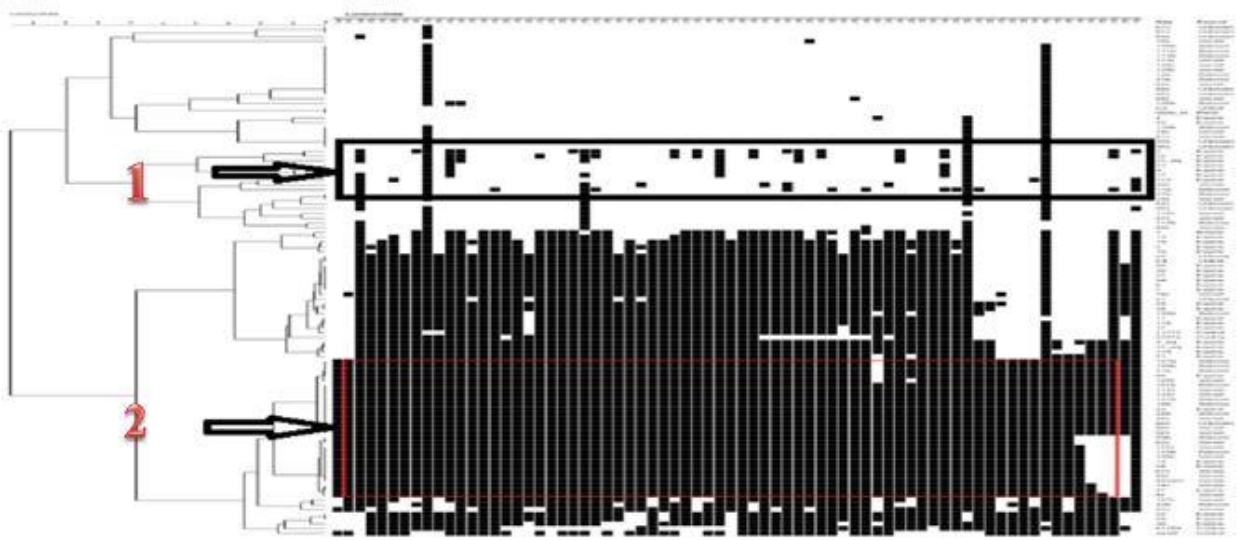


Figure 22: A phylogenetic tree of the CRISPR 1 and 2 loci patterns showing interrelatedness of all 95 *Salmonella* isolates analysed using the Dice index on BioNumerics software

Discussion

Bead readings of amplified spacer regions were particularly low for all the strains as compared to reference strains in Europe that are used in international CRISPR analysis. Repeated analysis gave lower readings up until the point where continuous thawing of the DNA samples lead to its lysis. In the original research by Fabre *et al* (2012), mean fluorescence signals of 709 to 5,707 in the presence of the spacer, and of 52 to 193 in the absence of the spacer were recorded. In our research however, bead readings were diverse with larger standard deviations. Mean fluorescence signals of 200 to 2,100 in the presence of the spacer, and of 12 to 150 in the absence of the spacer were recorded. This could not be explained.



Unusual patterns were observed in some of both the confirmed and unconfirmed *Salmonella enterica* serovar Typhimurium strains whereby all spacer regions targeted were amplified thereby making the interpretation of the data generated impossible. Spacer regions that fail to amplify in test strains aid in tracking the epidemiological backgrounds of strains being analysed by comparison with reference strains with accurate epidemiological data. Thus, strains that have all spacer regions amplified cannot be analysed with data currently available since it is new to the system. The phylogenetic tree showed some strains that had all spacer regions amplified – making the interpretation of their genotypes difficult. Area 1 showed strains that had usual CRISPR 1 and 2 loci patterns whilst area 2 showed strains that had unusual CRISPR 1 and 2 loci patterns

This challenge could be due to several reasons discussed below.

Perhaps, these challenges can be associated with similar challenges faced by Carleton (2013) in a similar project using the CRISPR system in subtyping the 30 *Salmonella* serotypes through a combination of CRISPR elements and virulence genes. Various challenges were encountered in their analysis and listed in their report, similar to the experience in this research project.

First of all, it is difficult to design universal primers for all *Salmonella* serotypes. A *Salmonella* serotype is determined by the immunoreactivity of three surface antigens - O (LPS), H (flagellin protein), and Vi (capsule) (WHO, 2010). Over 2500 *Salmonella* serotypes, also referred to as serovars, are known and may potentially cause illness in humans and animals (FAO, 2002). This makes it practically impossible to design a primer that caters for all these serotypes. Even within serotypes, there are still variations that limit the use of some primers. It is suspected that the primers used in Europe might not be specific enough for southern African strains. Some of both the serologically confirmed Typhimurium and unconfirmed Typhimurium strains had amplifications of all spacer regions which implies that there are some observable differences between the strains in South Africa and those in Europe

Secondly, the same primer design does not work for all isolates within the same serotype. As earlier stated, strains serologically identified as Typhimurium were expected to produce results for interpretation and genotyping. However, some of these strains showed amplifications in all spacer regions. Variations within serotypes would reduce primer/probe specificity for the spacer regions being studied and this would result in such unique results. Lack of specificity in the CRISPR/*cas* system due to the presence of multiple DNA sequences highly homologous to target DNA sequences have been experienced in gene editing (Zhang, 2014) and have resulted in off-target mutations.

There are reported issues with CRISPR region variation and size. As more spacers are added the CRISPR region becomes larger, pushing the limits of what can be feasibly sequenced. The particular results generated in this project could also be attributed to the number of spacer regions targeted by the current assay. This is because spacer regions keep increasing as phage attacks on the bacteria increases. Thus strains from South Africa might have more spacer regions than those studied – leading to the amplifications of all regions studied. It is believed that the parental strain of *Salmonella* is of African origin (Feasay *et al.,* 2012). This could be the reason why the strains

would have more spacer regions as they are expected to have been more exposed to phage attacks than the European counterparts.

Conclusions

The use of the CRISPR/*cas* system in genotyping is a cost-effective, rapid method, which when standardised, would be efficient in monitoring *Salmonella* outbreaks in especially developing countries by determining interrelatedness of outbreak strains for epidemiological purposes.

Genotyping of South African Typhimurium strains on this system have not previously been attempted but is important. Further research is required to determine the specificity of the primer and probes used in the research. In addition, since CRISPR genotyping has only been done on Typhimurium strains from humans, more research should be done on strains from veterinary sources to determine if there are any observable differences.

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