Wild and domestic animals as reservoirs of antibiotic resistant *Escherichia coli* in South Africa

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Thesis presented in fulfilment of the requirements for the degree of
Master of Science: Microbiology

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January 2017

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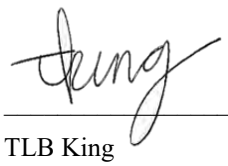

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Declaration 2 – Supervisor's Declaration

Submitted in fulfilment of the academic requirements for the degree of Master of Science in the School of Life Sciences, Discipline of Microbiology, University of KwaZulu-Natal, Pietermaritzburg campus

January 2017

As the candidate's supervisor, I have approved this thesis for submission.

Supervisor

Signed:  Name: S. Schmidt Date: January 2017

Declaration 3 – Publications and Conference Proceedings

Details of publications/conferences that form part and/or include research presented in this thesis:

1. **South African Society for Microbiology 19th Biennial Congress**

   Oral Presentation: Screening of *Escherichia coli* isolated from wild herbivores and farm animals for multidrug resistance and presence of resistance genes

   January 2016; Durban, South Africa

2. **European Congress for Clinical Microbiology and Infectious Diseases**

   Oral Presentation: Assessment of indigenous South African herbivores as potential reservoirs for multidrug-resistant *Escherichia coli*

   April 2016; Amsterdam, the Netherlands

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05 January 2017

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Abstract

Antibiotic resistance is a global health concern, addressed by the World Health Organization, the CDC, EFSA and the United States White House. This issue is primarily a consequence of the overuse and misuse of antibiotic agents in both human and veterinary medicine. However, as of 2016, information regarding the presence of antibiotic resistant bacteria in different environments in South Africa is limited. The objective of this research was to provide an insight into the potential for South African herbivores to serve as reservoirs or vectors for antibiotic resistant *Escherichia coli.* The antibiotic resistance profiles for *E. coli* isolated from wildebeest, zebra and giraffe faeces were established, in addition to resistance profiles of *E. coli* from both pet and farm pig faeces. A standard MPN guideline procedure was used to quantify and isolate total and faecal coliforms and *E. coli.* Twelve antibiotics representing seven antibiotic classes were selected, and profiles for 30 confirmed *E. coli* isolates per animal were determined using the European Committee on Antimicrobial Susceptibility Testing (EUCAST) disk diffusion procedure.

The $\log_{10}$MPN values per gram of animal faeces for total/faecal coliforms were between 4.51/4.11 and 5.70/5.50 $\log_{10}$MPN/g, while the results for *E. coli* were in the range of 3.23-5.14 $\log_{10}$MPN/g for all animal faeces. There was no incidence of carbapenem or glycylcycline resistance for any of the 150 *E. coli* isolates; and only one farm pig *E. coli* isolate presented resistance to the fluoroquinolone antibiotics norfloxacin and ciprofloxacin. Antibiotics to which the largest proportions of resistance occurred were amoxicillin-clavulanic acid, ceftazidime and tobramycin. 47% of *E. coli* isolates from zebra were multidrug resistant (MDR, resistant to at least one antibiotic from three or more different classes), with no giraffe or wildebeest faecal *E. coli* isolates demonstrating MDR. Of *E. coli* isolated from farm and pet pig, 7% and 10% were MDR, respectively.

Of 150 *E. coli* isolates screened for antibiotic resistance, 102 (68%) demonstrated resistance to at least one β-lactam antibiotic (ampicillin, amoxicillin-clavulanate, ceftazidime, aztreonam or a combination thereof). These isolates were screened for the presence of the β-lactamase gene bla_{TEM}. The bla_{TEM} gene was only detected in three farm pig *E. coli* isolates. All of these 102 *E. coli* isolates were screened for β-lactamase activity using the colorimetric substrate nitrocefin, and the specific β-lactamase activity was determined for a selection of isolates. Of the tested β-lactam-resistant *E. coli* isolates, 64 were positive for β-lactamase activity (63%)

using nitrocefin as test substrate, with 38 isolates failing to hydrolyse nitrocefin (37%). The specific β-lactamase activity was determined spectrophotometrically for ten *E. coli* isolates from a combination of herbivore and pig faeces with diverse phenotypic resistance profiles and nitrocefin hydrolysis results. For those *E. coli* isolates unable to hydrolyse nitrocefin (2 pet pig isolates), the resultant specific activity was <1 nmol × min^{-1} × mg^{-1}. Five nitrocefin-positive *E. coli* isolates from herbivore faeces had specific activities of between 2 and 3 nmol × min^{-1} × mg^{-1}, while the specific β-lactamase activity of three farm pig *E. coli* isolates containing the bla_{TEM} gene was between 81 and 168 nmol × min^{-1} × mg^{-1}.

The results of this study indicated that South African herbivores serve as reservoirs and thus potential vectors of antibiotic resistant *E. coli*. Furthermore, this study confirmed that companion (pet pig) as well as farm animals (farm pig) in South Africa may harbour antibiotic resistant bacteria, with some carrying β-lactam resistance determinants such as the bla_{TEM} gene. Lastly, while an isolate may present phenotypic β-lactam resistance, it does not automatically indicate that a β-lactamase enzyme confers the resistance; but rather the presence of alternate mechanisms including reduced expression of porin genes or mutated porins.

Acknowledgements & Dedication

I would like to extend my sincere gratitude to the following people and institutions for their contributions and support during this degree:

Prof. Stefan Schmidt for his vital assistance, support and sense of humour as my supervisor;

Dr. Maike Claußen for valuable editing assistance and encouragement;

The National Research Foundation (NRF) and the University of KwaZulu-Natal for financial support;

My friends and lab-mates: Kimberley, Jus, Alex, Tash and Sel for emotional support and great memories I will always appreciate;

To my family: Dave, Cooks, Baboo, Andrew, Shasha, Courtney, Nathan and Jackie - I will forever be greatful for the endless love and encouragement you have all shown me.

An extra special mention to my mom, Jackie, who is quite simply the strongest woman I know. You inspire me daily.

This thesis is dedicated to my dad, Edmund King. I will always cherish your constant encouragement and belief in me. I love and miss you.

CONTENTS

This thesis represents a compilation of manuscripts where each chapter is an individual entity, formatted as per the European Journal of Wildlife Research. Some repetition between chapters has thus been unavoidable.

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Introduction and Literature Review

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1. Antibiotic resistance

Antibiotic resistance, or the ability of a microorganism to withstand the effects of such a drug, has developed into a global health issue, recognized and addressed by organizations including the World Health Organization (WHO), the Centres for Disease Control, the United States White House and the European Food and Safety Authority (EFSA 2012; Tadesse et al. 2012; WHO 2014; White House 2014). As a result, surveillance programs have emerged with the intention of estimating the extent of antibiotic resistance as well as tracking the evolution thereof.

According to the WHO 2014 global antimicrobial resistance surveillance report (2014), high resistance rates are frequently observed in bacteria commonly associated with healthcare and community-acquired infections, across all WHO regions. This report amalgamated data from 114 countries representing the six WHO regions including Africa, the Americas, Europe, South-East Asia, the Eastern Mediterranean and the Western Pacific. With regard to regional African data, there are significant gaps in surveillance for many countries, including South Africa, which is entirely absent from the report. Furthermore, the issue of global non-consistency concerning methodology and data collection protocols presents a problem when surveillance data are compared. In the latest 2014 surveillance report, the African region was poorly represented with only 8 out of 47 member countries submitting data - a 17% participation rate (WHO 2014). The report highlighted that selected bacteria such as *Escherichia coli* are showing resistance to a combination of fluoroquinolones and third generation cephalosporins in a number of African countries. It further reported antibiotic resistance among *Staphylococcus aureus* and enteric bacteria including *Klebsiella pneumoniae* and *Shigella* spp. (WHO, 2014). It is widely accepted and acknowledged that the overuse of antibiotics in both human and veterinary medicine is a key reason for the increasing emergence of both resistant bacteria and the dissemination of matching resistance genes. In addition to indiscriminate antibiotic use among humans, the misuse of such agents in food animals is also a contributing factor to the horizontal transfer of resistance genes and thus the increase of antibiotic resistant bacteria (Allen et al. 2010; Wellington et al. 2013). The interconnectivity of factors including the environment, farming practices including animals and crops, wild and companion animals, and humans with antibiotic resistance transmission is summarised in Figure 1.

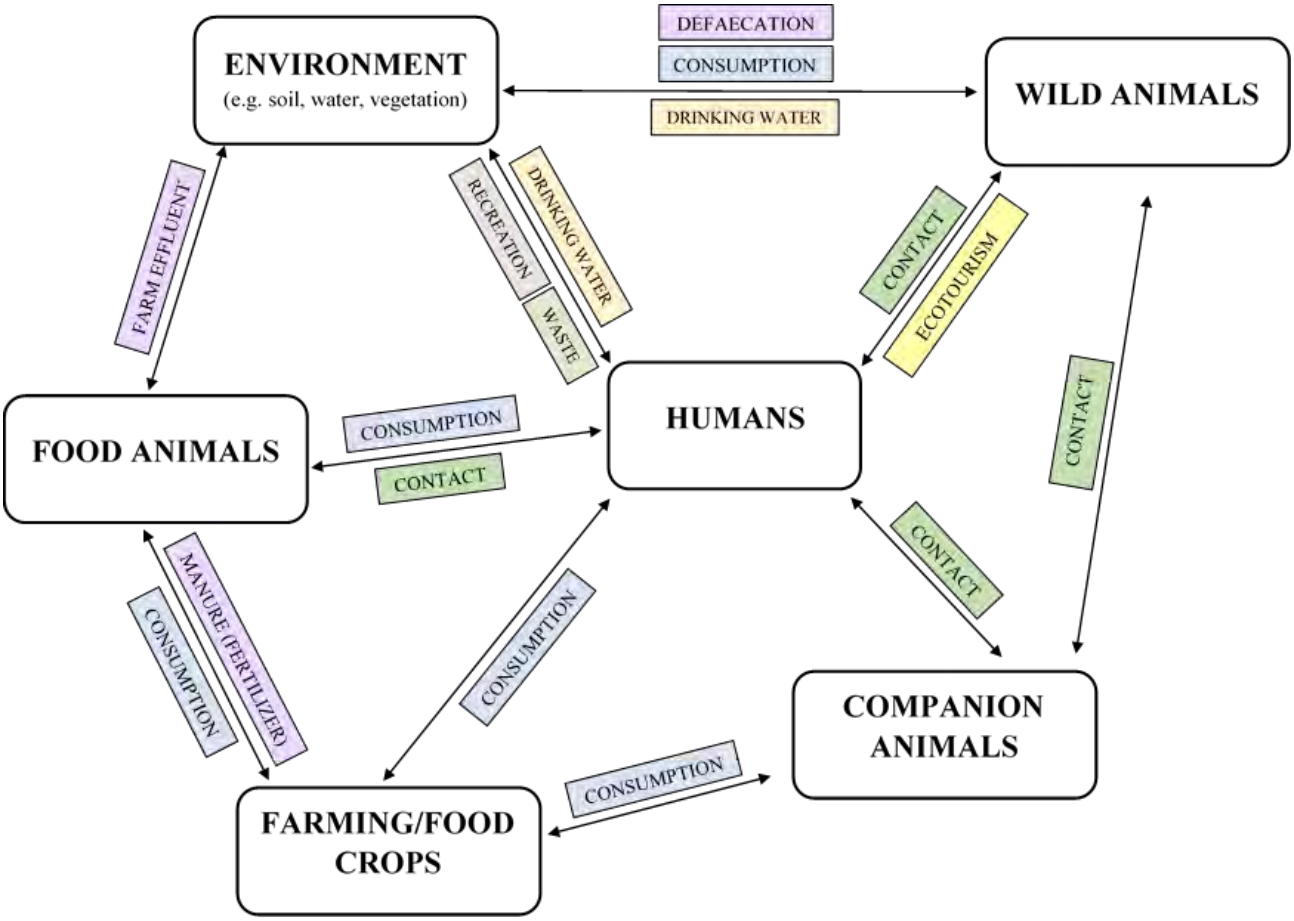

FIGURE 1. Potential connections between farming practices, companion and wild animals, humans and the environment and antibiotic resistance transmission

Developing countries (including South Africa) are particularly affected by spreading antibiotic resistance. This is in part due to widespread poverty and lack of adequate sanitation and clean water (Schellack et al. 2011), which in turn can lead to untreatable common diseases. Farm animals, whether exposed through antibiotic prophylaxis or via growth-promoters, may serve as a reservoir for harmful antibiotic resistant zoonotic bacteria (Sørum and L'Abée-Lund 2002; Silbergeld et al. 2008; EFSA 2016). Direct human contact with the animals or ingestion of animal by-products may cause acquisition of infections caused by zoonotic antibiotic resistant bacteria [Figure 1] (Hammerum and Heuer 2009). Apart from emergence in clinical and farm environments, antibiotic resistance has also been observed in what would be considered "pristine habitats" - for example in unexposed environments including forests, deserts and savannahs. Primates, small mammals and birds of prey from such "pristine" environments have been analysed in recent years for the presence of antibiotic resistant bacteria (Rolland et al.

1985; Rwego et al. 2008; Radhouani et al. 2009 & 2012; Pesapane et al. 2013; Jobbins and Alexander 2015), with a variety of resistant and multidrug resistant (resistance to antibiotics from three or more different classes) bacterial phenotypes emerging. The monitoring of resistance profiles of bacteria isolated from animals in the "wild" is important as increased anthropological activity and encroachment into the habitats of wild animals could lead to an increase in interspecies disease transmission, involving hard to treat antibiotic resistant bacteria [Figure 1] (Katakweba et al. 2015). In addition, as animals inhabiting pristine environments could serve as reservoirs or vectors for the transmission of antibiotic resistant bacteria and corresponding resistance genes; an assessment of the current situation could curtail further transfer.

2. Animals as reservoirs and vectors for the dissemination of antibiotic resistance

The misuse of antibiotics in disease treatment has resulted in the development of resistances by bacteria (Radimersky et al. 2010). Although bacterial antibiotic resistance is widely observed in human and domestic animal populations, studies have acknowledged the presence of antibiotic resistance in commensal *Escherichia coli* isolated from wild animals not directly exposed to antibiotic treatment (Čížek et al. 2007; Literak et al. 2007; Radimersky et al. 2010; Vittecoq et al. 2016). Antibiotic agents exert a selective pressure on both pathogenic and commensal bacteria colonizing the human and animal gastrointestinal tract. The emergence and subsequent spread of resistances within and between bacterial genera can be attributed to, among other factors, the intensive use of antibiotic drugs in medicine, livestock production, agriculture and aquaculture (Silva et al. 2010; Graham et al. 2016). *E. coli* is a common hygiene indicator organism, useful to "track the evolution of antimicrobial resistance in different ecosystems" (van den Bogaard and Stobberingh 2000; Leclerc et al. 2001), and thus an excellent model organism for such studies. Although only certain strains of *E. coli* are pathogenic, *E. coli* may serve as a reservoir of antibiotic resistance genes with the potential for transfer to a variety of pathogenic bacteria. This in turn could result in diseases caused by pathogenic microorganisms rendered untreatable by common antibiotics.

The use of antibiotics in animals has been a long-standing global controversy, particularly with regard to those used for growth promotion or to increase feed-efficiency (Furtula et al. 2010). Similarly, certain classes of antibiotics including the β-lactams and fluoroquinolones have prominent use in human medicine, however these are further utilized in veterinary environments. The widespread and unrestricted use of antibiotics in human and animal environments causes selection of antibiotic resistant bacteria and the eventual emergence and distribution of these bacteria and their resistance genes throughout the food chain (Meyer et al. 2013). Table 1 depicts selected antibiotic categories which are of importance in human and veterinary disease treatment as well as agriculture; and summarizes the modes of antibiotic action and the resistance mechanisms that have developed.

TABLE 1. Important antibiotic families with selected representatives and their principal resistance mechanisms

Antibiotic family	Antibiotic examples	Mode of antibiotic action	Resistance mechanism
β-Lactams	Ampicillin, Cefotaxime, Aztreonam	Inhibition of cell wall biosynthesis	Enzymatic inactivation of the antibiotic (β-lactamases), and mutation of penicillin binding proteins; porins
Fluoroquinolones	Norfloxacin, Ciprofloxacin	Inhibition of DNA gyrase during DNA replication	Mutation of DNA gyrase and drug efflux
Aminoglycosides	Gentamycin, Tobramycin	Impairment of codon-anticodon interaction causing accumulation of defect proteins	Enzymatic inactivation/ modification of the antibiotic
Tetracyclines/ Glycylcyclines	Tigecycline	Inhibition of protein synthesis (affect t-RNA binding to 30S ribosome)	Efflux of drug and enzymatic inactivation of the antibiotic

Walsh 2000; Chopra and Roberts 2001; Hooper 2001; McDermott et al. 2002

2.1 Farm and companion animals

The increasing demand and sheer scale of livestock production has resulted in an intensification of production systems, leaving no tolerance for the possibility of disease outbreak (Barton 2014; Rushton 2015). Concurrently, Van Boeckel et al. (2015) predicted that the annual raising and consumption of pigs in China totals approximately half the entire planet's pork industry.

With the yield of pork increasing every year, the amount of manure produced increases, and thus the potential for excretion of antibiotic residues into the environment (Larson 2015). Antibiotic prophylaxis in such intense livestock production systems occurs for purposes including maintaining the health of the pigs as well as growth promotion, and although regulations to curtail antibiotic use have been implemented in many countries (Landers et al. 2012), direct use of antibiotics in animal feed in many countries is still poorly controlled worldwide. A molecular typing study undertaken by Hu et al. (2013) involved *E. coli* isolated from pig faeces and nearby water sources. Results indicated that all isolates presented resistance to the cephalosporin antibiotic cefotaxime, and approximately 65% of isolates were resistant to the fluoroquinolone antibiotic ciprofloxacin (Hu et al. 2013). The latter in particular is a concerning statistic considering ciprofloxacin is a second-generation fluoroquinolone antibiotic with less than twenty years of FDA approved clinical use thus far (Goossens et al. 2007). Moreover, the overuse of antibiotics by humans worldwide contributes to the introduction of antibiotic compounds into rivers via waste water treatment plants, as antibiotic residues are not sufficiently removed by waste-water treatment plants (Xu et al 2007). Consequently, an eventual dissemination of transferable antibiotic resistance genes may filter into drinking water supplies, surface water, rivers and dams - thus potentially impacting marine life or the recreational activities of humans [Figure 1] (Korzeniewska et al. 2013).

There is a strong correlation between antibiotic use and the extent of antibiotic resistant *E. coli* isolated from livestock worldwide (Barton 2014). The use of antibiotics as growth promoters in swine is considered particularly controversial. Herein, the growth promoters are often of the same antibiotic families as those valuable or of critical importance in the treatment of human infections. The additives to pig feed are most often prepared by companies on the instruction of the farmer with no veterinary input (Barton 2014). The liberal use of such feed additives creates an ideal environment for selection of antibiotic resistant bacteria and the spread of such genes within the intestinal tract. Antibiotic growth promoters received a blanket ban by the European Union (for EU countries) in 2006, with other European countries following suit since (Maron et al. 2013). A second use of antibiotics in swine raising is prophylactic (individual animals receive antibiotic treatment) or metaphylactic (an entire pen receiving treatment) for the purpose of disease prevention (Barton 2014). A veterinarian-prescribed medicated feed is employed in a metaphylactic treatment plan, and is only utilized when a disease outbreak among the herd/flock emerges as a threat. Lastly, swine raising may utilize antibiotics for

therapeutic reasons, where injections or an oral dose is administered to individual pigs in the case of specific bacterial infections.

Due to the general absence of stringent guidelines as to the appropriate type of antibiotic to use and recommended dosage, widespread resistance has developed to agents including those belonging to the tetracycline, aminoglycoside, fluoroquinolone and penicillin classes (Hu et al. 2013; Wasyl et al. 2013). Callens et al. (2012) reported that due to a lack of prudent prescription guidelines in Belgium, antibiotics used in agriculture were administered at inadequate doses, and moreover belonged to classes considered important in human medicine. In Denmark, prescription of tetracyclines increased between 2002 and 2008. However, due to increased surveillance and stricter guidelines, the prescription of both fluoroquinolones and cephalosporins decreased within the same time period (Vieira et al. 2011). Furthermore, through the elimination of non-therapeutic antibiotic use in agriculture and animal raising, there has been an observed decline in the detection of resistance genes including $bla_{\text{CTX-M}}$ in soils (Graham et al. 2016). In the United States, tetracyclines remain the most prescribed antibiotics in animal husbandry, with Canada mirroring this trend (Apley et al. 2012; Glass-Kaastra et al. 2013). However, it is difficult to reliably quantify and describe antibiotic usage in animals in South Africa, as usage is controlled by two different national Acts, and sensitive information is closely guarded by pharmaceutical companies (Eagar et al. 2012). A 2012 study found that mean antibiotic sales for veterinary/agricultural use between 2002 and 2004 amounted to over 1.5 million kilograms (~ 1500 metric tons) of active ingredient (Eagar et al. 2012). The global consumption of antibiotics in food animal production alone is projected to increase by 67% from 2010 to 2030, with approximately 95 million kilograms (~ 95 000 metric tons) of antibiotics consumed by 2030 (Van Boeckel et al. 2015).

The use of poultry manure and litter as fertilizer has become common practice for farms in South Africa and overseas (Materechera 2010). In British Columbia (Canada), poultry litter is composted for use in organic crop farming and mushroom farming (Furtula et al. 2010). Of growing concern is the fact that growth-promoting antibiotics may be present in the manure, as many antibiotics are not fully absorbed by the animal gut and are thus excreted into the environment via the faeces [Figure 1] (Boxall et al. 2003; Kumar et al. 2005[a]). Veterinary pharmaceuticals present in the environment pose a potential health risk to humans, especially considering evidence suggesting that antibiotic agents may bioaccumulate in certain plants - a serious concern for food crops (Kumar et al. 2005[b]; Khan et al. 2007). The use of growth-

promoting antibiotics in feed may lead to a modification of the intestinal flora of poultry and other livestock, consequently creating a selective pressure favouring antibiotic resistant bacteria including *E. coli*. In turn, antibiotic resistant *E. coli* may be disseminated into the environment and food chain through faecal excretions and in the slaughtering and consumption process (Diarra et al. 2007; Furtula et al. 2010). Moreover, the avian intestine has been suggested to be a reservoir of *E. coli* with "zoonotic potential directly transferable to humans" (Ewers et al. 2009). Although *E. coli* is a commensal inhabitant of the human gastrointestinal system, pathogenic strains of *E. coli* have the ability to induce colibacillosis in chickens, as well as diseases including urinary tract infections, neonatal meningitis and septicaemia in humans (Amara et al. 1995; Ewers et al. 2004; Furtula et al. 2010). Some considerations with regard to attempts made to reduce the use of antibiotics in animals include the need to control infectious animals individually rather than employing a mass prophylaxis approach. In addition, housing and climate conditions of the animals should be optimized, and the level of biosecurity should be improved (Postma et al. 2016).

Companion animals are of importance in global antibiotic resistance research, particularly due to the consistent intimate contact they have with humans, and thus their putative role in the spread of zoonotic resistant bacteria (Ewers et al. 2012). While traditionally companion animals referred to dogs and cats, the term now encompasses a much wider variety of animals; from rabbits and mice to ferrets, reptiles and freshwater fish (Damborg et al. 2015). Although kept for company or oftentimes psychological support, the potential for colonization with a variety of zoonotic bacteria - and the likely transmission to humans - is concerning (Damborg et al. 2015). In the last decade, the number of reports on community and veterinary-associated infections has steadily increased. Resistance to antibiotics belonging to the third generation cephalosporins, fluoroquinolone and carbapenem classes has been detected in bacteria isolated from companion animals comprising dogs and horses in countries including Germany, the Netherlands and Australia (Platell 2010; Ewers et al. 2012; Stolle et al. 2013; Baede et al. 2015). Alternative antibiotic therapy has been suggested including glycopeptides and oxazolidinones, however as resistance emerges the antibiotic arsenal is reduced thus leaving few treatment options (Bengtsson and Greko 2014). A study analysing *E. coli* isolated from domesticated pets in China found almost 58% of isolates to be resistant to ciprofloxacin, a clinically important fluoroquinolone antibiotic (Yang et al. 2014). Moreover, resistance to antibiotics that have frequent use in farm environments (including fluoroquinolones) have been detected in *E. coli* isolates from companion animals. Of 1 263 *E. coli* isolates from livestock,

humans and companion animals (dogs, cats and horses) in Minnesota (USA), 31% presented resistance to tetracycline, of which 17% was attributed to isolates from companion animals (Bryan et al. 2004). Despite the scaling-back of β-lactam antibiotics for animal use in a number of countries (CDDEP 2016), resistance determinants including β-lactamase genes continue to emerge worldwide. The CTX-M enzymes (active on CefoTaXime, first isolated in Munich, elaborated upon later), in particular CTX-M-14, have been found to be the most prevalent type of β-lactamase enzymes detected in companion animal-sourced isolates from Asia (Ewers et al. 2012). Furthermore, the detection of two major β-lactamases, CTX-M-1 and SHV-12, were reported for the first time in *E. coli* from dogs and cats in Rome, Italy (Carattoli et al. 2005). The first detection of a New Delhi Metallo-β-lactamase-encoding gene (NDM-1) from companion animals occurred in the United States in 2013, in a meropenem resistant *E. coli* isolate recovered from dog urine (Shaheen et al. 2013). This finding is particularly concerning considering carbapenems' reputation as last resort drugs in human medicine, and emergence of resistance to such antibiotics may limit treatment options for humans who might contract *E. coli* infections from their pets (WHO 2012; Shaheen et al. 2013).

2.2 Wild animals

While indiscriminate antibiotic use in human and veterinary medicine is still considered a significant factor in the emergence and spread of antibiotic resistance, there has been particular focus on a potential co-emergence of resistant bacteria in animals and birds inhabiting wild ("pristine") environments. Such animals have proven to be reservoirs of bacteria harbouring antibiotic resistance genes, and in addition serve as vectors for the transfer of zoonotic bacteria between other animals and humans (Allen et al. 2010). Considering that the lifestyle habits of wild animals include an unrestricted capacity for relocation and migration, it has thus become vital to screen and continually monitor bacteria isolated from such animals for resistance determinants. The following table presents a summary of wild animals and birds from which resistant *E. coli* was isolated, along with corresponding antibiotics and the countries of origin.

TABLE 2. *Escherichia coli* isolated from various wild animals in different countries and antibiotic resistance profiles detected.

	Country of Origin	Animal	Antibiotic resistances detected *	Reference
Small mammals	Canada	Mice, Voles, Shrews	AMP; CHL; FOX; SIA STR; STX; TET	Kozak et al. 2009
	Canada	Mice, Voles, Shrews	AMC; AMP; FOX; CEF; CFX; GEN; KAN; STR; STX; TET	Allen et al. 2011
	Germany	Mice, Voles, Shrews	AMP; KAN; STR; STX; TET; TIC	Guenther et al. 2010
	Vietnam	Rats, Shrews	AMP; AMC; CHL; CIP; TET; TMP	Nhung et al. 2015
	Portugal	Rabbits	AMP; CIP; CHL; GEN; TET; TOB; STR	Silva et al. 2010
	Botswana	Banded Mongoose	AMP; CEF; CHL; CIP; DOX; GEN; NEO; STR; STX; TET	Pesapane et al. 2013
	Tanzania	Goat	AMP; AMC; STR; STX; TET; TMP	Mwanyika et al. 2016
	Mexico	Mexican Free-tailed Bat	AMP; NEO: STR	Souza et al. 1999
	Mexico	Howler Monkey	AMP; AMC; CHL; CIP; STX; TET	Cristóbal-Azkarate et al. 2014
	Australia	Brush-tailed Rock-wallaby	SPC; STR; TMP	Power et al. 2013
	Italy	Alpine Marmot	STR; TET; SUL	Caprioli et al 1991
	Canada	Raccoons (rural & urban)	AMC; AMP; CEF; CFX; CHL; CIP; FOX; GEN; NAL; SIA; STR; STX; TET; TMP	Jardine et al. 2012
Birds	Wales	Magpies	AMP; CHL; KAN; TET; TMP	Livermore et al. 2001
	Ireland	Gulls	PEN; STR; TET	Carroll et al. 2014
	Portugal	Seagulls	AK; AMC; AMP; CHL; CIP; GEN; NAL; STR; STX; TET; TOB;	Radhouani et al. 2009
	Portugal	Raven	CHL; STR; SXT; TET	Pinto et al. 2010
	Japan	Waterfowl	AMP; CHL; DSM; KAN; OXY; SDMX	Tsubokura et al. 1995

Large herbivores and omnivores	Czech Republic	Wild Boars	AMP; AMC; NAL; STR; STX; TET	Literak et al. 2010[a]
	Ireland	Deer	PEN; STR; TET	Carroll et al. 2014
	Mexico	Deer	ATM; CAZ; CFX; CFZ; CPM; PIP	Carrillo-Del Valle et al. 2016
	South Africa	Impala	TET	Mariano et al. 2009
	South Africa	Impala	AMP; CHL; STR	Marè 1968
	Kenya	Baboons	AMP; KAN; TET	Rolland et al. 1985
	Botswana	Elephants, Baboons, Impala, Warthogs, Waterbuck	AMP, DOX, STR; STX; TET	Jobbins & Alexander 2015
	Tanzania	Wildebeest, Buffalo, Zebra	AMC; AMP; CTX; ENO; GEN; STX; TET	Katakweba et al. 2015
Carnivores	Spain	Iberian Lynx	STR; STX; NAL; TET	Gonçalves et al. 2013
	Portugal	Iberian Wolf	CHL; CIP; GEN; NAL; STR; SXT; TET; TOB	Gonçalves et al. 2012
	United States	Bottlenose Dolphin	AMP; AMC; CHL; ERY; PIP; TET	Schaefer et al. 2009
	Poland	Lion, Dingo, Wildcat (zoo)	AK; AMC; AMP; CEP; CHL; DOX; GEN; NAL; NEO; STX; TET	Baldy-Chudzik and Stosik 2007

* AK Amikacin; AMC Amoxicillin-clavulanic acid; AMP Ampicillin; ATM Aztreonam; CAZ Ceftazidime; CEF Ceftiofur; CEP Cephalothin; CHL Chloramphenicol; CFX Ceftriaxone; CFZ Cefazolin; CIP Ciprofloxacin; CPM Cefepime; CTX Cefotaxime; DOX Doxycycline; DSM Dihydrostreptomycin; ENO Enrofloxacin; ERY Erythromycin; FOX Cefoxitin; GEN Gentamicin; KAN Kanamicin; NAL Nalidixic Acid; NEO Neomycin; OXY Oxytetracycline; PEN Penicillin; PIP Piperacillin; SDMX Sulfadimethoxine; SIA Sulfisoxazole; SPC Spectinomycin; STR Streptomycin; STX Sulfamethoxazole/trimethoprim; SUL Sulphathiazole ; TET Tetracycline; TIC Ticarcillin; TMP Trimethoprim; TOB Tobramycin

A 2001 study by Osterblad et al. (2001) compared the faecal *Enterobacteria* of mice from rural England to those of Finnish wild deer, revealing that ninety percent of bacteria isolated from the mice were resistant to various β-lactam antibiotics whereas almost no resistance was observed in bacteria isolated from the Finnish deer. As a result of Finland being less densely populated than England, it may be inferred that human proximity and resulting anthropological activity influence the antibiotic resistance observed in the gut microbiota of wild animals.

Research by Silva et al. (2010) illustrated the antibiotic resistance of *E. coli* isolated from healthy wild European rabbits. As a recognised food source for both humans and other wild animals, it was deemed important to identify the resistance genes present, with the potential for transfer. *E. coli* isolates demonstrated resistance to ampicillin, tetracycline, streptomycin, gentamycin, tobramycin, ciprofloxacin and chloramphenicol, with resistance genes including bla_{TEM}, *aad*A, *aac*(3)-II and *tet*A being detected (Silva et al. 2010). Some resistances detected in this study belong to classes of antibiotics utilized in human medicine, thus making the results obtained all the more concerning. Moreover, the failure to treat human diseases can contribute to longer infectivity periods thus increasing the severity of infections.

As illustrated in Table 2, the detection of *E. coli* with antibiotic resistance may occur in numerous wild animal groups - from those with specific dietary and habitat patterns (carnivores, water-dwelling animals), to those encompassing wide-ranging lifestyle factors, for example omnivores or migratory birds. A study conducted by Jobbins and Alexander (2015) in Botswana surveyed the potential for antibiotic resistance in *E. coli* isolated from a variety of animals; both large and small herbivores and carnivores including African elephant, crocodile, spotted hyena, waterbuck, leopard and hippopotamus. At least one *E. coli* isolate from all above-mentioned animals was resistant to at least one (albeit unspecified) antibiotic (Jobbins and Alexander 2015). Moreover, a high variability of multidrug resistance was observed despite the apparently "pristine" wild habitat; largely attributed to dietary habits (whether conforming to an herbivorous, omnivorous or carnivorous diet), association with water (fully-dependent or only for drinking water) and proximity to urban or anthropogenic areas (Jobbins and Alexander, 2015). Further proof of such a phenomenon was observed in a Czech Republic wild boar study undertaken by Literak et al. (2010[a]). Isolated faecal *E. coli* contained extended spectrum β-lactamases (ESBL), likely due to the omnivorous diet of the boars, and their tendency to consume human waste from encroaching anthropogenic habitats (Literak et al. 2010[a]). Additionally, research concerning antibiotic resistance in African primates, wild birds and birds of prey has been conducted (Rolland et al. 1985; Rwego et al. 2008; Radhouani et al. 2009 & 2012), with the detected resistances being attributed to proximity to human habitats and waste. Katakweba et al. (2015) conducted a study evaluating the antibiotic resistance profiles of *E. coli* isolated from buffalo, wildebeest and zebra in Tanzania. It was inferred that increased anthropological activity within the animal's habitats resulted in a notable increase in interspecies' resistance gene transmission (Katakweba et al. 2015).

In addition to mammals, wild birds have demonstrated the potential for dissemination of antibiotic resistance genes over great distances. Seasonal migration between habitats will also contribute to resistance gene dissemination (Allen et al. 2010). The feral pigeon population of Europe totals more than 9.3 million nesting pairs, with pigeons observed in both rural villages and large urban cities (Johnston and Janiga 1995; Hudec and Šťastný 2005). Their diet may consist of cereal grains and vegetable waste (Radimersky et al. 2010). Although feral pigeons would not be directly exposed to antibiotics, they may be considered potential reservoirs of antibiotic resistant enteric bacteria, as a result of their migratory habits and proximity to anthropogenic activity. Although *E. coli* present in pigeon gastrointestinal tracts would generally be considered innocuous, stress or adenoviral infection may result in the emergence of disease causing *E. coli* (Kimpe et al. 2002). In an Arctic bird study, 8% of *E. coli* isolated from gut samples presented resistance to at least one of seventeen antibiotics, with 4% of isolates resistant to four or more antibiotics (Sjölund et al. 2008). As a result of the "pristine" nature of an Arctic habitat as well as the fact that Arctic birds may migrate between six continents, the presence of these resistances illustrates the vast geographical distances resistance genes may travel (Allen et al. 2010).

Observations that have been made regarding the detection of antibiotic resistance in pristine wild environments are that selective pressure (allowing for selection of antibiotic resistant bacteria) is more common at ground level; i.e. more likely to be found in animals that have frequent interaction with soil and earth (Cristóbal-Azkarate et al. 2014). Geophagy is a recognized practice performed by animals including herbivores (Mahaney et al. 1999); and the resultant potential uptake of secondary metabolites produced by soil microorganisms such as streptomycetes may cause antibiotic resistance in isolated enteric bacteria.

As previously mentioned, proximity of animals to human activity will greatly increase the prevalence and potential for mobility of antibiotic resistant bacteria. Moreover, the awareness that humans, the environment, wild and companion animals and farming practices are not isolated domains but rather a complex interconnected network is integral in understanding the issue of antibiotic resistance [Figure 1] (Calistri et al. 2013; Cantas and Suer 2014; Cristóbal-Azkarate et al. 2014). While the habitat of wild animals may be envisioned as "pristine" with no direct antibiotic usage, the flooding of septic tanks or runoff of storm-water may cause entry of human faecal material into waterways and soil of such habitats (McCarthy et al. 2004). Furthermore, it was established that direct association of wild animals with water bodies

(aquatic or semi-aquatic life-history) led to a greater level of multidrug resistance being observed, as was the case in bacteria isolated from crocodile, hippopotamus, otter and waterbuck faeces in a study from Botswanan (Jobbins and Alexander, 2015). Such resistances were attributed to the exposure and consumption of water by the animals, as well as water-associated vegetation and sediment. Similarly, a local study that took place in the Kruger National Park (South Africa) inferred that surface water bodies (e.g. watering holes) could be a source of antibiotic resistance and thus allow for spread of tetracycline resistance genes to previously unaffected animal populations, in this case to impala (Mariano et al. 2009).

3. Antibiotics employed in animal medicine and agriculture and relevant resistance mechanisms

Antibiotics, since the first discovery of the activity of Penicillin-G in vitro by Alexander Fleming in 1929, have revolutionised medicine and for a period of time, were viewed as a miracle invention (Fleming, 1929). While several antibiotic classes comprising many antibiotics have emerged over the years, there has been a significant innovation gap for antibiotic production since the late 1960s, particularly for broad spectrum antibiotics [Figure 2].

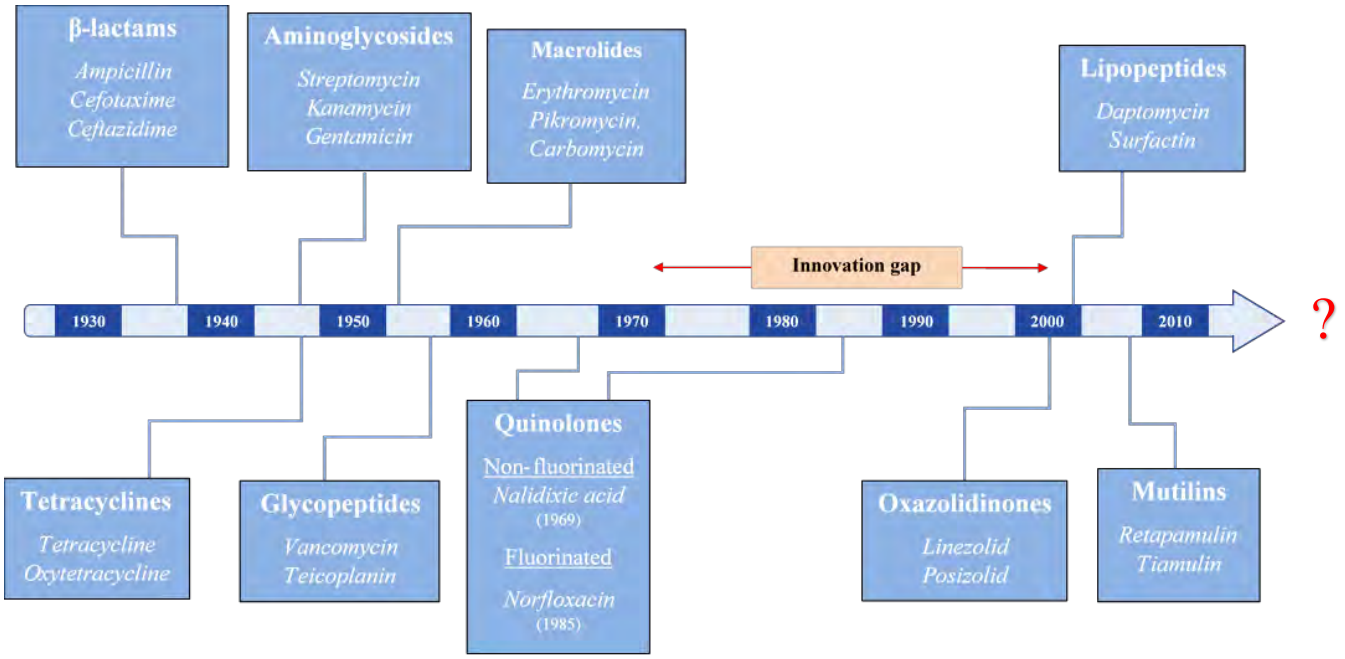

FIGURE 2. Timeline illustrating the discovery of prominent antibiotic classes and examples, emphasizing the innovation gap between ~1972 and 2000

Adapted from: Bozdogan and Appelbaum 2004; Jones et al. 2006; Fischbach and Walsh 2009

This, in conjunction with emerging resistance mechanisms, means antibiotics cannot be viewed and applied in the same indiscriminate way as before. Moreover, antibiotic use in animals has far-reaching consequences, further than simply that of the animal itself or the habitat it occupies. It is particularly concerning in agricultural settings, as dissemination of resistance determinants into the food chain impacts humans upon consumption or interaction with such animals. However, it is not only animals incorporated in the food chain that play a role in the spread of antibiotic resistance, but even birds and wild animals devoid of direct human contact. Antibiotics that have found extensive (though often justified) use in humans and animals (both the agriculture and veterinary sectors), in conjunction with the relevant resistance mechanisms that have subsequently emerged will be briefly addressed in this section.

3.1 β-Lactams

The β-lactam antibiotics represent one of the most highly utilized classes of antibiotics with widespread use in both human and veterinary medicine. Henton et al. (2011) reported that between 2002 and 2004 in South Africa, the β-lactam antibiotics encompassed more than 11% of all antibiotics sold. The report further commented on usage indicators, and that these antibiotics were most frequently utilized as growth promoters in poultry and pigs (Henton et al. 2011; Eagar et al. 2012). It has been established that β-lactam antibiotics are most commonly prescribed in Europe in the veterinary sector, with a decrease in livestock prescription having been observed over time (NORM/NORM-VET 2010; Guenther et al. 2011; DANMAP 2013).

The β-lactam family consists of four major groups of antibiotic agents: penicillins, cephalosporins, monobactams and carbapenems [Figure 3]. All antibiotics within the four groups contain a β-lactam ring; with the classes being differentiated by their additional structural moieties viz. a thiazolidine ring for penicillins and a dihydrothiazolidine ring structure with the sulphur atom replaced by a carbon plus a double-bond between carbon 2 and 3 for the carbapenem antibiotics. The cephalosporins have a dihydrothiazine ring attached to the β-lactam ring, and the monobactams comprise a monocyclic β-lactam molecule (Birnbaum et al. 1985; Neu 1986; Donowitz and Mandell 1988; Drawz and Bonomo 2010). Clavulanic acid, although not an effective antibiotic on its own, when combined with antibiotics of the penicillin class can function as an effective β-lactamase inhibitor [Figure 3].

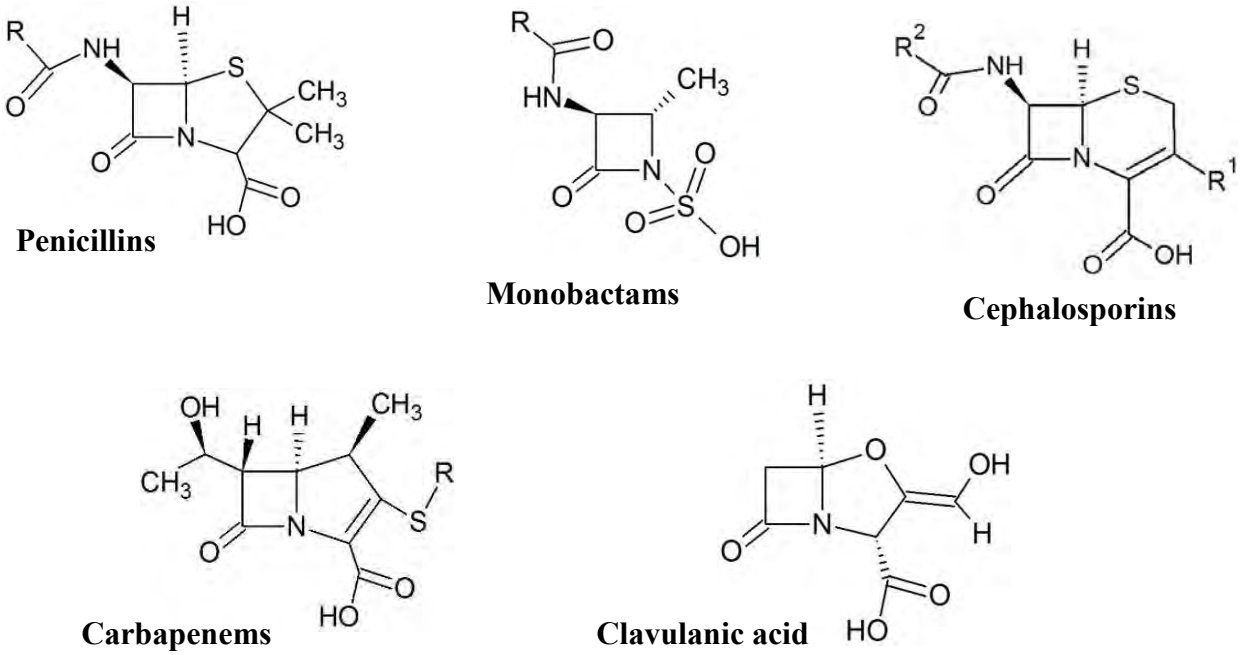

FIGURE 3. Respective base structures for the major β-lactam antibiotic sub-classes

The mechanism of action of β-lactam antibiotics is the inhibition of bacterial cell wall biosynthesis, through inhibition of transpeptidation (Drawz and Bonomo 2010). The cell wall consists of a peptidoglycan layer, which in the case of Gram negative bacteria is located between the outer and the cytoplasmic membrane, maintaining the cell shape and protecting against osmotic forces (Schleifer and Kandler 1972; Guenther et al. 2011). The β-lactam ring [Figure 3] of the antibiotics is sterically similar to the terminal D-alanine-D-alanine residue in the N-acetylmuramic acid (NAM) pentapeptide (a constituent of bacterial peptidoglycan). As a result of the similarity, the penicillin binding proteins (PBPs), such as transpeptidase, pair with the β-lactam antibiotics, thus causing acylation and inhibition of the transpeptidase enzyme, rendering further cell wall synthesis impossible (Fisher and Mobashery 2009; Drawz and Bonomo 2010). Attachment of the β-lactam antibiotic to certain penicillin binding proteins induces release of cell wall hydrolases and increased permeability and eventual deterioration of the cell wall (Zapun et al. 2008). The β-lactam family includes the first ever discovered antibiotic, penicillin-G (Fleming, 1929), as well as those that are more recent including the first to fourth generation cephalosporins (Guenther et al. 2011).

3.1.1 β-Lactamases

The hydrolysis of β-lactam antibiotics by β-lactamase enzymes is a primary mechanism of resistance for such antibiotics. Over 400 β-lactamase enzymes are currently specified, with all of these enzymes showing the same underlying catalytic activity - hydrolysis of the β-lactam ring, but with varying substrate specificity and susceptibility to β-lactamase inhibitors (Guenther et al. 2011). A number of β-lactam antibiotics are considered a preferred first line of defence for several infections caused by Gram positive and negative bacteria, however the rise in resistance to these antibiotics has resulted in a predicted reduction of their efficacy to zero by 2023 (Wellington et al. 2013). A variety of β-lactamase enzymes have been characterized from *E. coli* isolates and have been assigned to specific classes using the Ambler and Bush/Jacoby/Medeiros schemes [Table 3].

TABLE 3. Summary of major β-lactamase enzymes found in *Escherichia coli* according to the Ambler and Bush/Jacoby/Medeiros classification schemes

Ambler classification [a]	**Bush/Jacoby/ Medeiros classification** [b]	**β-lactam substrates hydrolysed**	**Enzyme types and examples**	
A	2b, 2be, 2br, 2c, 2e, 2f	Penicillins 3rd generation Cephalosporins	ESBL *	TEM, SHV, CTX-M
			Carbapenemases	KPC, GES, SME
B	3	All β-lactams	Carbapenemases	IMP-1, VIM-1, NDM
C	1	Cephamycins 3rd generation Cephalosporins	Cephamycinases	CMY, DHA, MOX FLOX, ACC
D	2d	Penicillins 3rd generation Cephalosporins	ESBL and Carbapenemases	OXA-1

[a] Ambler et al. 1991

[b] Bush et al. 1995; Bush and Jacoby 2010

* ESBL - Extended Spectrum β-lactamase

3.1.1.1 Extended spectrum β-lactamases: TEM, CTX-M and SHV

Extended spectrum β-lactamases (ESBLs) are hydrolytic enzymes produced by some *Enterobacteriaceae* with the capability of inactivating numerous extended-spectrum β-lactam antibiotics before they reach the penicillin-binding proteins (Falagas and Karageorgopoulos

2009). ESBLs were first reported in the mid-1980s, and although initially only detected in *K. pneumoniae* and *E. coli,* they are now found in numerous other species (Bradford 2001; Mesa et al. 2006). Although a large portion of research is dedicated to ESBLs produced by *Enterobacteriaceae* isolated from nosocomial human samples, there has been evidence of such from community-acquired human, companion and wild animal samples (Otero et al. 2003; Brinãs et al. 2005; Mesa et al. 2006). Extended spectrum β-lactamases derived from the original TEM or SHV-1 type β-lactamases occur through point mutations in the parent enzymes (Jacoby and Munoz-Price 2005). A number of clinically relevant ESBL enzymes exist today, including but not limited to the TEM, SHV and CTX-M types.

The TEM-type β-lactamase was originally discovered in an *E. coli* isolate from a Greek patient named Temoneira in 1965 (Datta and Kontomichalou 1965). More than 200 TEM-type β-lactamases have since been characterised, a large proportion of which are extended spectrum β-lactamases (Kocsis and Szabó 2013). All TEM-type β-lactamases are point mutation derivatives of the TEM-1 types (Smet et al. 2008). A number of research groups have described the existence of CMT ("complex mutant TEM") type enzymes, which have the ability to hydrolyse even 3rd generation cephalosporins and β-lactamase inhibitors, for example cefotaxime and clavulanic acid respectively (Fiett et al. 2000; Neuwirth et al. 2001). The origins of TEM β-lactamases vary widely, with such ESBLs being detected in clinical environments like hospitals and nursing homes (Sabaté et al. 2002), as well as in animals and food sources (Shooter et al. 1971; Prats et al. 2003; Brinãs et al. 2005). However, even wild animals have been sources for TEM type β-lactamases, evidenced by the detection of the TEM-1 enzyme in *E. coli* isolated from foxes and birds of prey in Portugal (Costa et al. 2006), wild geese from Belgium (Garmyn et al. 2011), and waterbird faeces from the Baltic Sea coast of Poland (Literak et al. 2010^{b}).

The first report of a CTX-M ESBL was from an *E. coli* strain isolated from a laboratory dog in 1989 (active on CefoTaXime, first isolated in Munich) (Matsumoto et al. 1988; Peirano and Pitout 2010). A worldwide dissemination of CTX-M producing *Enterobacteriaceae* has since been observed, with subtypes CTX-M-1 and CTX-M-9 occurring most frequently in clinical environments and agriculture. There have however been observations of rarer CTX-M types including CTX-M-2, 8 and 14 in pigs, cattle and other livestock from countries including Japan, Spain and Denmark respectively (Cavaco et al. 2008; Shiraki et al. 2004; Escudero et al. 2010). Furthermore, studies have identified a correlation between the type of CTX-M enzymes

detected in *Enterobacteriaceae* isolated from pigs and proximate humans, suggesting such CTX-M-producing strains may span niches including the environment, community as well as wild animals (Dohmen et al. 2015). *E. coli* carrying CTX-M type β-lactamases have been detected in poultry and rabbit faeces in Spain (CTX-M-9 and 14) (Blanc et al. 2006), swine faeces in China (CTX-M-1 and 9) (Hu et al. 2013), in *E. coli* isolated from Glaucous winged gulls in Russia (CTX-M-15) (Hernandez et al. 2010) and *E. coli* isolated from rats in Germany (CTX-M-9) (Guenther et al. 2012).

The SHV-type β-lactamases were first identified by Pitton (1972) as a derivative of a chromosomal penicillinase isolated from *K. pneumoniae.* The SHV β-lactamases are plasmid encoded, and over 23 variants exist, most of which possess extended spectrum activity against 3rd and 4th generation cephalosporins and monobactams (Arakawa et al. 1986; Tzouvelekis and Bonomo 1999). Furthermore, many SHV-type β-lactamases share functional and structural similarity with TEM β-lactamases [Table 3]. Although frequently observed in *K. pneumoniae*, clinical *E. coli* strains on occasion present SHV-type ESBLs (Ling et al. 1994). Although there is no clear geographical pattern of distribution, the SHV-type β-lactamases are most often observed in highly selective nosocomial and ICU environments (Sanders and Sanders 1992). However, detection in livestock samples (including *E. coli* from poultry meat in Tunisia) has been reported (Jouini et al. 2007). With regard to wild environments, SHV-type enzymes have been observed in *E. coli* isolated from wild birds; including black-headed gulls from the Czech Republic (SHV-2 and 12) (Dolejska et al. 2009), Blackcap warblers from the Azores Archipelago (SHV-12) (Silva et al. 2011), and wild geese from Belgium (SHV-12) (Garmyn et al. 2011). Moreover, the SHV-12 enzyme was detected in *E. coli* isolated from Iberian wolf faecal samples [Table 2] (Gonçalves et al. 2012), as well as in *E. coli* from Norwegian rat faeces (Čížek et al. 1999). In addition to *E. coli,* the SHV-12 enzyme has been detected in *Citrobacter freundii* isolates from healthy swine faeces in Portugal (Machado et al. 2008).

3.1.1.2 Carbapenem hydrolysing enzymes: KPC and MBL

Carbapenem hydrolysing enzymes (carbapenemases) are a class of β-lactamases belonging to classes A, B and D designated by the Ambler system of classification, and classes 1 and 2 according to Bush/Jacoby/Medeiros classification [Table 3]. The enzymes have the ability to inactivate all hydrolysable β-lactam agents, including carbapenems (Queenan and Bush 2007).

KPC (*Klebsiella pneumoniae* Carbapenemase) is dominant among carbapenem hydrolysing enzymes in terms of ubiquity (Yigit et al. 2001). This enzyme (belonging to Ambler class A and Bush/Jacoby/Medeiros class 2f, Table 3) has a broad substrate spectrum including third generation cephalosporins, carbapenems and aztreonam (Bush and Fisher 2011). The KPC carbapenemases have been commonly associated with multidrug resistant Gram negative infection outbreaks, with particular attention paid to those occurring in hospitals. The epidemiology of KPC enzymes has included countries as far apart as the United States and Israel, with global spread being observed in recent years (Bratu et al. 2005; Leavitt et al. 2007; Wei et al. 2007).

The MBL enzymes (Metallo-β-Lactamases) belong to Ambler Class B and Bush/Jacoby/Medeiros group 3a [Table 3] (Kocsis and Szabó 2013). With regard to *Enterobacteriaceae*, metallo-β-lactamase genes are transferred via plasmid conjugation (Kocsis and Szabó 2013). The first detected MBL was the IMP (active on IMiPenem) phenotype in 1991, from *Pseudomonas aeruginosa*, which was detected a few years later in *Enterobacteriacea* (Watanabe et al. 1991; Osano et al. 1994). Currently, the most important MBL enzyme is the New-Delhi Metallo-β-lactamase or NDM enzyme, first reported by Yong et al. in 2009. The first report originated from a carbapenem resistant *K. pneumoniae* strain isolated from a Swedish patient afflicted with a urinary tract infection, following travel to New Delhi, India (Yong et al. 2009). The isolate possessed metallo-β-lactamases, but was negative for all previously known MBL genes (Yong et al. 2009). NDM-1 has since been isolated from a number of *K. pneumoniae* and *E. coli* isolates spanning several countries, often associated with travel to India, and usually related to clinical cases involving invasive hospital treatment or extended nursing home stays (Kumarasamy et al. 2010; Poirel et al. 2014; Tijet et al. 2015; Park et al. 2016). Furthermore, detection of NDM type enzymes in *E. coli* from companion animals has occurred, including NDM-1 from dogs and cats in the USA (Shaheen et al. 2013), and NDM-9 in an *E. coli* isolate from a dog in Algeria (Yousfi et al. 2015). The detection of such enzymes in isolates from wild animals is rarely reported, however one study described the presence of the NDM-1 enzyme in a *Salmonella enterica* subsp. *enterica* serovar Corvallis isolate from a black kite (*Milvus migrans*) in Germany (Fischer et al. 2013).

3.1.2 Porins and efflux pumps

Water-filled pores are present in the outer membrane of bacteria, serving the purpose of facilitating the movement of hydrophilic molecules including nutrients into the periplasmic space of the cell (Koebnik et al. 2000). The proteins that cause formation of these channels are called porins, and were first described in *E. coli* in 1976 (Nakae 1976). Porins serve as a molecular "sieve" rendering the outer membrane permeable to certain hydrophilic compounds (smaller than a specific size exclusion limit), determined by the diameter of the channels present. There are multiple porin classes, ranging from general porins (involved in permeability of the cell), specific porins that may enable the uptake of specific molecules, and iron-regulated outer membrane proteins involved in enabling uptake of iron complexes via siderophores (Fernández and Hancock 2012).

Bacterial cells may have up to 10^6 porins per cell (Achouak et al. 2001), with exclusion limits approaching that of the size of many antibiotics thus limiting the diffusion rate. To gain entry into a cell, antibiotics must first pass through the outer membrane barrier, provided one is present in the target bacterium (Hancock 1984). One such route is through porins, the number and type of which will determine the permeability and thus the antibiotic resistance or susceptibility of the cell. Mutations in porin encoding genes will affect the expression and structure of porins and thus directly impact the susceptibility of bacteria to antibiotics, either through the loss of the porin, lower level of expression or modification in the size or charge of the porin channel. The earliest example of porin-mediated resistance was reported in 1981, with the OmpF porin conferring resistance in *E. coli* K12 mutants against β-lactam antibiotics including carbenicillin (Harder et al. 1981). Beceiro et al. (2011) described clinical *E. coli* isolates resistant to cefpirome and cefepime, lacking OmpC and OmpF concomitant with increased levels of TEM-1 and OXA-1 β-lactamase (Beceiro et al. 2011). Any mutations causing the loss or alteration of porins can impact resistance as the rate of entry of the antibiotic will be limited, thus enhancing the influence of secondary resistance mechanisms - if applicable (e.g. β-lactamases).

Efflux pumps, present in both Gram positive and Gram negative microorganisms (van Bambeke et al. 2000; Piddock, 2006), can essentially be divided into two groups based on their specificity. Broad spectrum pumps export members of structurally distinct molecular classes, while the substrate specific pumps export only one type of molecule (Cox and Wright, 2013). With regard to β-lactam antibiotics, there may be presence of an ATP binding cassette ("ABC")

type pump system, a resistance-nodulation division ("RND") family, or multidrug efflux systems (Webber and Piddock, 2003; Piddock, 2006). The efflux pumps conferring intrinsic antibiotic resistance in Gram negative bacteria predominantly belong to the RND family. The "AcrAB-TolC RND" is an efflux system found primarily in *E. coli* strains, conferring resistance to various β-lactam antibiotics (Piddock, 2006). Because one efflux pump has the ability to mediate resistance to multiple structurally discrete antibiotics, inhibition of such a pump could restore the antibacterial effects of several antibiotics (Lomovskaya et al. 2001; Blair and Piddock 2009; Cox and Wright 2013).

3.2 Fluoroquinolones

The first report of transferable resistance to fluoroquinolones was published by Martínez-Martínez et al. (1998). Three mechanisms of transferable resistance have been identified so far. The first involves Qnr protein families consisting of proteins Qnr (Quinolone resistance) which function to protect DNA gyrase and topoisomerase IV from quinolone antibiotics (Yang et al. 2014; Poirel et al. 2012). The second transferable resistance mechanism is based on the *aac*(6')Ib-cr gene, encoding an aminoglycoside acetyltransferase with the ability to acetylate fluoroquinolones with a piperazinyl substituent (Ruiz et al. 2012[a]; Yang et al. 2014). Another mechanism providing fluoroquinolone resistance is the activity of multidrug efflux pumps QepA and OqxAB, which confer increased resistance to agents including quinoxalines and fluoroquinolones (Rodriguez-Martinez et al. 2011; Yang et al. 2014).

3.2.1 Qnr protein families

Crystallography studies indicate that Qnr proteins are folded into a right hand β helix, with nine complete coils (Vetting et al. 2011; Ruiz et al. 2012[a]). Five families of Qnr proteins have been described: QnrA, B, S, C and D (Minarini et al. 2008; Jacoby et al. 2008; Wang et al. 2009; Ruiz et al. 2012[a]). Where the function of a quinolone antibiotic is to bind the DNA-enzyme complex, stabilize the cleavage and ultimately lead to lethal double-stranded breaks, the Qnr proteins have demonstrated binding to topoisomerase IV and DNA gyrase, thus destabilizing the cleavage complex between the antibiotic, DNA and enzyme (Martínez-Martínez et al. 1998;

Robicsek et al. 2006; Quiroga et al. 2007; Jacoby et al. 2008; Guo et al. 2011). Origins of the Qnr families vary widely, with some being proposed to have derived from chromosomal ancestors of waterborne *Shewanellaceae* or *Vibrionaceae* (Poirel et al. 2005[a]). Moreover, the QnrA family presents >70% homology with four chromosomally encoded proteins of *Shewanella algae* (Poirel et al. 2005[b]). The detection of such *qnr* genes has occurred in *E. coli* isolated from a variety of animals including; waterbird faeces from the coast of Poland (*qnr*S) (Literak et al. 2010[b]), urban brown rats in Berlin (*qnr*B) (Guenther et al. 2012) and red foxes in Washington DC (*qnr*S) (Ahmed et al. 2007).

3.2.2 Aminoglycoside acetyltransferase

The AAC(6')Ib-cr enzyme is a bifunctional acetyltransferase variant. The -cr variant in particular has two amino acid substitutions: Trp102Arg and Asp179Tyr, which are essential in the acetylation of quinolones (Jacoby et al. 2014). Agents to which such an enzyme presents activity include a variety of aminoglycoside antibiotics including amikacin, kanamycin and tobramycin, as well as fluoroquinolones including ciprofloxacin and norfloxacin (Robicsek et al. 2006). The corresponding *aac*(6')Ib-cr gene is generally observed within a multiresistance plasmid, in a cassette as part of an integron (Jacoby et al. 2014). Although also found in organisms such as *P. aeruginosa*, prevalence of the *aac*(6')Ib-cr gene is associated with *Enterobacteriaceae* and *E. coli* in particular; often more ubiquitous than *qnr* alleles (Pitout et al. 2008; Ruiz et al. 2012[b]; Ciesielczuk et al. 2013). In addition, the *aac*(6')Ib-cr gene is commonly found in correlation with the extended spectrum β-lactamase CTX-M-15, particularly in *E. coli* isolates from nosocomial environments including urine and blood samples (Pitout et al. 2008; Baudry et al. 2009; Perilli et al. 2009). Although not widespread in wild animals, the *aac*(6')Ib-cr gene has been detected in *E. coli* isolated from gulls from Portugal and Sweden (Vredenburg et al. 2014) and primates in China (Wang et al. 2012).

3.2.3 Multidrug efflux pumps

The multidrug efflux pump OqxAB was first identified as a member of the PMQR (plasmid mediated quinolone resistance) determinants by Strahilevitz et al. (2009), as a means of

resistance to the antibiotic olaquindox in *E. coli* isolated from farm pigs (Sorensen et al. 2003; Strahilevitz et al. 2009). Although the mode of action was initially related to conferring resistance to quinoxaline antibiotics, OqxAB has a considerable substrate specificity, including fluoroquinolones ciprofloxacin and norfloxacin, as well as other antibiotics like chloramphenicol, nalidixic acid and trimethoprim (Hansen et al. 2007). Plasmid mediated *oqx*AB have been observed primarily from clinical *K. pneumoniae* and *E. coli* isolates, however the gene was detected in *E. coli* from pigs inhabiting farms in China (Zhao et al. 2010). In addition, co-occurrence of *oqx*AB with genes associated with plasmid-borne CTX-M alleles is common (Liu et al. 2013).

The second major efflux pump is QepA - a plasmid-encoded pump which controls the decrease in susceptibility to hydrophilic fluoroquinolones including ciprofloxacin and norfloxacin. Zhao et al. (2010) indicated that *qep*A positive *E. coli* isolates had a higher frequency of topoisomerase mutations than that of Qnr-producing isolates, inferring that efflux pumps (OqxAB and QepA) may favour the selection of high level quinolone resistance compared to those selected by Qnr proteins.

The detection of *qep*A has been the subject of numerous studies involving *E. coli* isolates from clinical samples (human urine), swine manure and livestock (chicken spleen), from countries including Nigeria, China and South Korea respectively (Ogbolu et al. *2*011; Xu et al. 2015; Oh et al. 2016). In addition, detection of the *qep*A gene in *E. coli* isolated from a gorilla in a protected nature reserve in the Central African Republic has occurred (Janatova et al. 2014).

3.3 Aminoglycosides

Aminoglycoside resistance is thought to have originated from the mutation of normal cellular genes, acquisition of resistance genes as well as from aminoglycoside producing organisms. Moreover, as discovered by Shaw et al. (1993), resistance may also arise when the regulation of usually quiescent cellular genes is altered to high-level expression. Primarily however, aminoglycoside resistance is frequently attributed to enzymatic inactivation by acetyltransferases, phosphotransferases and nucleotidyltransferases.

3.3.1 Aminoglycoside modifying enzymes

The three aminoglycoside modifying enzyme classes causing inactivation of aminoglycoside antibiotics, include phosphotransferases (APH) catalyzing O-phosphorylation, acetyltransferases (AAC) catalyzing N-acetylation (as mentioned above in *3.2.2*), and nucleotidyltransferases (ANT) catalyzing O-adenylation. Within these three categories exist more than 50 different enzymes, combinations of which have been found in both Gram positive and negative bacterial pathogens (Shaw et al. 1993; Davies and Wright 1997). With regard to acetyltransferases, four principal classes exist, which modify aminoglycosides in the 1-, 3-. 6'- and 2'- amino groups (Benveniste and Davies 1973; Lovering et al. 1987; Shaw et al. 1993), conferring resistance to a combination of gentamicin, tobramycin, amikacin, fortimicin and apramycin (Hedges and Shannon 1984; Shimizu et al. 1985; Lovering et al. 1987). Genes encoding aminoglycoside resistance (including *aad*A and *str*A) have been detected in *E. coli* isolated from gulls in the Czech Republic (Dolejska et al. 2009) and rodents in Canada (Allen et al. 2011).

3.4 Tetracyclines

Tetracycline antibiotics include a large variety of broad spectrum agents such as tetracycline, oxytetracycline and doxycycline, capable of inhibiting the growth of both Gram positive and negative organisms. These antibiotics act through blocking the binding of the aminoacyl tRNA to the A site of the 30S ribosomal subunit (Chopra 1985; Chopra and Roberts 2001). First discovered in the 1940s, tetracyclines have since found extensive use in agriculture and clinical and veterinary medicine, primarily due to the favourable antibiotic properties and absence of harmful side effects (Dancer et al. 1997; Chopra and Roberts 2001). Such extensive use of tetracyclines over the decades has allowed for the emergence of resistance, predominantly based on the genetic acquisition of *tet* genes (Paulsen et al. 1996; Chopra and Roberts 2001). A number of mechanisms conferring resistance to such antibiotics exist, including the presence of proteins encoding efflux genes, and ribosomal protection proteins.

3.4.1 Efflux proteins

The tetracycline efflux proteins are considered the most ubiquitous method of tetracycline resistance. The proteins encoded by the *tet* efflux genes are membrane-associated, and allow for the export of tetracycline from the bacterial cell allowing for reduction of the intracellular drug concentration. All efflux genes belong to the major facilitator superfamily, and within the genus *Escherichia,* include *tet*A, *tet*B, *tet*C, *tet*D, *tet*E, *tet*I and *tet*Y (Chopra and Roberts 2001). In addition, the Gram negative efflux genes are normally associated with conjugative plasmids, which often carry multiple other antibiotic resistance genes. This therefore means that selection for any one of the included resistance factors, selects for the plasmid, potentially leading to a dramatic increase of multidrug resistant bacteria (Falkow 1975; Levy 1992; Paulsen et al. 1996; Chopra and Roberts 2001). Tetracycline resistance genes are a common finding in *E. coli* from wild animals; inclusive of deer in the USA (*tet*B) (Bryan et al. 2004), wild boars in Europe (*tet*A and B) (Literak et al. 2010[a]) and a number of wild birds in Europe (*tet*A and B) (Literak et al. 2010[b]).

3.4.2 Ribosomal protection proteins

A number of cytoplasmic ribosomal proteins (e.g. TetM and TetO), act to protect the ribosomes from tetracycline, and thus confer resistance to antibiotics including doxycycline and minocycline (Burdett 1996; Taylor and Chau 1996; Chopra and Roberts 2001). The mechanism of action concerns the binding of the ribosomal protein to the ribosome, altering the conformation of the ribosome and preventing the binding of tetracycline antibiotics. Moreover, the spectrum of resistance in terms of number of tetracycline agents is larger than that seen with bacteria carrying tetracycline efflux proteins (Chopra and Roberts 2001).

4. Methods employed for antibiotic resistance detection

Rapidity and precision are essential characteristics of diagnostic methods employed in the detection and classification of antibiotic susceptibility (Sundsfjord et al. 2004). Both genotypic and phenotypic methods can be used. Advantages of genotypic methods include the fact that the result is a matter of presence or absence of a particular resistance gene or integron, which can be detected using PCR-based methods if appropriate primers are available. Furthermore, by bypassing conventional culture methods, detection time as well as potential biohazard risks can be decreased (Sundsfjord et al. 2004). The major disadvantage of genotypic detection of antibiotic resistances not encountered in the disk diffusion method is the fact that screening is limited to known genes. Therefore, novel or unknown resistance mechanisms will go undetected (Sundsfjord et al. 2004). In addition, the genotypic detection of resistances will not provide information on expression of the resistance. False-positive and false-negative results due to the presence of silent or pseudogenes, or mutations in primer binding sites are therefore possible (Sundsfjord et al. 2004).

4.1 Phenotypic methods

4.1.1 Disk diffusion method

A standardized disk diffusion method was first suggested by Bauer et al. (1966). It is a cost-effective and time-efficient procedure to determine the antibiotic resistances of a wide range of organisms to various antibiotic compounds. The method involves the preparation of specific agar, generally Mueller-Hinton, followed by surface inoculation using the organism of interest (Bauer et al. 1966). Commercially available antibiotic-impregnated disks are then placed on the agar surface and incubated for a specified amount of time depending on the experimental guidelines being followed (Jorgensen and Ferraro 2009).

Due to the immediate diffusion of the antibiotic out of the disk, a gradient is created wherein the highest concentration of the antibiotic is found closest to the disk (Bauer et al. 1966). Following the specified incubation time, the diameter of the area showing no growth (i.e. the "zone of growth inhibition"), is measured. This zone is a representation of the minimum antibiotic concentration sufficient to inhibit the growth of the tested microorganism (Jorgensen

and Ferraro 2009). Following consultation with standard pre-established breakpoint tables (for example EUCAST or CLSI), the tested isolate would then be classified as either susceptible, intermediate or resistant to the tested antibiotic (Jorgensen and Ferraro 2009; CLSI 2016; EUCAST 2016). Homogeneity as well as thickness or depth of the agar must remain strictly constant across all tests to ensure consistent diffusion of the antibiotic (Matuschek et al. 2013). Furthermore, physiochemical factors including the pH and hydration of the agar are important to ensure batch to batch consistency (Gould 1960). Furthermore, as recently addressed in several reports and international conferences, the quality of the antibiotic disks is integral in maintaining uniformity as well as obtaining correct and reliable results; as investigation has revealed significant differences in antibiotic activity in disks from a variety of manufacturers (Åhman et al. 2015; EUCAST 2015). It is thus imperative that in-house quality control testing is regularly performed for consistency in all laboratories utilizing the EUCAST guidelines and breakpoint tables (EUCAST 2015; Kahlmeter 2016).

4.1.2 MIC broth dilution method

Minimum Inhibitory Concentration (MIC), defined as the lowest concentration of an antibiotic that will result in inhibition of microbial growth after overnight incubation, has been previously described as a gold standard for determining an organism's antibiotic susceptibility (Andrews 2001). MICs may be utilized in diagnostic laboratories to confirm resistances, as well as in research institutions to determine in vitro activity of new antibiotics, for the establishment of breakpoints (Jorgensen and Ferraro 2009). Furthermore, the MIC method is utilized when other methods such as disk diffusion are not appropriate, as is the case when determining the susceptibility of coagulase-negative staphylococci to teicoplanin (Andrews 2001).

Although the generation of a quantitative result in the form of the MIC is advantageous, this method can be tedious and both reagent and space expensive (Jorgensen and Ferraro 2009). MIC testing may therefore be miniaturized in the form of "microdilution" trays (96 well microtitre plates), where approximately 12 antibiotics can be tested in a range of 8 two-fold dilutions per tray (Phillips et al. 1991). Following appropriate incubation, the MIC of each antibiotic at each concentration is determined through spectrophotometric inspection of the plate wells (Jorgensen and Ferraro 2009).

4.1.3 Enzyme activity

With the advancement of antibiotic resistance and the need for the development of rapid and definitive tests, several colourimetric and acidimetric tests have been devised. In particular, techniques exist for the detection and quantification of β-lactamase activity in bacteria. Some of these tests may serve purely as a screening method through the use of various "spot-tests". However, others can quantify the amount of β-lactamase activity. Colourimetric assays have been regularly utilized since first description in 1968 (Sargent 1968), and have been improved to include flow cytometric methods (Sparbier et al. 2012; Farias-Ramos et al. 2013).

One widely used method involves the use of nitrocefin, a chromogenic cephalosporin that undergoes a colour change from yellow to red upon hydrolysis of the β-lactam ring, first suggested by O'Callaghan et al. (1972). Biomass of colonies may be used for direct testing, where a colony sample of the organism is suspended in a mixture of nitrocefin and phosphate buffer, and a change in colour within 30 minutes at 30°C denotes the presence of β-lactamase activity (Livermore and Brown 2001). Although predominantly used in fastidious Gram negative organisms, this method is not appropriate for detection of staphylococcal penicillinases and ROB-1 present in *Haemophilus* spp., as the uninduced β-lactamase levels are inadequate for a visible colour reaction (O'Callaghan et al. 1972; Livermore 1995; Livermore and Brown 2001).

A second chromogenic substrate is CENTA, which is readily prepared from the (commercially available) antibiotic cephalothin. This substrate can be applied in kinetic studies of β-lactamases as well as for detection in various crude extracts. CENTA is however not suitable for direct detection using agar plates or slides, as the absorption spectrum of the leaving group is not presented as a visible colour change (Bebrone et al. 2001).

Iodometric tests utilize a reaction wherein iodine is reduced by the hydrolysis of benzylpenicillin yielding penicilloic acid, which in turn is decolourizes a starch-iodine complex (Livermore and Brown 2001). The method may be carried out by means of test tubes or drops on filter paper strips or glass slides. Following mixing of culture material with the iodine reagent, decolourization after 5 minutes denotes the presence of β-lactamase activity (Livermore and Brown 2001). This test is not as sensitive as the nitrocefin assay but is less expensive to run (Livermore and Brown 2001).

Lastly, there may be implementation of pH dependent tests where a pH indicator, for example phenol red, is applied. As the hydrolysis of the β-lactam ring is generating a carboxyl group, causes acidification takes place which can be confirmed using a suitable pH indicator. Paper strips or glass test tubes may be used, and upon testing a change in colour to yellow (in the case of phenol red) indicates β-lactamase activity (Livermore and Brown, 2001).

4.1.4 MALDI-TOF MS

MALDI-TOF MS, (Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry), is considered an analytical method that can be implemented for antibiotic resistance detection. MALDI-TOF MS has been successfully used to detect β-lactamase enzyme proteins (Kostrzewa et al. 2013), as the cleavage of the β-lactam ring (the mechanism of action of a β-lactamase enzyme) is characterized by addition of a water molecule, which results in an increase of the molecular weight of the antibiotic (Kostrzewa et al. 2013).The reaction product is monitored by MALDI-TOF MS, and strains lacking β-lactamases would therefore not form product due hydrolysed ring cleavage (Sparbier et al. 2012). This technique has been successfully utilized for a number of β-lactam antibiotics including ampicillin, ceftazidime, cefotaxime, ertapenem and meropenem. Furthermore, the organisms that have been successfully analysed using this technique comprise representatives from various genera, including *E. coli, K. pneumoniae, Salmonella* spp*., P. aeruginosa* and *Acinetobacter baumannii* (Burckhardt and Zimmermann 2011; Hrabák et al. 2011; Sparbier et al. 2012; Kostrzewa et al. 2013).

An additional advantage of this method is the potential of identification directly from specimen samples (urine or blood cultures for example), thus increasing speed and efficiency of analysis (La Scola and Raoult 2009; Ferreira et al. 2010; Kostrzewa et al. 2013). MALDI-TOF MS has in addition been applied to monitor the effects of applying different antibiotics to resistant and susceptible bacterial strains. Limitations of this methods may include the presence of new or unknown resistance mechanisms which may be overcome to some degree through the use of whole genome sequencing if affordability and availability exist (Tyson et al. 2015).

4.2 Genotypic methods

4.2.1 Polymerase chain reaction (PCR)

The Polymerase Chain Reaction (PCR) was first described almost thirty years ago by Mullis and Faloona (1987), and only one year thereafter was first published as a method for diagnostic application (Saiki et al. 1988[a]). PCR involves three principle steps: denaturation of the DNA via heating, annealing of the primers and lastly elongation of the (target specific) primers by (originally) a thermostable DNA polymerase isolated from *Thermus aquaticus* ("Taq" DNA polymerase) (Saiki et al. 1988[b]; Fluit et al. 2001). PCR has been identified as a useful tool to detect antibiotic genes in organisms isolated from a wide range of environments (Sundsfjord et al. 2004).

A multiplex PCR protocol can be implemented, wherein several primer sets can simultaneously detect several antibiotic resistance genes in a single PCR reaction. This is a time and cost-efficient method for screening multiple samples against a variety of resistance genes (Henegariu et al. 1997; Bii et al. 2005). Moreover, a real-time PCR protocol allows for monitoring of the accumulation of the amplicon using labelled primers and fluorescing amplicons to provide detectable signals (Mackay 2004; Sundsfjord et al. 2004). The presence or absence of known β-lactamase genes can be detected through the use of PCR using specifically designed primers to amplify the target genes.

5. Outlook

Antibiotic resistance threatens the prevention and treatment of an ever-increasing spectrum of diseases and infections caused by bacteria. Although antibiotics are essential to save lives, the inappropriate way in which they have been applied over time has resulted in a number of previously manageable infections rendered untreatable. While antibiotic resistance is observed in environments with extensive antibiotic use or exposure to these compounds - for example in clinical settings - the emergence and dissemination of antibiotic resistance in farm and wild animal habitats is of tremendous concern. Furthermore, the identification of bacteria with similar resistance profiles in an array of diverse interconnected ecological niches substantiates how widespread the issue has become. Therefore, strategies to address this challenge have been

discussed at an international level, with efforts aimed at reducing the amount of antibiotics sold and used. This is achieved through the implementation of policies for both sale and application of antibiotics in clinical and farm settings, many of which have already been brought into effect.

While governmental (e.g. WHO, CDC, EU and the US White House) policies and reports promoting appropriate antibiotic use exist, it is in addition necessary to consider other strategies to tackle the issue of antibiotic resistance. Many of these institutions are therefore advocating a holistic approach, in accordance with the "One World, One Heath" initiative. The improvement of antibiotic resistance monitoring, sanitary measures in clinical and food production facilities, as well as stringent guidelines with regard to the use of antibiotics in food processing and animal raising will allow for reduction of antibiotic resistant bacteria and limit the emergence and dissemination of antibiotic resistant strains.

Considering the knowledge gaps for South Africa regarding antibiotic resistances as illustrated by the WHO 2014 surveillance report, the research outlined in the following chapters sought to provide a first insight into the status of wild animals as a potential reservoir of bacteria exhibiting antibiotic resistance. This study therefore focused on indigenous South African animals inhabiting what would be considered a "pristine" habitat and screened for *Escherichia coli* from faecal samples of three selected indigenous herbivores from a local nature reserve in Pietermaritzburg, KwaZulu-Natal, South Africa. Furthermore, *E. coli* was isolated from faecal samples of farmed pigs and from a "pet" (domestic) pig.

The resultant antibiotic resistance profiles of *E. coli* isolates were established by employing the EUCAST disk diffusion procedure. Thereafter, the results were verified using genotypic PCR screening for a selected β-lactamase gene, followed by qualitative and quantitative analysis of β-lactamase activity in isolates displaying phenotypic resistance to relevant β-lactam antibiotics.

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Assessment of three indigenous South African herbivores as potential reservoirs and vectors of antibiotic resistant *Escherichia coli*

Due to the limited data available on the presence of antibiotic resistant bacteria in faeces of wild herbivores in South Africa, this study analysed resistance patterns for *Escherichia coli* isolates from wildebeest, zebra and giraffe in addition to pet and farm pig faeces. Total and faecal coliforms and *E. coli* were quantified in faecal matter using a MPN guideline procedure. Antibiotic resistance profiles against twelve selected antibiotics representing seven antibiotic classes were determined for 30 randomly selected *E. coli* isolates from each animal using the European Committee on Antimicrobial Susceptibility Testing (EUCAST) disk diffusion procedure. While MPN values per gram of animal faeces for total/faecal coliforms ranged from 4.51/4.11 to 5.70/5.50, the *E. coli* MPN values were in a range of 3.43-5.14. About 47% of *E. coli* isolates from zebra faeces were categorized as multidrug resistant (MDR), while for wildebeest and giraffe no MDR isolates were detected. In comparison, 10% of *E. coli* isolates from pet pig and about 7% from farm pig faeces presented an MDR phenotype. Although most MDR isolates were resistant to at least one β-lactam antibiotic, only one MDR isolate from farm pig faeces was resistant to both norfloxacin and ciprofloxacin, the two fluoroquinolones tested. However, no resistance was detected to the tested carbapenems and tigecycline. The results of this study indicate that indigenous South African herbivores may serve as potential reservoirs and vectors for the dissemination of antibiotic resistant *E. coli* strains.

Introduction

The extensive use of antibiotic compounds in human and veterinary medicine has led to the presence of antibiotic residues in food and the environment (Welch 1957; Kümmerer 2003; Kemper 2008; Martinez 2009). Consequently, recent research has focused on the transfer and detection of antibiotic resistant microorganisms and their genes in the environment (Sørum and L'Abée-Lund 2002; Garcia-Alvarez et al. 2012). Only recently, the World Health Organization (WHO), the European Food and Safety Authority (EFSA) and even the President of the United States of America highlighted antibiotic resistance as a serious global health problem (EFSA 2016; WHO 2014; White House 2014). As zoonotic bacteria can acquire resistance to antibiotics typically utilized in human and veterinary medicine, farm animals can serve as a potential reservoir for antibiotic resistant bacterial strains as well as potential vectors for transferring such resistant bacteria or their genes into the environment (Sørum and L'Abée-Lund 2002; Silbergeld et al. 2008; EFSA 2016). Zoonotic bacteria presenting resistance to antibiotics critical in human medicine such as fluoroquinolones and 3rd and 4th generation cephalosporins are of particular concern, as they may compromise the effective treatment of infections in humans (Hammerum and Heuer 2009; WHO 2012). Humans can acquire infections with such antibiotic resistant zoonotic bacteria either via contact with animals or through ingestion of contaminated food products of animal and non-animal origin.

Escherichia coli is an intestinal bacterium commonly associated with the digestive system of animals and therefore considered a useful hygiene indicator (Leclerc et al. 2001). However, pathogenic strains of *E. coli* can be a serious threat to public health as was observed in 2011 and 2016, when Shiga-toxin producing strains of *E. coli* caused outbreaks in European countries, ultimately attributed to the consumption of sprouts made from contaminated fenugreek seeds (EFSA 2011) and soft cheese (Peron et al. 2016). The pathogenic *E. coli* strain causing the 2011 outbreak exhibited multidrug resistance (MDR) against ampicillin, ceftriaxone, streptomycin and tetracycline (King et al. 2012), further highlighting the problem of MDR *E. coli*.

Antibiotic usage in the diets of food animals through prophylactic or therapeutic means can promote the horizontal transfer of antibiotic resistance genes (Silbergeld et al. 2008; Wellington et al. 2013), which might even take place in the absence of selective pressure imposed by the use of antibiotics (Allen et al. 2010). Human-livestock contact taking place in rural areas in developing countries might promote exposure to and transfer of such zoonotic

bacteria due to the close contact between the population and livestock (Rwego et al. 2008; Klous et al. 2016). Additionally, studies in South Africa by Schellack et al. (2011) indicated that social issues including widespread poverty and lack of access to safe potable water and proper sanitation might stimulate the dissemination of multidrug resistant bacterial strains. However, antibiotic resistant bacteria are even found in the faeces of wild animals, ranging from primates (Rolland et al. 1985) to rodents (Guenther et al. 2010), wild boars (Literak et al. 2010) and various wild birds (Costa et al. 2008), with even extended spectrum β-lactamase (ESBL) containing *E. coli* being detected (Literak et al. 2010). It was assumed that the presence of such antibiotic resistant bacteria is most likely due to anthropogenic activities or contact with anthropogenic waste material; for example seagulls that scavenge food wastes (Cole et al. 2005; Radhouani et al. 2009) or wild baboons feeding on human refuse (Rolland et al. 1985). The ribotyping patterns of *E. coli* isolates from seagulls in New Hampshire (USA) had a >90% similarity to those of *E. coli* strains isolated from proximate wastewater treatment plants and landfill sites (Nelson et al. 2008), suggesting a potential for transfer of faecal bacteria to recreational areas frequented by humans. Moreover, considering the ability of long distance migration, birds can act as efficient transporters for the dissemination of antibiotic resistant bacteria and mirror the spectrum of such microorganisms found in humans and human waste materials (Radhouani et al. 2009).

Antibiotic resistance profiles of *E. coli* isolates from various African wild animals - ranging from buffalo, eland, and primates to the smaller mongoose and warthog - have been previously established and reported (Rolland et al. 1985; Skurnik et al. 2006; Pesapane et al. 2013; Jobbins and Alexander 2015; Katakweba et al. 2015). However, to the best of our knowledge, no information on the resistance profiles of *E. coli* from South African herbivores zebra, giraffe and wildebeest is available. To estimate the extent of antibiotic resistance among bacteria associated with these herbivores, which might serve as potential reservoirs and vectors, surveillance is necessary. Therefore, this study reports initial screening data pertaining to antibiotic resistance profiles in *E. coli* isolates from the three selected indigenous herbivores zebra, giraffe and wildebeest, in comparison to farm and pet (“domestic”) pig.

Materials and methods

Sample collection

Faecal samples of giraffe (*Giraffa camelopardalis*), zebra (*Equus burchellii*) and wildebeest (*Connochaetes taurinus*) were obtained from the Bisley Valley Nature Reserve (S29°66'18.99" E30°39'10.95"), while pig (*Sus scrofa domesticus*) faeces were collected from a commercial pig farm in KwaZulu-Natal and a private household (pig kept as a pet) situated in Pietermaritzburg (KwaZulu-Natal, South Africa). All samples were collected between February and May 2015 (the dry autumn season) using sterile, labelled plastic bags, and immediately transferred on ice to the laboratory with sample analysis taking place within 12 hours.

Quantification of total and faecal coliforms and *Escherichia coli* from faecal samples

Enumeration of total and faecal coliforms as well as *E. coli* was carried out in a single analysis for each of the five faecal samples according to the Most Probable Number (MPN) guideline procedure MFHPB-19 (Health Canada 2002). Confirmation of presumptive *E. coli* isolates was done via biochemical confirmation (GIMViC) and by PCR as described previously (Gemmell and Schmidt, 2012). For the PCR based detection of the *gad*A (glutamate decarboxylase A) gene, cells were harvested by centrifugation (13 500 x g, 5 min) from 1 ml of overnight cultures of presumptive *E. coli* isolates (nutrient broth, 35°C, 120 rpm), re-suspended in 100 µl sterile water followed by DNA extraction using a simple freeze and thaw technique (Gemmell and Schmidt 2012). The amplification reactions using the primers reported by Kim et al. (2006), were done in 25 µl volumes, containing 1.5 µl template DNA, 0.5 µl of each primer (10 µM, Inqaba South Africa), 12.5 µl 2x DreamTaq Green PCR Master Mix (Thermo Scientific) and nuclease free water (KAPA), with the following cycling conditions (Labnet MultiGene II thermocycler): initial denaturation at 94°C for 2 minutes followed by 25 cycles at 94°C for 30 seconds, annealing at 55°C for 30 seconds, 72°C for 1 minute and a final extension cycle at 72°C for 7 minutes. PCR products were analysed by electrophoresis on 1% (w/v) agarose gel with addition of SYBR® Safe stain (Life technologies) and visualized under UV light using a Gbox Chemi XRQ system (Syngene) and GeneSnap software. A ready-to-use 100 bp DNA ladder (KAPA) was used as size marker, *E. coli* ATCC 8739 served as positive PCR control with *Salmonella* Typhimurium ATCC 14028 and nuclease free water employed as negative controls. The expected size of the *gad*A amplicon was 680 bp.

Antibiotic susceptibility disk diffusion assay

The disk diffusion method (Version 5, 2015) from the European Committee on Antimicrobial Susceptibility Testing (EUCAST 2015) was used to assess the antibiotic resistance patterns of all *E. coli* isolates. The following twelve antibiotics were tested employing ready to use 6 mm disks (Oxoid): ampicillin (AMP - 10 µg), amoxicillin-clavulanic acid (AMC - 20/10 µg), cefotaxime (CTX - 5 µg), ceftazidime (CAZ - 10 µg), ertapenem (ERT - 10 µg), meropenem (MEM - 10 µg), aztreonam (ATM - 30 µg), ciprofloxacin (CIP - 5 µg), norfloxacin (NOR - 10 µg), gentamicin (GEN - 10 µg), tobramycin (TOB - 10 µg) and tigecycline (TIG - 15 µg). Antibiotics utilized in this study were categorized into antibiotic classes based on therapeutic relevance, as suggested by Magiorakos et al. (2012). For the disk diffusion assay, suspensions from each *E. coli* isolate were prepared from nutrient broth cultures after incubation at 35°C (120 rpm, 14 hours) and adjusted to approximately 2 x 10^8 cells per ml (corresponding to approximately 0.5 McFarland standard suggested by EUCAST) using a Helber type bacterial counting chamber (Marienfeld, Germany). 100 µl cell suspension was spread plated onto Mueller-Hinton (MH) agar (Oxoid) plates containing 25 ml of agar per sterile 90 mm Petri dish. Four different antibiotic test disks (adjusted for approximately 1 hour to ambient temperature), were firmly placed equidistantly onto the agar surface. Plates were sealed using Parafilm and incubated inverted in stacks of two at 35°C for 20 hours as specified (EUCAST 2015). Inhibition zones were measured to the nearest mm using digital Vernier callipers (Marshall Tools, India) and analysed using the latest EUCAST antibiotic breakpoint tables (EUCAST 2016). All isolates were analysed in duplicate against all twelve antibiotics.

Statistical analysis

Fisher's exact was used for analysis of the significant differences between the pairwise resistance profiles of *E. coli* isolated from various animals, by using SPSS for Windows (Version 23.0; SPSS Inc., Chicago, IL, USA).

Chemicals

Unless otherwise stated, all chemicals were of the highest purity commercially available.

Results

In this study, both total and faecal coliforms as well as *E. coli* were quantified in faeces collected from zebra, wildebeest, giraffe, farm and pet pig. The MPN values of total coliforms for wild herbivores were in the range of 4.51 to 5.36 $\log_{10}$ MPN/g, while values for pig faeces were slightly higher at 5.50 and 5.70 $\log_{10}$ MPN/g [Table 1]. As expected, faecal coliform levels were lower than the corresponding total coliform values, ranging from 4.11 (wildebeest) to 5.50 $\log_{10}$MPN/g (pet pig). The $\log_{10}$MPN/g values for *E. coli* in zebra, wildebeest and giraffe faeces were 4.69, 3.43 and 4.89 $\log_{10}$MPN/g respectively, while the values for pet and farm pig faeces were slightly higher at 5.11 and 5.14 $\log_{10}$ MPN/g [Table 1].

TABLE 1. Quantification of total and faecal coliforms and *Escherichia coli* (with 95% confidence intervals) in pooled faecal samples obtained from wild herbivores and pet and farm pigs.

Animal faeces	**Total coliforms** ($\log_{10}$ MPN/g)	95% CI (lower/ upper limit)	**Faecal coliforms** ($\log_{10}$ MPN/g)	95% CI (lower/ upper limit)	***E. coli*** ($\log_{10}$ MPN/g)	95% CI (lower/ upper limit)
Zebra	5.11	4.66 / 5.57	4.89	4.40 / 5.38	4.69	4.19 / 5.19
Wildebeest	4.51	4.04 / 4.99	4.11	3.65 / 4.56	3.43	3.06 / 3.80
Giraffe	5.36	4.89 / 5.83	5.11	4.66 / 5.57	4.89	4.40 / 5.38
Farm pig	5.70	5.19 / 6.20	5.34	4.95 / 5.72	5.14	4.74 / 5.54
Pet pig	5.50	5.03 / 5.97	5.50	5.03 / 5.97	5.11	4.66 / 5.57

From each of the five tested animal faecal samples, 30 randomly selected confirmed *E. coli* isolates were further analysed for their antibiotic resistance patterns. In total 106 out of 150 selected *E. coli* isolates from all animal faeces were resistant to at least one of the 12 antibiotic compounds tested [Table A1]. The highest proportion of *E. coli* isolates showing resistance to at least one of the twelve antibiotics tested was found in zebra faeces (93%) followed by isolates from pet pig and wildebeest faecal samples, with 90% and 80% resistance respectively. In contrast, isolates from farm pig and giraffe showed the lowest proportion of resistances with 47% and 43%, respectively [Table 2]. More than half (56.6%) of all 106 *E. coli* isolates from wild herbivore and pig faeces presenting antibiotic resistance possessed only one resistance,

followed by about 23% (24/106 isolates) with a combination of two antibiotic resistances. The remaining 22 *E. coli* isolates showed resistances ranging from three (19 isolates) up to even five (one isolate) antibiotic compounds. With a few exceptions (4 isolates), all antibiotic resistant *E. coli* isolates possessed resistance against at least one of the following tested β-lactam antibiotics: ampicillin, amoxicillin-clavulanic acid, ceftazidime, cefotaxime or aztreonam [Table A1]. Of all antibiotics tested, amoxicillin-clavulanic acid was the least effective as a large proportion (94 isolates) of the resistant *E. coli* isolates obtained from all five animals showed resistance against this compound [Figure 1]. However, no resistances were observed for the two tested carbapenem antibiotics ertapenem and meropenem, the cephalosporin antibiotic cefotaxime and the glycylcycline tigecycline [Table A1]. Interestingly, only 6 out of all 106 resistant *E. coli* isolates were resistant to ampicillin, half of which were isolated from farm pig faeces [Table A1]. The proportion of ceftazidime resistant *E. coli* isolates from zebra faeces was clearly higher than that observed in all other animals [Figure 1]. Among the non β-lactam antibiotics tested, the least effective antibiotic was the aminoglycoside compound tobramycin and, to a lesser degree, gentamicin [Figure 1]. Noticeably, one out of all 150 *E. coli* isolates - from farm pig faeces - presented resistance to both ciprofloxacin and norfloxacin, the two fluoroquinolone antibiotics tested.

TABLE 2. Prevalence of resistances and multidrug resistance (MDR) for *Escherichia coli* strains isolated from faeces of wild herbivores and pet and farm pigs.

Animal faeces	***N*** [a]	**Resistant** [b]	**Multidrug resistant (MDR)** [c]
Zebra	30	93% (28/30)	47% (14/30)
Wildebeest	30	80% (24/30)	0% (0/30)
Giraffe	30	43% (13/30)	0% (0/30)
Pet pig	30	90% (27/30)	10% (3/30)
Farm pig	30	47% (14/30)	7% (2/30)
Total	**150**	**71% (106/150)**	**13% (19/150)**

[a] Number of *E. coli* isolates analysed

[b] % of *E. coli* isolates resistant to one or more antibiotics (number of isolates)

[c] % of *E. coli* isolates resistant to 3 or more antibiotics representing at least 3 different antibiotic classes (number of isolates)

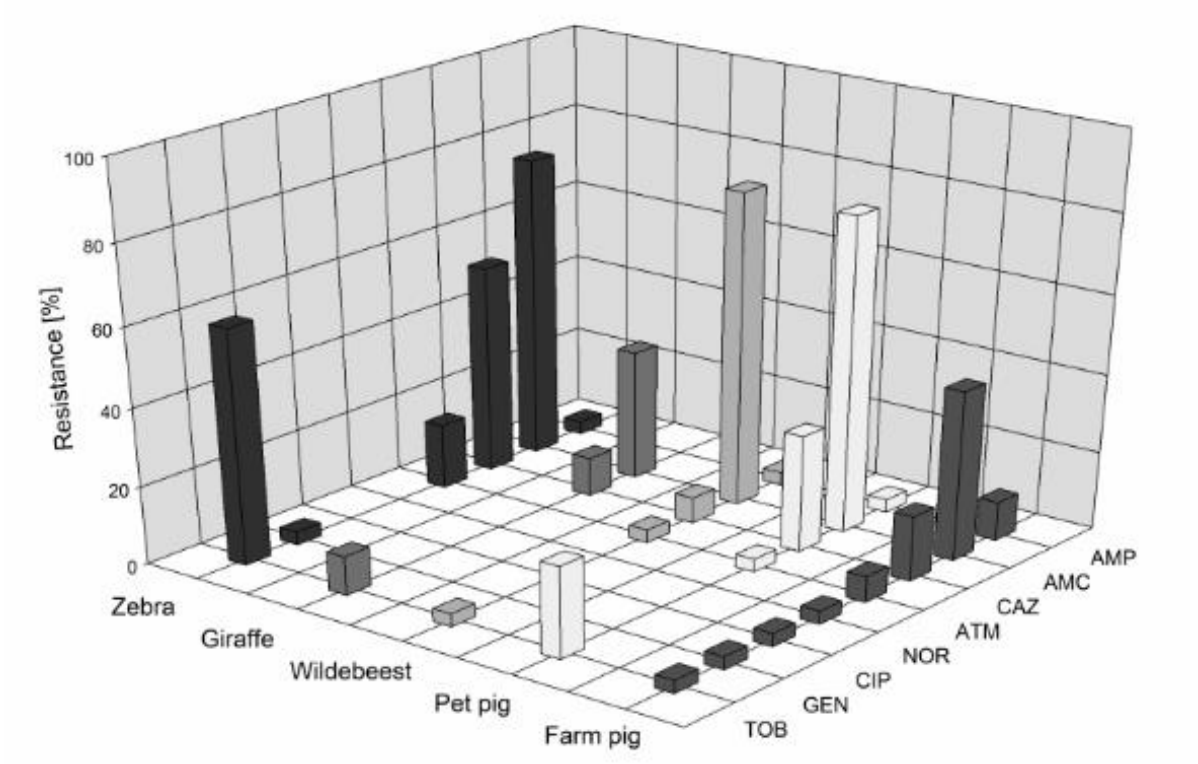

FIGURE 1. Incidence of resistances observed against selected antibiotics for *Escherichia coli* isolates obtained from zebra, giraffe, wildebeest and farm and pet pig faeces. Antibiotics for which no resistance was detected (cefotaxime, ertapenem, meropenem and tigecycline) are not shown.

AMP, ampicillin; AMC, amoxicillin-clavulanic acid; CAZ, ceftazidime; ATM, aztreonam; NOR, norfloxacin; CIP, ciprofloxacin; GEN, gentamicin; TOB, tobramycin

The prevalence of multidrug resistant (MDR) *E. coli* isolates - those showing resistance to antibiotics from three different classes - was considerably lower with 19 MDR isolates detected out of the total 150 *E. coli* isolates analysed [Table 2]. Approximately 73% (14/19) of these MDR isolates were obtained from zebra faeces, while the analysis of giraffe and wildebeest faeces resulted in no *E. coli* isolates with multiple drug resistance. The analysis of pet pig faeces established 10% (3 out of 30) MDR isolates. Interestingly, the proportion of MDR in *E. coli* isolates obtained from farmed pig was also in the lower range at about 7% (2 out of 30). The phenotypic resistance profiles for all multidrug resistant *E. coli* isolates are summarized in Table 3. Nearly all MDR *E. coli* isolates (18 out of 19) featured resistance against the antibiotic amoxicillin-clavulanic acid, and eleven isolates (8 isolates from zebra and 3 from pet pig) showed the same resistance profile of amoxicillin-clavulanic acid, ceftazidime and tobramycin (AMC-CAZ-TOB). One MDR *E. coli* isolate from farm pig faeces was resistant to five

different antibiotics: amoxicillin-clavulanic acid, ampicillin, aztreonam, norfloxacin and ciprofloxacin (AMC-AMP-ATM-NOR-CIP). Moreover, this was the only *E. coli* isolate displaying resistance to both fluoroquinolone antibiotics tested.

TABLE 3. Antibiotic resistance profiles detected for all multidrug resistant (MDR) *Escherichia coli* isolates from faeces of wild herbivores and pet and farm pigs.

Host species (number of isolates)	Phenotypic multidrug resistance profile
Zebra (8); Pet Pig (3)	AMC-CAZ-TOB
Zebra (2)	AMC-ATM-TOB
Zebra (1)	AMC-ATM-CAZ
Zebra (1)	ATM-CAZ-TOB
Zebra (1)	AMC-CAZ-GEN
Zebra (1)	AMC-ATM-CAZ-TOB
Farm Pig (1)	AMC-AMP-CAZ-GEN
Farm Pig (1)	AMC-AMP-ATM-NOR-CIP

AMP, ampicillin; AMC, amoxicillin-clavulanic acid; CAZ, ceftazidime; ATM, aztreonam; NOR, norfloxacin; CIP, ciprofloxacin; GEN, gentamicin; TOB, tobramycin

However, high levels of intermediate resistance were observed for the aminoglycoside antibiotics gentamicin (136 isolates) and tobramycin (116 isolates), as well as for the β-lactam antibiotics ceftazidime (101 isolates) and aztreonam (98 isolates) [Table A1]. In contrast to this, only one *E. coli* isolate - from wildebeest faeces - presented an intermediate resistance phenotype for tigecycline. In addition, less than 10 isolates showed an intermediate phenotype for the carbapenem antibiotic meropenem and the fluoroquinolone antibiotics norfloxacin and ciprofloxacin [Table A1].

The pairwise comparison between the observed antibiotic resistances *E. coli* isolates from different animals showed that the number of resistant *E. coli* isolated from giraffe was significantly lower than that isolated from zebra for the antibiotics amoxicillin-clavulanic acid, ceftazidime and tobramycin; and zebra was significantly higher than wildebeest for amoxicillin-clavulanic acid [Table S1].

Discussion

This study compared the number of total coliforms, faecal coliforms and *E. coli* in faecal matter of the five different animals tested. As expected, the MPN values established for total and faecal coliforms were higher in faeces from all five animals than the values for *E. coli*, with the highest numbers detected in pig faeces. The level of *E. coli* in pet and farm pig faeces in this study was 5.11 and 5.14 $\log_{10}$ MPN/g respectively, which is similar to the maximum counts of 5.11 $\log_{10}$ CFU/g reported for *E. coli* in fresh pig slurry from a Bulgarian pig farm (Petkov et al. 2006). The numbers of *E. coli* that were established for faeces of zebra, giraffe and wildebeest in the present study were somewhat lower in a range of 3.43 to 4.89 $\log_{10}$ MPN/g faeces. A similar scenario was observed by Smati et al. (2015) when comparing *E. coli* counts in wild and domesticated animal faeces, which was attributed to the variation in diet. Katakweba et al. (2015) quantified *E. coli* in cattle and wildlife faeces in Tanzania, obtaining counts for *E. coli* in wildlife faeces that were consistently higher than those obtained for cattle faeces. These authors established *E. coli* counts in faecal samples as 4.25 $\log_{10}$ CFU/g for zebra, 4.60 $\log_{10}$ CFU/g for wildebeest, 4.84 $\log_{10}$ CFU/g for buffalo and 3.14 $\log_{10}$ CFU/g for cattle. These values are almost identical to the values obtained for zebra (4.69 $\log_{10}$MPN/g), wildebeest (3.43 $\log_{10}$ MPN/g) and giraffe (4.89 $\log_{10}$ MPN/g) faeces from South Africa.

In addition, the 30 *E. coli* isolates obtained each from zebra, giraffe, wildebeest, pet pig and farm pig faecal samples were analysed to establish their antibiotic resistance profiles against twelve antibiotics using the EUCAST disk diffusion assay. The degree of resistances to one or more antibiotics observed in this study varied from 43%, for *E. coli* isolates from giraffe faeces, to 93% for *E. coli* isolates from zebra faeces. Similarly, Katakweba et al. (2015) detected antibiotic resistant *E. coli* in wildebeest and zebra faeces in Tanzania, with resistances to ampicillin, amoxicillin-clavulanic acid, cefotaxime and gentamicin identified. These authors identified 58.8% of *E. coli* isolates from wildebeest, 47.6% from zebra and 38.7% from buffalo as ampicillin resistant. The highest proportion of ampicillin resistant *E. coli* isolates from the three South African herbivores was therefore 3.3% lower, but closer to the percentage value of 9% reported for ampicillin resistant *E. coli* isolates from cattle in Serbia (Knezevic and Petrovic 2008).

In contrast to the data established for the South African pet and farm pig, higher rates of ampicillin resistance were detected in *E. coli* isolates from pet animals (dogs and cats) and bulls and horses in Spain at 31%, while 29% of isolates from farmed pigs in Spain presented

resistance (Sáenz et al. 2001). However, resistance rates for amoxicillin-clavulanic acid in the current study were higher at over 70% among *E. coli* isolates from South African zebra and wildebeest, than amoxicillin-clavulanic acid resistance rates reported recently for wildebeest (14.7%) and zebra (11.9%) in Tanzania (Katakweba et al. 2015). Similarly, for pet and farm pig from South Africa, amoxicillin-clavulanic acid resistance for *E. coli* isolates amounted to 80% and 43% respectively, while for pets and farm pigs from Spain only 6% of isolates were resistant to amoxicillin-clavulanic acid (Sáenz et al. 2001). As the EUCAST breakpoint tables (2016) do not provide an intermediate resistance category for ampicillin and amoxicillin-clavulanic acid, intermediate resistance phenotypes were not established for these two antibiotics.

E. coli isolates resistant to the cephalosporin antibiotic cefotaxime were not detected in the faeces from any of the five South African animals although this has been reported for *E. coli* isolates from herbivores in Tanzania (Katakweba et al. 2015). Similar to our results, cefotaxime resistance was not detected in pet and farm pigs analysed in Spain (Sáenz et al. 2001). However, resistant *E. coli* isolates were identified for the cephalosporin antibiotic ceftazidime and the monobactam antibiotic aztreonam. In the case of ceftazidime, this ranged from about 7% (wildebeest) to 53% (zebra) and for aztreonam from 0% (giraffe) to about 17% (zebra). While no resistance was detected for cefotaxime, the results of the EUCAST disk diffusion assays showed the presence of *E. coli* isolates displaying intermediate resistance to cefotaxime as defined by the breakpoint tables (EUCAST, 2016). Intermediate resistance phenotypes might indicate a likelihood of therapeutic failure under specific clinical treatment conditions (Rodloff et al. 2008). Therefore, the EUCAST expert rules suggest categorizing certain intermediate resistances as resistant to avoid potential treatment failure (Leclercq et al. 2013). It is therefore concerning that 32 out of 150 isolates exhibited intermediate resistance for cefotaxime and that 98 and 101 out of 150 isolates showed intermediate resistance for ceftazidime and aztreonam respectively, particularly as these antibiotics are defined as critically important antibiotics by the WHO (2012).

No resistance was observed for any of the 150 *E. coli* isolates against the carbapenem antibiotics meropenem and ertapenem. The absence of resistance is encouraging and might be attributed to the fact that carbapenems are intended for use in human medicine while their application in veterinary medicine should be avoided (WHO 2012; Poirel et al. 2014). Similar to our results, Costa et al. (2008) did not detect isolates with resistance to the carbapenem

imipenem when analysing 112 *E. coli* isolates from a large range of wild animals from nature reserves in Portugal. However, 6 (meropenem) and 30 (ertapenem) out of 150 *E. coli* isolates with intermediate resistance were nevertheless detected in our study.

Similarly, low numbers of resistant and intermediate phenotypes were detected for the fluoroquinolone antibiotics norfloxacin and ciprofloxacin [Figure 1 and Table A1]. The only incidence of resistance to both ciprofloxacin and norfloxacin was detected for one *E. coli* isolate from farm pig faeces, with two additional isolates from the same animal showing intermediate resistance for ciprofloxacin and a ciprofloxacin/norfloxacin combination, respectively. This is in line with Sáenz et al. (2001), who identified 3% of *E. coli* isolates from farm pigs as ciprofloxacin resistant, which may be due to the use of fluoroquinolones for treating food animals (Collignon 2005). Other *E. coli* isolates showing an intermediate phenotype for fluoroquinolones were mainly present in zebra faeces [Table A1], which is surprising as they are not under veterinary surveillance.

For the *E. coli* isolates from South African wild herbivores and pigs, the resistance rates for tobramycin (30/150: 18 from zebra, 7 from pet pig, 3 from giraffe, one each from wildebeest and farm pig) were elevated while those detected for gentamicin (2/150: one each from zebra and farm pig) were clearly lower [Figure 1]. For *E. coli* isolated from zebra and wildebeest in Tanzania, 23.8% and 20.6% of isolates showed resistance to gentamicin while this amounted to only 3.2% in buffalo (Katakweba et al. 2015). For farm pigs Sáenz et al. (2001) reported that 7% of *E. coli* isolates showed resistance to these two aminoglycoside antibiotics while a study from Serbia reported that only 1% of *E. coli* isolated from swine presented resistance to gentamicin and tobramycin (Knezevic and Petrovic 2008), which is in line with our results for farm pig faeces. The somewhat higher incidence of tobramycin resistance in pet pig isolates (7/30) might therefore be due to veterinary treatment or close contact to humans. It is noteworthy that the levels of intermediate resistance among all 150 *E. coli* isolates were particularly high for the aminoglycoside antibiotics gentamicin (about 91%) and tobramycin (about 77%), both of which are approved for use in veterinary medicine in South Africa (Eagar et al. 2012). Only three isolates from giraffe and one from farm pig were sensitive to both aminoglycoside antibiotics.

Out of all 150 *E. coli* isolates analysed in this study, no isolate was resistant to tigecycline and only one isolate from wildebeest faeces presented an intermediate resistance phenotype. Sáenz et al. (2001) and Knezevic and Petrovic (2008) reported that more than 60% of *E. coli* isolates

from farmed pigs in Spain and Serbia were resistant to tetracycline, and tetracycline resistance was reported as well for *E. coli* isolates from impala (Mariano et al. 2009), wildebeest, zebra and buffalo in Africa (Katakweba et al. 2015). However, the antibiotic tigecycline used in our study is a newer generation tetracycline type antibiotic, having only received approval for use by the FDA in 2005 (Noskin 2005) and not usually considered for use in veterinary medicine (Papich 2012). Even in the presence of tetracycline resistance, tigecycline evades common tetracycline resistance mechanisms (Fluit et al. 2005) although recent work demonstrated that mutations in tetracycline resistance genes can confer increased MIC (minimum inhibitory concentration) values for tigecycline in *E. coli* (Linkevicius et al. 2016). Nevertheless, among more than 360 clinical isolates of *E. coli* from South Africa, no tigecycline resistance phenotype was detected, highlighting the efficiency of this antibiotic (Kanj et al. 2014).

Nearly half (46 out of 106) of the antibiotic resistant *E. coli* isolates obtained from faecal samples possessed resistance against more than one antibiotic, with a total of 19 isolates exhibiting multidrug resistance (MDR), hence presenting resistance to 3 or more antibiotics belonging to at least 3 different antibiotic classes. The highest MDR proportion among all animal samples was observed for zebra faeces with 47% (14 out of 30 isolates, Table 2) displaying such a phenotype. However, no MDR *E. coli* isolates were detected in faecal samples from wildebeest and giraffe. The remaining five MDR *E. coli* isolates came from pet pig (3 isolates) and farm pig (2 isolates) faeces. Other studies in Africa also showed a large variability in the degree of MDR among *E. coli* isolates from different wild animals. Pesapane et al. (2013) established 40% of faecal *E. coli* isolates from banded mongoose in the Chobe National Park in Botswana as MDR, attributing this to the close contact of mongoose with guest and staff accommodations of the National Park. Similarly, Jobbins and Alexander (2015) analysed resistance patterns of *E. coli* isolated from a variety of animals in Botswana, including herbivores such as giraffe, impala and waterbuck as well as carnivores such as leopard and spotted hyena. Similar to our results, no multidrug resistant *E. coli* were obtained from giraffe faeces (Jobbins and Alexander 2015). Moreover, the percentage of MDR isolates varied greatly among different animals in Botswana, with some exhibiting 100% MDR, as in the case of isolates from otter and spotted hyena faeces, and some with low to non-existent MDR phenotypes such as those isolated from warthog, impala and bushbuck faeces (Jobbins and Alexander 2015). The high variability of MDR prevalence between different animals was attributed to dietary factors such as whether the animal is naturally a herbivore, omnivore or carnivore, water-proximity and association with urban areas influencing the observed

resistance profiles (Jobbins and Alexander, 2015). Another study that surveyed the prevalence of MDR *E. coli* isolates among farm pigs from the Mekong Delta in Vietnam found almost 87% of isolates displaying a multidrug resistant phenotype (Nhung et al. 2015). However, these authors additionally analysed the difference between the prevalence of MDR in wild mammals trapped on farms, versus those trapped in nearby forests and rice fields. The results indicated that MDR phenotypes were almost eight times more abundant among *E. coli* isolates from wild mammals trapped on farms than among those *E. coli* isolates from mammals trapped in forests or fields, again highlighting that antibiotic resistance is likely driven in part by antibiotic usage on farms and contact to humans. This matches a study from Canada, where levels of resistant *E. coli* from wild mammals trapped on a farm were more than five times higher than in wild animals trapped in natural areas (Kozak et al. 2009). In the present study, the proportion of antibiotic resistant *E. coli* in farm pig faeces was in the lower range with only 14 resistant isolates, which is similar to the number of isolates detected for giraffe but much lower than the pet pig resistance percentage of 90%, which included 3 MDR isolates. A possible reason for this comparably high percentage of antibiotic resistant *E. coli* isolates in pet pig faeces might be due to its close and regular contact with humans. In addition, the intensive care it receives (including regular veterinary care and possible treatments to maintain its health) is similar to the treatment companion animals such as dogs and cats receive, which is known to potentially elevate resistance levels among bacterial isolates (Guardabassi et al. 2004).

Although the presence of antibiotic resistant bacteria in wildlife inhabiting habitats devoid of apparent antibiotic pressure may seem an unlikely phenomenon, such incidences were observed even decades ago. In 1978, Sato et al. analysed *E. coli* isolated from feral and kept pigeons, and discovered the presence of conjugative R-plasmids, conferring resistance against chloramphenicol. Furthermore, research by Tsubokura et al. (1995) reported the presence of antibiotic resistant *E. coli* in migratory waterfowl in Japan between the years of 1983 and 1986. Research concerning the resistance profiles of *E. coli* isolated from over 77 wild mammal species was carried out in Australia (Sherley et al. 2000), with a low but widespread prevalence of antibiotic resistance being observed. In the current study, the incidence of *E. coli* isolates from wild animals resistant to at least one antibiotic was remarkably high for those isolated from zebra (28/30) and wildebeest (24/30), with a much lower incidence detected in *E. coli* isolates from giraffe faeces (13/30).

The presence of *E. coli* with extended spectrum β-lactamases (ESBL) in wild boars was attributed to the omnivorous diet of the wild boar and the tendency to consume animal and human waste (Literak et al. 2010). As residents of Pietermaritzburg and surrounds can visit the park for recreational purposes, the wild herbivores are potentially exposed to humans and human waste, enabling the transfer of resistant bacteria to the herbivores. In fact, even contaminated clothing or contamination of the animal keeper were suggested as vectors for the spread of antibiotic resistant bacteria (Bosman et al. 2014; Poirel et al. 2014). A recent study in Tanzania demonstrated that the presence of antibiotic resistant *E. coli* in zebra faeces was linked to frequent contact between the animals and staff and visitor lodging areas (Katakweba et al. 2015).

An additional factor potentially contributing to the presence of antibiotic resistant *E. coli* in faeces from wild herbivores in the current study is the proximity of the wild animals to a horse stable adjacent to the Bisley Nature Reserve. Although zebra, wildebeest and giraffe are not known to receive antibiotic treatment, the horses in the stables - which might enter the nature reserve - likely receive regular veterinary attention.

Only recently, herbicides such as glyphosate were identified as a factor causing elevated antibiotic resistance levels in bacteria (Kurenbach et al. 2015). Strains of *E. coli* and *S.* Typhimurium showed several fold higher MIC values for certain antibiotics after exposure to sub-lethal concentrations of both antibiotics (including ampicillin and tetracycline) and herbicides (Kurenbach et al. 2015). The use of such herbicides in domestic and agricultural settings in the proximity of the nature reserve or the application of such herbicides within the park might therefore induce antibiotic resistant phenotypes in bacteria present.

Finally, it is well established that animals - including herbivores - perform geophagy, which might lead to the uptake of secondary metabolites such as antibiotics produced by microorganisms present in the soil consumed (Mahaney et al. 1999). This and the fact that zebra and wildebeest preferentially graze close to the soil - with possible exposure to human waste present - whilst giraffe would preferentially practice browsing leaves from trees, might explain to some degree why the level of antibiotic resistance was significantly higher in zebra and wildebeest *E. coli* isolates than in *E. coli* isolates from giraffe [Table S1].

The data obtained in this study show that wild herbivores from KwaZulu-Natal serve as potential reservoirs for antibiotic resistant and even multidrug resistant *Escherichia coli* strains. This may be due to association with anthropogenic activity and proximity to a neighbourhood. However, further research is required in order to address which factors are involved in establishing the presence of antibiotic resistant bacteria in the herbivores targeted in this study.

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SUPPLEMENTARY TABLE S1. Pairwise comparison (Fishers exact test, two-tailed) of the antibiotic resistance profiles of *Escherichia coli* isolated from zebra, wildebeest, giraffe, pet pig and farm pig faeces resistant to twelve different antibiotics. *

	AMP	AMC	CTX	CAZ	ATM	ERT	MEM	NOR	CIP	GEN	TOB	TIG
Zebra & giraffe	>0.9999	0.0016	>0.9999	0.0006	0.0522	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	0.0001	>0.9999
Zebra & wildebeest	>0.9999	>0.9999	>0.9999	0.0001	0.1945	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	0.0001	>0.9999
Zebra & pet pig	>0.9999	>0.9999	>0.9999	0.1154	0.1945	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	0.0082	>0.9999
Zebra & farm pig	0.6120	0.0169	>0.9999	0.0061	0.4238	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	0.0001	>0.9999
Giraffe & wildebeest	>0.9999	0.0006	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	0.6120	>0.9999
Giraffe & pet pig	>0.9999	0.0006	>0.9999	0.1042	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	0.2990	>0.9999
Giraffe & farm pig	0.2373	0.5959	>0.9999	0.7065	0.4915	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	0.6120	>0.9999
Wildebeest & pet pig	>0.9999	>0.9999	>0.9999	0.0419	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	0.0522	>0.9999
Wildebeest & farm pig	0.6120	0.0073	>0.9999	0.4238	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999
Pet pig & farm pig	0.6120	0.0073	>0.9999	0.3604	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	0.0522	>0.9999

* Pairs were considered significantly different if $p \leq 0.05$, indicated by green highlight

β-lactamase gene screening and determination of specific enzyme activity in *Escherichia coli* isolated from wild South African herbivores and farm and pet pigs

Escherichia coli presenting resistance to at least one β-lactam antibiotic (ampicillin, amoxicillin-clavulanate, ceftazidime, aztreonam or a combination thereof), originating from faecal samples of wild herbivores (zebra, giraffe and wildebeest) and farm and pet pigs in KwaZulu-Natal, South Africa, were screened for the presence of one selected β-lactamase gene and β-lactamase activity. The *bla*$_{TEM}$ gene was detected in three farm pig *E. coli* isolates. All 102 isolates were thereafter tested for β-lactamase activity using a nitrocefin hydrolysis spot-test. 64 β-lactam-resistant isolates tested colourimetrically positive for β-lactamase activity (63%), with 38 isolates failing to hydrolyse nitrocefin (37%). Thereafter, the specific β-lactamase activity using nitrocefin was determined for 10 *E. coli* isolates (from herbivore and pig faeces) displaying a variety of phenotypic β-lactam resistance profiles and nitrocefin hydrolysis reactions. Two pet pig isolates that failed to hydrolyse nitrocefin presented specific activities of <1 nmol × min^{-1} × mg^{-1}, while three farm pig isolates containing the *bla*$_{TEM}$ gene had specific activities of between 81 and 168 nmol × min^{-1} × mg^{-1} - the highest observed among all 10 isolates. The results obtained demonstrate the presence of the *bla*$_{TEM}$ gene in farm pigs in KwaZulu-Natal, South Africa. However, detection of *E. coli* containing the gene in wild or "pristine" environments was not observed. Furthermore, the data show that while genotypic methods may effectively determine the presence or absence of resistance genes, it is worthwhile to include additional phenotypic and enzymatic tests to verify the resistance profiles.

Introduction

Since the pioneering discovery of the first β-lactam, penicillin-G, in the late 1920s, β-lactam antibiotics have found extensive use in both clinical and veterinary environments (Fleming 1929; Henderson 1997; Guenther et al. 2011). Following the introduction of penicillin as an antibiotic agent available to the public in the early-1940s, the first cephalosporin antibiotic was approved for clinical use in 1964 (Greenwood 2008). The frequent and favoured use of these broad-spectrum antibiotics can be largely attributed to their safety as pharmaceutical agents, as serious side-effects are rare (Moellering et al. 1989; Drawz and Bonomo 2010).

Although initially hailed as a "miracle drug", resistance to penicillin by *Staphylococcus aureus* was observed already in 1940 (Abraham and Chain 1940), initially in hospitals before spread to the community occurred (Rammelkamp and Maxon 1942; Kirby 1944). The increase in bacterial resistance as a result of widespread and often indiscriminate use of these antibiotics in turn causes a reduction or even elimination of the agents' efficacy in treating infections (Essack 2001). Resistance is most commonly mediated by the inactivation of the antibiotic itself through β-lactamases, as well as by reduced cell membrane permeability through the loss or alteration of porins and alteration of the antibiotic target sites (i.e. the penicillin-binding proteins) (Nikaido 2000; Essack 2001; Poole 2004).

β-lactamases are enzymes that hydrolyse the amide bond of the β-lactam ring, rendering the antibiotic ineffective (Birnbaum et al. 1985; Majiduddin et al. 2002). In Gram-negative bacteria, β-lactamases are located in the periplasmic space situated between the outer and cytoplasmic membranes, and may be plasmid or chromosomally encoded depending on the enzyme type and bacterial species (Livermore, 1986). The efficacy of this resistance mechanism is influenced both by the enzyme's affinity for the β-lactam antibiotic as well as the quantity of enzyme produced (Neu 1986; Livermore 1995). There are currently more than 400 characterized β-lactamases; including ESBLs, carbapenemases and cephamycinases (Guenther et al. 2011; Graham et al. 2016).

With the escalation of antibiotic resistance, it has become essential to develop methods to efficiently and accurately detect and characterize resistances in bacteria isolated from human, animal and environmental sources. Phenotypic methods include the well-established classical disk-diffusion procedure where pre-established breakpoint tables are used to determine the resistance profile of an isolate to a number of antibiotics (Bauer et al. 1966; Jorgensen and

Ferraro 2009). This method in particular is very cost-effective and flexible. A potential disadvantage is the lack of automation in the case of large numbers of samples and antibiotics. Alternatively, more sophisticated genotypic methods like PCR can be utilized to screen bacterial isolates for the presence of known resistance genes. While this method can screen for specific genes encoding β-lactamases, selecting from the large number of known β-lactam resistance genes may prove difficult for routine laboratories. Furthermore, the presence of silent or pseudogenes may lead to false-positive results (Sundsfjord et al. 2004). Other methods that exist for β-lactam resistance detection include chromogenic tests. Established is the iodometric test, where iodine is reduced as a result of benzylpenicillin hydrolysis. The subsequent yield of penicilloic acid decolourizes the starch-iodine complex (Livermore and Brown 2001). Nitrocefin is a chromogenic β-lactam substrate, characterized by its structural similarity to the cephalosporin antibiotics, routinely used in the qualitative and quantitative detection of β-lactamases (Livermore and Brown 2001). Upon hydrolysis, nitrocefin changes from yellow to red, making it an effective chromogenic substrate (O'Callaghan et al. 1972; McManus-Munoz and Crowder 1999). Nitrocefin can be applied qualitatively as a reagent in a visual spot-test, or quantitatively in enzyme assays (O'Callaghan et al. 1972; Livermore and Brown 2001). Although an expensive substrate, the nitrocefin hydrolysis method is considered more sensitive and reliable than the iodometric test (Livermore and Brown 2001).

The aim of this study was therefore to confirm and supplement the phenotypic profiles of β-lactam-resistant *Escherichia coli* from South African wild herbivores and farm and pet pigs (Chapter 2), using genotypic and enzymatic methods. All β-lactam-resistant *E. coli* isolates were screened for the presence of one specific β-lactamase gene: bla_{TEM}. Additionally, all isolates were subjected to a qualitative nitrocefin spot-test to determine the presence of β-lactamase activity. Finally, the specific β-lactamase activity was determined for ten selected *E. coli* isolates, displaying a variety of phenotypic β-lactam resistance profiles.

Materials and methods

Bacterial strains

A total of 102 β-lactam-resistant *Escherichia coli* strains isolated from faecal matter of herbivores and pet and farm pigs, as described in Chapter 2 above, were used for the following experiments. All isolates were resistant to at least one or more of the following β-lactam antibiotics: ampicillin, amoxicillin-clavulanate, cefotaxime, ceftazidime and aztreonam.

Bacterial cultivation

Isolates utilized for PCR analysis were grown overnight in 20 ml of nutrient broth (Oxoid), at 37°C and 120 rpm. For preparation of cell-free crude extract, 10 selected bacterial strains were grown in 100 ml of nutrient broth in 500 ml Erlenmeyer flasks, at 37°C and 120 rpm until an OD_{600} of ~ 2 was achieved.

Crude extract preparation

Cells were harvested by centrifugation at 10 000 x g for 10 minutes at 4°C (Beckman Avanti J-26 XPI), washed twice in sodium-phosphate buffer (0.5 M, pH 7), and finally centrifuged again at 10 000 x g for ten minutes followed by re-suspension in 1 ml of the same buffer. To obtain the crude extract, cells were disrupted using an ultrasonic treatment with a maximum output of 7 W (VirTis Virsonic 60, Polychem), consisting of 15 seconds sonication per 1 ml aliquot, followed by a 30 second cooling break on ice. The procedure was repeated ten times per isolate with manual mixing between each sonication. Cell debris was removed by centrifugation at 10 000 x g for 10 minutes at 4°C, followed by a final centrifugation at 40 000 x g for 45 minutes at 4°C. The clear and cell-free supernatant was collected and stored at –20°C until required.

Protein determination

The protein content of the crude extracts was determined according to Spector (1978), using Bovine Serine Albumin (Oxoid) as a standard.

PCR screening for the *bla*$_{TEM}$ gene

Cells were harvested by centrifugation at 13 500 x g for 5 min (Progen GenFuge 24D) and the pellets re-suspended in 100 µl sterile distilled water. DNA was extracted using a freeze-thaw technique (Gemmell and Schmidt 2012). The following primers (Inqaba, South Africa) were used as described by Ahmed et al. (2007): *bla*$_{TEM}$-F (5'-ATAAAATTCTTGAAGACGAAA-3'), *bla*$_{TEM}$-R (5'- GACAGTTACCAATGCTTAATC-3'). The amplification reactions were performed in 25 µl volumes with 1.5 µl template DNA, 0.5 µl of each primer (10 µM), 12.5 µl 2x DreamTaq Green PCR Master Mix (Thermo Scientific) and nuclease free water (Kapa). The PCR cycling conditions involved (Labnet MultiGene II thermocycler): initial denaturation step at 94°C for 2 minutes; 25 cycles of 94°C for 30 seconds; annealing at 55°C; extension at 72°C for 1 minute and a final extension cycle at 72°C for 7 minutes. PCR products were analysed by electrophoresis on 1% (w/v) agarose gel using a ready-to-use 1kb DNA ladder (ThermoScientific) as a size marker with addition of SYBR® Safe stain (Life technologies), and were visualized under UV light using a Gbox Chemi XRQ system (Syngene) and GeneSnap software. As negative controls, *E. coli* ATCC 8739 and water were used. The expected size of the *bla*$_{TEM}$ amplicon was 1080 bp.

Nitrocefin spot-test

One loop of colony biomass from each isolate grown on nutrient agar (Oxoid) at 37°C overnight was transferred to a well of a microtitre plate containing 20 µl of a 0.5 mg/ml nitrocefin (Oxoid). The plate was manually agitated by gently rotating it on the work surface for one minute and then incubated in the dark at 30°C for 30 minutes. Development of a distinct red colour within 30 minutes constituted a positive result for nitrocefin hydrolysis. Heat inactivated *E. coli* ATCC 8739 cells and *S.* Typhimurium ATCC 14028 served as negative controls in the spot-test. In addition, one *E. coli* isolate from each animal group presenting susceptibility to all β-lactam antibiotics was tested using nitrocefin as a negative control.

β-lactamase activity

Crude extracts of ten selected isolates with positive (8) and negative (2) nitrocefin spot-test results were used to spectrophotometrically determine the specific β-lactamase activity. The hydrolysis of 10 µg of nitrocefin (Oxoid) in a total volume of 1 ml in the presence of 10-100 µg of protein was monitored at 485 nm at 30 °C (BioRad SmartSpec™ Plus) using phosphate

buffer (0.5 M, pH 7) as described by O'Callaghan et al. (1972). The specific β-lactamase activities were determined using a molar extinction coefficient of 17 420 $M^{-1}cm^{-1}$ for the nitrocefin hydrolysis product as suggested by McManus-Munoz and Crowder (1999). The specific activity was established as nanomols of nitrocefin hydrolysed per minute per milligram of protein.

To confirm hydrolysis of the β-lactam ring by nitrocefin, β-lactamase activity was demonstrated by measuring the absorbance spectra over time in the presence of crude extract from a representative *E. coli* isolate from farm pig, FP5 (Shimadzu UV1800 Spectrophotometer). In a total volume of 1 ml, analysis of 10 µg of nitrocefin incubated in the presence of 43 µg protein from farm pig isolate FP5, in 0.5 M sodium-phosphate buffer (pH 7.0) at 30°C occurred.

Chemicals

Unless otherwise stated, all chemicals were of the highest purity commercially available. The nitrocefin stock solution was prepared as per the manufacturer's instructions (Oxoid).

Results

A total of 102 *Escherichia coli* faecal isolates from three herbivores and pet and farm pigs (Chapter 2) presenting phenotypic resistance to either ampicillin (AMP), amoxicillin-clavulanic acid (AMC), ceftazidime (CAZ), aztreonam (ATM) or a combination thereof - [Table A1], were screened using PCR. Only three farm pig *E. coli* isolates (FP5, FP6 and FP29) tested positive for the *bla*$_{TEM}$ gene [Figure 1].

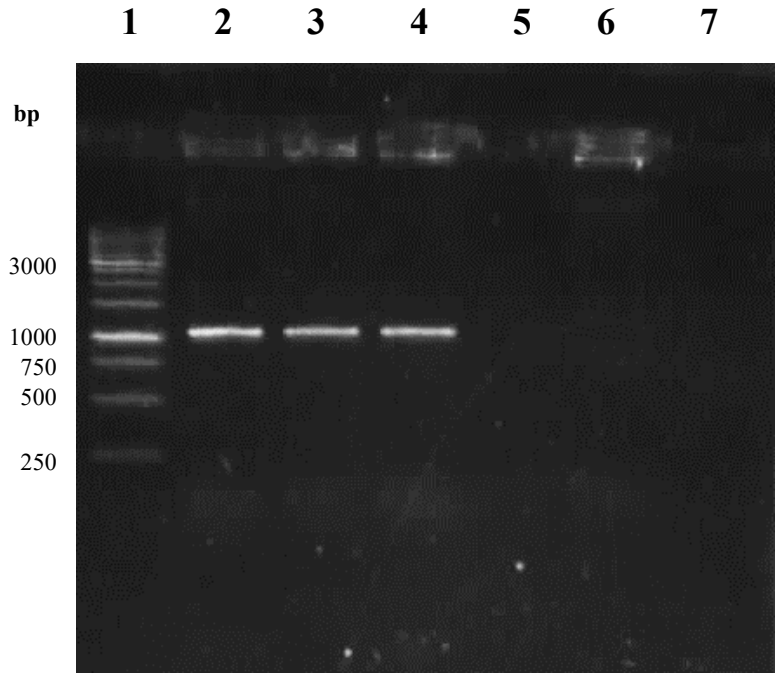

FIGURE 1. Representative 1% agarose gel image depicting results of PCR: amplified *bla*$_{TEM}$ gene (1080 bp) from *Escherichia coli* isolated from farm pig. *E. coli* ATCC 8739 served as a negative control.

Lane:
1 - 1 kb Molecular Weight Marker; **2** - FP5 (farm pig); **3** - FP6 (farm pig); **4** - FP29 (farm pig)
[**5** - Empty]
6 - *E. coli* ATCC 8739; **7** - Nuclease-free water

All 102 *E. coli* isolates were qualitatively screened for β-lactamase activity by means of a nitrocefin spot-test, with representative results shown in Figure 2. Of 102 β-lactam-resistant isolates, 64 (63%) tested positive for β-lactamase activity in the nitrocefin spot-test, while 38 tested negative (37%) [Table A1]. All 6 *E. coli* isolates presenting resistance to ampicillin hydrolysed nitrocefin [Table A1]. The 6 ampicillin resistant *E. coli* isolates (one each from zebra, wildebeest and domestic pig, and three from farm pig), were all resistant in combination with amoxicillin-clavulanic acid, and either ceftazidime or aztreonam. Of 102 isolates, 61 (from both herbivores and pigs) were resistant to only amoxicillin-clavulanic acid with 38 of these 61 (62%) testing positive for β-lactamase. Among the 38 *E. coli* isolates resistant to only amoxicillin-clavulanic acid, 7 of 9 giraffe isolates (78%) and 18 of 20 wildebeest isolates (90%) were nitrocefin positive. Conversely, only 3 of 7 zebra isolates (43%), 9 of 18 pet pig isolates (50%) and 1 of 7 farm pig isolates (14%) tested positive for nitrocefin hydrolysis. Phenotypic resistance to just one cephalosporin antibiotic, ceftazidime, occurred in 5 of 102 *E. coli* isolates, of which 3 (from zebra and giraffe faeces) were nitrocefin positive, while 2 isolates from pet pig were nitrocefin negative.

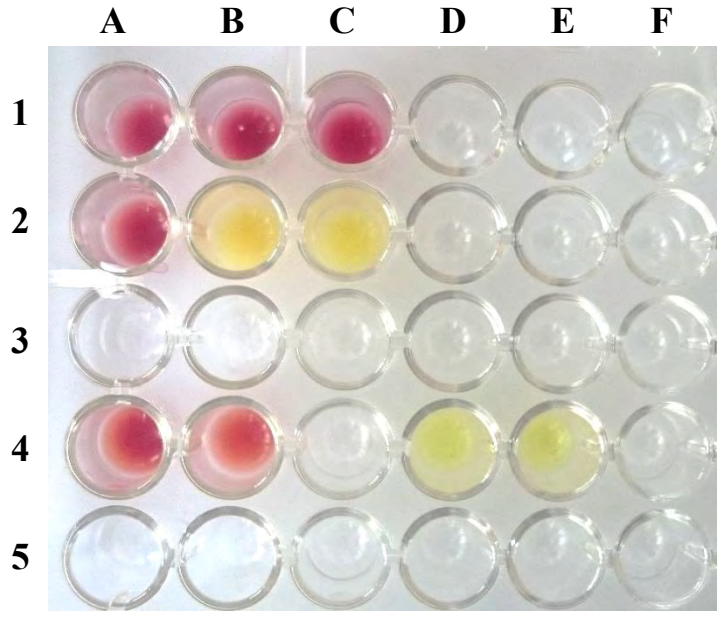

FIGURE 2. Nitrocefin spot-test for 8 randomly selected representative *Escherichia coli* isolates and two controls. The test was performed in a microtitre plate as indicated in the materials and methods section.

Red indicates a positive reaction; **Yellow** indicates a negative reaction

A1: FP5; **B1**: FP6; **C1**: FP29 (farm pig)
A2: DP6; **B2**: DP5; **C2**: DP7 (pet pig)
A4: Z2; (zebra) **B4**: W9 (wildebeest)
D4: Heat inactivated *E. coli* ATCC 8739; **E4**: *S.* Typhimurium ATCC 14028

Ten selected *E. coli* isolates displaying a variety of phenotypic resistance profiles and nitrocefin hydrolysis results were then further evaluated for specific β-lactamase activity [Table 1]. The *E. coli* isolates chosen represented herbivore, farm pig and pet pig isolates. Out of these 10 isolates, 8 tested positive and 2 tested negative in the nitrocefin spot-test. The specific β-lactamase activity was determined for all 10 isolates. For the nitrocefin-negative isolates (DP5 and DP7 - pet pig isolates), the specific activity was <1 nmol × min^{-1} × mg^{-1} [Table 1]. Isolates containing the *bla*$_{TEM}$ gene (FP5, FP6 and FP29 - farm pig isolates) showed specific activities of 144, 81 and 168 nmol × min^{-1} × mg^{-1} respectively - the highest specific β-lactamase activity of all 10 isolates. The tested herbivore isolates Z2 and Z19 (zebra); W9 and W26 (wildebeest) and G23 (giraffe), showed specific activities of between 2 and 3 nmol × min^{-1} × mg^{-1}.

TABLE 1. Nitrocefin spot-test results, *bla*$_{TEM}$ gene detection and specific β-lactamase activity (nmol × min^{-1} × mg^{-1}) for ten selected *Escherichia coli* isolates isolated from zebra, giraffe, wildebeest, pet pig and farm pig faeces in KwaZulu-Natal, South Africa.

Isolate	Nitrocefin spot-test result [a]	*bla*$_{TEM}$ gene [b]	Specific activity (nmol × min^{-1} × mg^{-1}) [c]
FP5	+	+	144
FP6	+	+	81
FP29	+	+	168
Z2	+	-	2
Z19	+	-	2
W9	+	-	3
W26	+	-	2
G23	+	-	2
DP5	-	-	<1
DP7	-	-	<1

[a] + denotes a change in colour in the nitrocefin spot-test from yellow to red within 30 minutes incubation at 30°C in the dark

[b] + denotes the expected amplification product (1080 bp) for the *bla*$_{TEM}$ gene was obtained

[c] The specific activity stated for each isolates is the average of three independently performed experiments

The hydrolysis of nitrocefin by β-lactamase activity was confirmed by UV-Vis spectrometry. [Figure 3]. The absorbance maximum due to the presence of nitrocefin at 390 nm decreased over time, and a new maximum, due to the hydrolytic cleavage of the β-lactam ring by nitrocefin, emerged at 485 nm. The isosbestic point was established at approximately 440 nm.

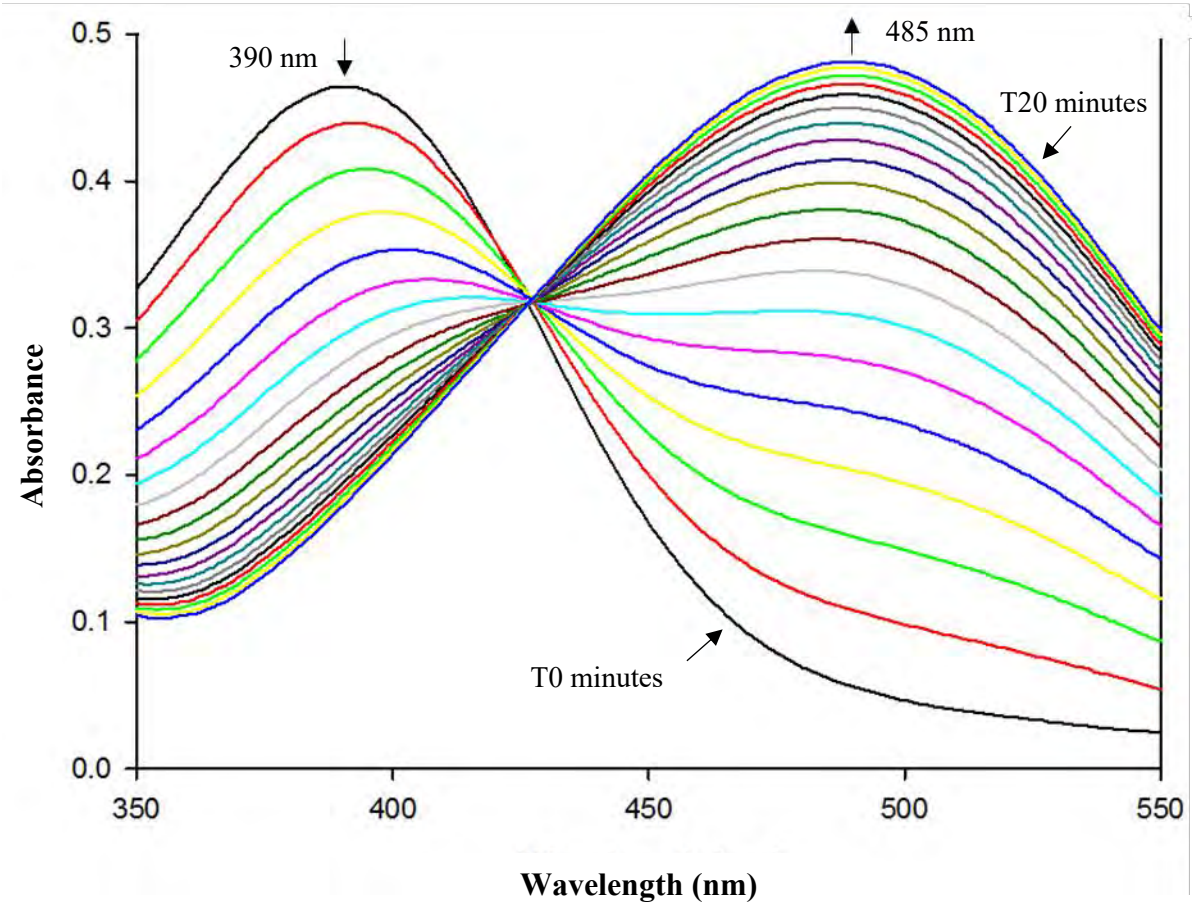

FIGURE 3. UV-Vis overlay spectra of a β-lactamase assay containing in a total volume of 1 ml; 43 µg of protein from farm pig *Escherichia coli* isolate FP5 and 10 µg of nitrocefin in 0.5 M sodium-phosphate buffer (pH 7.0) at 30°C.

Discussion

β-lactamases have been identified as one of the most important mechanisms of resistance to β-lactam antibiotics, including penicillins, cephalosporins and monobactams, in bacteria including *Escherichia coli, Klebsiella* spp. and other *Enterobacteriaceae* (Bush 2001; Mesa et al. 2006; Bush and Jacoby 2010; Guenther et al. 2011). The genes encoding such enzymes can be found on both chromosomes and plasmids, the latter with the ability to be readily transferred between different bacteria (Bradford et al. 1994). The TEM-1 β-lactamase has been termed as the progenitor of the majority of extended spectrum β-lactamases, and genes encoding TEM-1 are ubiquitous determinants of β-lactam resistance among bacterial isolates from diverse environments; including soil and water bodies, animal/agricultural communities and the clinic (Roy et al. 1992; Briñas et al. 2002; Guenther et al. 2011). The TEM-type β-lactamases were frequently found in *E. coli* isolated from the faeces of livestock including cattle and pigs in a number of countries including South Korea, Switzerland and China (Rayamajhi et al. 2008; Geser et al. 2012; Dahms et al. 2015; Gao et al. 2015). In this study, three *E. coli* isolates from farm pig faeces contained the *bla*$_{\text{TEM}}$ gene (FP5, FP6 and FP29) [Figure 1, Table A1]. Given the rather relaxed guidelines and laws regarding the use and prescription of antibiotics in farm environments in South Africa, the presence of such TEM-type resistance genes in *E. coli* from farm environments is therefore not unexpected (Eagar et al. 2012). Considering the poor representation of the African region in the 2014 WHO global surveillance report (WHO 2014), there may be an abundance of resistances due to β-lactamase genes in South African farm environments that are not identified or reported, potentially due to lack of infrastructure, personnel and efficient monitoring. A 2008 African study reported the presence of such β-lactamase genes in *E. coli* isolated from farm pig faeces in Nigeria but did not specify the types and proportions of each β-lactamase gene found (Akujobi et al. 2008). In addition, genes encoding both the TEM-1 and TEM-20 type enzymes have been previously detected in *E. coli* strains isolated from cattle meat in Tunisia (Slama et al. 2010).

According to the current literature, there has been no incidence of the *bla*$_{\text{TEM}}$ gene found in *E. coli* isolated from South African wild herbivores. However, the detection of this gene has been reported for wild birds (for example seagulls) and animals including wild rabbits and wild boars; on continents other than Africa (Literak et al. 2010; Radhouani et al. 2009; Silva et al. 2010). The three isolates containing the *bla*$_{\text{TEM}}$ gene in the current study were all resistant to ampicillin, amoxicillin-clavulanic acid and either ceftazidime or aztreonam; together

comprising three of the six ampicillin-resistant isolates in total [Table A1]. Such genes have been previously associated with *E. coli* isolates resistant to ampicillin in particular. Briñas et al. (2002) found that where 83% of ampicillin-resistant *E. coli* isolated from humans and healthy animals in Spain contained a TEM-type β-lactamase. Furthermore, Gonçalves et al. (2013) detected the *bla*$_{TEM}$ gene in ampicillin-resistant *E. coli* isolates from an Iberian lynx in Spain. In a study by Kaye et al. (2004), 52% of amoxicillin-clavulanic acid-resistant *E. coli* isolated from human urine contained the *bla*$_{TEM}$ gene, and the resistance observed was attributed to TEM type β-lactamase hyperproduction frequently observed as a mechanism of resistance to β-lactam/β-lactamase inhibitor combinations (Henquell et al. 1994; Nicolas-Chanoine 1997; Kaye et al. 2004). Furthermore, these isolates displayed rapid hydrolysis of nitrocefin, suggesting that TEM gene hyperproduction plays an important part in mediating amoxicillin-clavulanic acid resistance (Kaye et al. 2004).

The fact that the *bla*$_{TEM}$ gene was not detected in any herbivore *E. coli* isolate in the current study may be explained by the environment from which the isolates were obtained. Given the apparently more "exotic" habitat, the genes that could be conferring resistance against β-lactam antibiotics may not be among those resistance genes that are typically tested in wild animal-based settings. Moreover, it may be an indication that resistance mechanisms other than β-lactamase encoding genes are involved in conferring the observed β-lactam resistance, for example porins.

The chromogenic cephalosporin nitrocefin [Figure 4] is a well-established substrate used in the colourimetric assessment of β-lactamase activity in bacterial isolates, including *E. coli* (O'Callaghan et al. 1972; Livermore and Brown 2001). β-lactamase enzymes can catalyse the hydrolysis of the β-lactam ring of nitrocefin, causing a subsequent colour change from yellow to red.

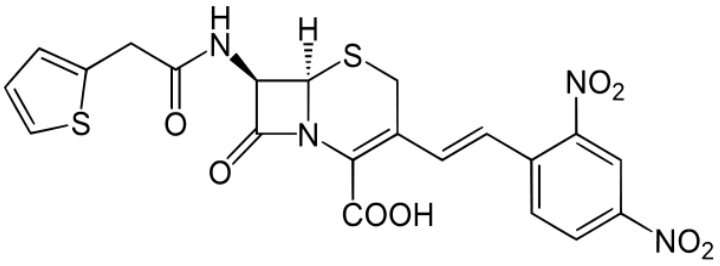

FIGURE 4. Structure of Nitrocefin.

In the nitrocefin spot-test [Figure 2], 63% (64/102) of *E. coli* isolates tested positive for the presence of β-lactamase activity, with 38 of these 64 isolates resistant to only amoxicillin-clavulanic acid. Wildebeest-faecal isolates constituted a large proportion thereof (18 amoxicillin-clavulanic acid resistant and nitrocefin positive isolates), which might be related to the dietary habits of the animal - it is a grazer and would likely ingest soil particles while feeding, or perform geophagia. Moreover, given that clavulanic acid is produced by *Streptomyces clavuligerus*, a typical soil bacterium, the presence of resistances to such antibiotics is not unexpected (Higgens and Kastner 1971; Reading and Cole 1977).

A number of *E. coli* isolates in this study presented phenotypic resistance to one or more β-lactam antibiotic but when tested for nitrocefin hydrolysis presented a negative result [Figure 2; Table A1]. This may be due to alternative mechanisms conferring resistance to β-lactam antibiotics, such as porins, or that nitrocefin is not an appropriate substrate for the β-lactamase (or β-lactamases) present. While an isolate may present phenotypic resistance to one or more β-lactam antibiotics, a negative result for nitrocefin hydrolysis does not automatically eliminate the possibility that β-lactamases may be the mechanism conferring resistance. This was illustrated in a study conducted by Miriagou et al. (2003), where the Class B metallo-β-lactamase VIM-1 was undetectable in *E. coli* when nitrocefin was employed as a substrate. Similarly, Villadares et al. (1996) utilized nitrocefin as a substrate for a variety of β-lactamases including CphA (a Class B β-lactamase) from *Aeromonas hydrophila*. The resultant kinetic parameters of the CphA enzyme were determined, and a very low k_{cat}/K_m value of 0.0003 $\mu M^{-1}s^{-1}$ was obtained (Villadares et al. 1996), suggesting that nitrocefin was not effectively hydrolysed in the reaction. The same enzyme (CphA) was analysed using an alternative chromogenic substrate, CENTA, and again no detectable hydrolysis was observed (Bebrone et al. 2001). Two additional studies analysed the hydrolysis of nitrocefin by Class A β-lactamases TEM-1 and SHV-1 in *E. coli*. The catalytic efficacy differed by a factor of 20 between the β-lactamases, with TEM-1 measuring 18 $\mu M^{-1}s^{-1}$ and SHV 0.84 $\mu M^{-1}s^{-1}$ (Raquet et al. 1994; Bouillenne et al. 2000). These results confirm that certain β-lactamases may be present in an isolate, but are unable to efficiently hydrolyse nitrocefin and other chromogenic cephalosporins.

Nitrocefin [Figure 4] was chosen as the substrate for both the spot-test and β-lactamase activity quantification in this study, due to the distinctive colouration of the hydrolysed (red) and unhydrolysed (yellow) species, allowing for efficient monitoring of the reaction and thus

determination of isolates' β-lactamase activity profiles. In the current study, the specific β-lactamase activity of the nitrocefin-positive representative herbivore *E. coli* isolates (Z2, Z19, W9, W26 and G23 - zebra, giraffe and wildebeest faecal isolates) were between 2 and 3 nmol × min^{-1} × mg^{-1} [Table 1]. In contrast, the specific β-lactamase activities of the nitrocefin-positive representative farm pig *E. coli* isolates - FP5, FP6 and FP29 - were much higher, at 81 to 168 nmol × min^{-1} × mg^{-1} [Table 1]. In a study assessing the β-lactamase activity of a TEM-producing clinical *E. coli* strain, the measured specific activity was 320 nmol × min^{-1} × mg^{-1} (Payne et al. 1994). The highest specific β-lactamase activities were detected in the presence of a TEM β-lactamase gene in the current study, as the three farm pig *E. coli* isolates (the only 3 of 102 to carry the bla_{TEM} gene) presented high specific β-lactamase activities. Händel et al. (2013) determined the specific activities of both amoxicillin-susceptible and amoxicillin-resistant *E. coli* cells, and reported that the amoxicillin-resistant cells had much higher specific activities - in this case attributed to strong upregulation of the *amp*C gene (Händel et al. 2013). The results obtained might infer that the expression of a TEM gene results in a greater measured specific β-lactamase activity, or that in fact the TEM resistance determinant is integral in conferring higher levels of β-lactamase activity among *E. coli* strains. The lower specific β-lactamase activities of the herbivore isolates may be explained by low expression, the presence of rare β-lactamases that are not readily hydrolysing nitrocefin - or in the case of nitrocefin-negative isolates - resistance is not conferred by β-lactamases but rather alternative mechanisms.

The results of the nitrocefin spot-test in this study demonstrated that nitrocefin can be an effective substrate for testing β-lactamase activity. However, it may miss certain β-lactamases in phenotypic screening, as observed when the phenotypic β-lactam resistance profiles did not always correlate with the spot-test results. It is therefore advisable when screening for potential β-lactam resistance to use multiple methods when testing for the presence of β-lactamases, particularly in lesser-known "wild" environments. There might be application of a phenotypic assessment using the disk diffusion test or the MIC method, the genotypic target of β-lactamase genes using PCR or, in addition to nitrocefin, another chromogenic cephalosporin like CENTA.

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General conclusions and future research potential

The issue of antibiotic resistance is not confined to humans and the clinic, but is in fact persistent in a large number of ecological niches; from the general surrounding environment (soil, water and vegetation), to animals inhabiting wild and domestic habitats. While the burden of antibiotic resistance has been well-characterized in both clinical and farm environments worldwide, it has recently emerged that animals inhabiting an apparently "pristine" environment, for example wild herbivores in nature reserves, may serve as reservoirs and/or vectors of antibiotic resistant bacteria. Such data is however particularly limited for South African herbivores.

Thus, this thesis aimed to screen *Escherichia coli* isolated from the faecal matter of different herbivores residing in a nature reserve in KwaZulu-Natal, South Africa; to assess the current status of local wild animals as potential reservoirs or vectors for antibiotic resistant bacteria. In addition, *Escherichia coli* from both pet (companion) and farm pig faeces was evaluated for comparative purposes.

The data established in this thesis show that South African wild herbivores do indeed serve as reservoirs and thus potential vectors of antibiotic resistant *E. coli.* Furthermore, the results obtained indicate that the feeding habits of the animals - whether they browse for food at the tops of trees (e.g. giraffes), or graze at ground-level (e.g. zebra and wildebeest) - apparently influence the observed antibiotic resistance profiles. This was evident from the significantly higher levels of antibiotic resistance observed for *E. coli* isolates from zebra and wildebeest faeces (grazers), when compared to resistance levels observed for isolates from giraffe faeces (browser). This may be due to geophagy, leading to uptake of secondary metabolites including antibiotics produced by soil microorganisms (Mahaney et al. 1999). An additional reason might be selective pressure imposed by the potential application of herbicides to ground-level shrubs and grasslands, where induction of antibiotic resistant phenotypes in bacterial pathogens can occur (Kurenbach et al. 2015). The usage of such agents in Bisley Park or the surrounding area is however unconfirmed.

The lack of resistance to carbapenem, fluoroquinolone or glycylcycline antibiotics in any of the wild herbivore *E. coli* isolates was not entirely unexpected, as such antibiotics are reserved for human (and in the case of fluoroquinolones, for farm and companion animal) use.

Considering the potential resistances that might exist faecal bacteria from horses in the adjacent stables, or companion animals entering the park - both of which may arise as a result of presumed veterinary attention - it is encouraging that such resistances have not yet been observed in *E. coli* isolated from the wild animals. There was no incidence of multidrug resistance (MDR - resistance to at least one antibiotic from at least three different classes) among those faecal *E. coli* isolates from giraffe or wildebeest faeces. However, a remarkably large proportion of *E. coli* from zebra (47%) presented a MDR phenotype. Although generally unanticipated, a high incidence of MDR from wild animals in Africa is not unheard of: two separate studies performed in Botswana screened faecal *E. coli* from a number of animals including banded mongoose, giraffe, otters, spotted hyena and impala; and reported instances of MDR *E. coli* isolates. In some cases, a 100% MDR rate was even observed, as for *E. coli* from otter and hyena faeces (Pesapane et al. 2013; Jobbins and Alexander 2015). The results from these studies were largely attributed to the animal's dietary habits (herbivore or omnivore), proximity to urban areas and frequency of water association. It might be important for future studies to screen the same herbivores (zebra, giraffe, wildebeest), but from diverse habitats - for example game reserves in different locations or grasslands around the country. Similarly, an investigation into different South African herbivores, but with identical dietary habits to the current herbivores (i.e. browser or grazer) might be enlightening. Such results would verify whether the findings from this study are confined to the Bisley Park Nature Reserve (KZN) and its inhabitants, or are indeed due to the dietary behaviour and feeding habits of the animals.

While herbivores and pet pigs presented similar resistance profiles in certain cases (more than 80% of *E. coli* isolates from each zebra, wildebeest and pet pig faeces presented resistance to at least one antibiotic), there was a significant difference between farm and pet pig with regard to amoxicillin-clavulanate resistance. One farm pig *E. coli* isolate (out of 30) presented resistance to the two tested fluoroquinolone antibiotics, ciprofloxacin and norfloxacin - not entirely unexpected considering the extensive use of fluoroquinolones in farm environments (Collignon 2005). *E. coli* isolated from pet pig faeces had the second highest rate of MDR, with 10% of isolates presenting such a phenotype. This is not surprising considering the "companion" nature of the pet pig; a lifestyle which includes regular veterinary attention and consistent interaction with humans and other domesticated pets on a residential property.

To supplement the disk diffusion methodology, additional PCR-based screening for one selected resistance gene was performed, as well as screening for the β-lactamase activity of β-lactam-resistant *E. coli* isolates. Upon analysis, only three farm pig *E. coli* isolates were found to carry the *bla*$_{TEM}$ gene. Considering the largely clinical nature of this particular gene, it is probably unsurprising that it was not detected in the "exotic" wild herbivore *E. coli* isolates. Upon investigation into β-lactamase activity, approximately 63% (64 out of 102 β-lactam-resistant isolates) tested positive for nitrocefin hydrolysis. Such isolates were from both herbivore and farm and pet pig faeces. All isolates testing positive for the *bla*$_{TEM}$ gene (3 *E. coli* isolates from farm pig faeces) exhibited phenotypic resistance to ampicillin, amoxicillin-clavulanic acid and ceftazidime. A number of phenotypically β-lactam-resistant isolates, when screened using nitrocefin, failed to hydrolyse this substrate. This may be attributed to one of two things: β-lactam resistance is conferred by a mechanism other than a β-lactamase, such as a porin or an efflux pump. Alternatively, there may be a β-lactamase that does not hydrolyse nitrocefin, as is the case for certain Class B enzymes including VIM-1 (Miriagou et al. 2003).

This highlights that PCR-based approaches for resistance profiling may not be sufficient when applied in isolation. Phenotypic methods such as the disk-diffusion assay and/or enzyme assays are essential to supplement the results. It might be practical to consider characterization of the types of β-lactamase enzymes present in those isolates testing positive for β-lactamase activity, through the selection of a greater number of appropriate β-lactamase genes. In contrast, for isolates that presented a negative nitrocefin hydrolysis result, it will be of interest to characterize the resistance mechanism conferring β-lactam resistance - be it a β-lactamase unable to hydrolyse nitrocefin, or another mechanism such as porins. The use of additional colourimetric substrates, for example the iodometric test or CENTA, could further verify the screening results from nitrocefin testing (Bebrone et al. 2001; Livermore and Brown 2001).

Ten isolates with distinct phenotypic resistance profiles and varied responses to the nitrocefin hydrolysis test were selected for determination of specific β-lactamase activity. The results indicated that the presence of the *bla*$_{TEM}$ gene was linked to high levels of specific nitrocefin hydrolysis activity in isolates containing the gene, as these activities were at least 20 times higher than those in nitrocefin positive isolates lacking the *bla*$_{TEM}$ gene. While crude extracts from herbivore-sourced *E. coli* isolates (2 from zebra, 2 from wildebeest and 1 from giraffe faeces) resulted in specific activities of between 2 and 3 nmol × min^{-1} × mg^{-1}, three *E. coli*

isolates from farm pigs with the *bla*$_{TEM}$ gene had specific activities ranging from 81 to 168 nmol × min^{-1} × mg^{-1}.

Additional research that could be performed to supplement the results described in this thesis may include the screening of *E. coli* isolated from faecal samples from the horses inhabiting the stables adjacent to Bisley Park Nature Reserve. Such an assessment may provide insight into whether matching resistance profiles occur between *E. coli* from the horses that can enter the park, and *E. coli* isolated from the herbivores. Furthermore, the isolation of *E. coli* and subsequent determination of antibiotic resistance profiles from the proximate soil and water-bodies in the park might reveal similarities to those obtained from the herbivores. Antibiotic resistance transmission relies on the interconnection of a number of biotic and abiotic factors; thus the additional screening would provide a necessary insight into the function of such factors.

A limitation of this study is that potential seasonal influences were not assessed. The samples analysed in the study were collected between March and April 2015 - the late summer/early autumn season in South Africa, while samples were not collected during the winter season. There may be a change in behaviour of the animals in the dry colder months - grazing may perhaps be less frequent, and there might also be an element of migratory birds (capable of transferring resistances via faeces) present in one season but not the next. If geophagy is indeed a potential explanation for the detection of resistance in *E. coli* isolated from wild herbivores, one might find that the colder temperatures influence the activity of streptomycetes present in the soil thus potentially impacting the antibiotic resistances detected in winter.

In conclusion, wild herbivores in South Africa indeed serve as reservoirs of antibiotic resistant *E. coli*, and thus may function as a vector for the transfer of resistance to other animals or even humans. Furthermore, farm pigs may contain bacteria resistant to important antibiotics from antibiotic classes including the fluoroquinolones and third-generation cephalosporins; as well as potentially harbour antibiotic resistance genes including *bla*$_{TEM}$. Considering the knowledge gap with regard to antibiotic resistance in South African wild animals, this research has provided a first insight into the role such animals may play in their environment as a reservoir or vector of antibiotic resistant *E. coli*.

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APPENDIX TABLE A1. Resistant, susceptible, and intermediate phenotypes for *Escherichia coli* isolated from zebra, wildebeest, giraffe, pet pig and farm pig faeces; including presence or absence of the *bla*$_{TEM}$ gene and nitrocefin spot-test results as described in Chapter 1 and 2 above.

		ANTIBIOTIC													
SAMPLE	**ISOLATE**	**AMP**	**AMC**	**CTX**	**CAZ**	**ATM**	**ERT**	**MEM**	**NOR**	**CIP**	**GEN**	**TOB**	**TIG**	***bla*$_{TEM}$ gene** [a]	**Nitrocefin spot-test** [b]
ZEBRA FAECES	**Z1**	S	R	S	R	S	I	I	S	S	I	I	S	-	+
	Z2	S	R	S	R	I	I	I	S	S	I	R	S	-	+
	Z3	S	R	S	R	I	I	I	S	S	I	R	S	-	-
	Z4	S	R	S	R	I	S	S	S	S	I	R	S	-	+
	Z5	S	R	S	R	I	I	S	S	S	I	R	S	-	-
	Z6	S	R	S	R	I	I	I	S	S	I	R	S	-	-
	Z7	S	R	S	R	S	I	S	S	S	I	R	S	-	+
	Z8	S	R	S	R	I	S	S	S	S	I	R	S	-	-
	Z9	R	R	S	R	I	S	S	S	S	I	I	S	-	+
	Z10	S	R	S	I	I	I	S	S	S	I	R	S	-	-
	Z11	S	R	S	I	R	S	S	S	S	I	R	S	-	+
	Z12	S	R	S	I	I	S	S	S	S	I	R	S	-	+
	Z13	S	R	S	I	I	S	S	S	S	I	I	S	-	-
	Z14	S	R	I	R	R	S	S	S	S	I	I	S	-	-
	Z15	S	R	S	R	I	S	S	S	S	I	I	S	-	+
	Z16	S	S	I	I	I	S	S	S	S	I	I	S	NT	-
	Z17	S	R	I	I	R	S	S	S	S	I	R	S	-	+
	Z18	S	R	I	R	I	I	S	S	S	R	I	S	-	+
	Z19	S	S	I	R	R	I	S	S	S	I	R	S	-	+
	Z20	S	R	I	R	R	S	S	I	I	I	R	S	-	-
	Z21	S	S	I	I	I	I	S	S	S	I	R	S	NT	NT
	Z22	S	S	I	I	I	S	S	S	I	I	R	S	NT	NT

	Z23	S	S	S	R	I	I	S	I	I	I	R	S	-	+
	Z24	S	R	S	R	I	I	I	I	I	I	R	S	-	-
	Z25	S	S	I	I	I	I	S	I	I	I	R	S	NT	NT
	Z26	S	R	S	I	S	S	I	S	S	I	I	S	-	+
	Z27	S	R	I	I	I	S	S	I	S	I	I	S	-	-
	Z28	S	R	S	I	I	S	S	S	S	I	I	S	-	-
	Z29	S	R	S	I	I	S	S	S	S	I	I	S	-	+
	Z30	S	S	S	I	I	S	S	S	S	I	I	S	NT	NT
GIRAFFE FAECES	**G1**	S	S	S	I	I	S	S	S	S	I	I	S	NT	-
	G2	S	R	S	I	I	S	S	S	S	I	I	S	-	+
	G3	S	S	S	I	I	I	S	S	S	I	I	S	NT	NT
	G4	S	R	S	I	I	S	S	S	S	I	I	S	-	+
	G5	S	S	S	I	S	S	S	S	S	S	S	S	NT	NT
	G6	S	S	S	S	S	S	S	S	S	S	I	S	NT	NT
	G7	S	S	S	S	S	S	S	S	S	S	I	S	NT	NT
	G8	S	S	S	S	S	S	S	S	S	S	S	S	NT	NT
	G9	S	S	S	I	S	S	S	S	S	I	I	S	NT	NT
	G10	S	S	S	I	S	S	S	S	S	S	I	S	NT	NT
	G11	S	S	S	S	S	S	S	S	S	I	I	S	NT	NT
	G12	S	S	S	I	S	S	S	S	S	S	S	S	NT	NT
	G13	S	S	S	S	I	S	S	S	S	I	R	S	NT	NT
	G14	S	S	S	I	I	S	S	S	S	S	I	S	NT	NT
	G15	S	R	S	I	S	S	S	S	S	S	I	S	-	+
	G16	S	S	S	I	I	S	S	S	S	I	I	S	NT	NT
	G17	S	R	I	I	S	S	S	S	S	I	R	S	-	+
	G18	S	S	S	I	I	S	S	S	S	I	I	S	NT	NT
	G19	S	S	S	S	S	S	S	S	S	I	I	S	NT	NT
	G20	S	R	S	I	I	S	S	S	S	I	I	S	-	+

	G21	S	R	S	I	S	S	S	S	S	I	R	S	-	+
	G22	S	S	S	I	I	S	S	S	S	I	I	S	NT	NT
	G23	S	S	S	R	S	S	S	S	S	I	I	S	-	+
	G24	S	S	S	I	S	S	S	S	S	I	I	S	NT	NT
	G25	S	R	S	R	S	S	S	S	S	I	I	S	-	-
	G26	S	S	S	I	I	S	S	S	S	I	I	S	NT	NT
	G27	S	R	S	I	I	S	S	S	S	I	I	S	-	+
	G28	S	R	S	I	I	S	S	S	S	I	I	S	-	-
	G29	S	R	S	I	S	S	S	S	S	I	I	S	-	-
	G30	S	S	S	R	S	S	S	S	S	I	I	S	-	+
WILDEBEEST FAECES	**W1**	S	R	I	I	I	I	S	S	S	I	R	S	-	+
	W2	S	R	S	I	I	I	S	S	S	I	I	S	-	+
	W3	S	R	S	I	I	I	S	S	S	I	I	S	-	+
	W4	S	R	S	I	I	S	S	I	S	I	I	S	-	+
	W5	S	S	I	I	I	I	S	S	S	I	I	S	NT	-
	W6	S	R	S	I	I	S	S	S	S	I	I	S	-	-
	W7	S	R	S	R	I	S	S	I	S	I	I	S	-	+
	W8	S	R	S	I	I	I	S	S	S	I	I	S	-	+
	W9	R	R	I	I	I	I	S	S	S	I	I	S	-	+
	W10	S	R	S	I	I	S	S	S	S	I	I	S	-	+
	W11	S	S	S	I	S	S	S	S	S	I	I	S	NT	NT
	W12	S	S	S	I	S	S	S	S	S	I	I	S	NT	NT
	W13	S	R	S	I	I	S	S	S	S	I	I	S	-	+
	W14	S	R	S	I	I	S	S	S	S	I	I	S	-	+
	W15	S	R	S	I	S	S	S	S	S	I	I	S	-	+
	W16	S	R	S	I	S	S	S	S	S	I	I	S	-	+
	W17	S	R	S	I	I	I	S	S	S	I	I	S	-	+
	W18	S	S	S	S	S	S	S	S	S	S	I	S	NT	NT

	W19	S	R	S	I	S	S	S	S	S	I	I	S	-	+
	W20	S	R	S	I	S	S	S	S	S	I	I	S	-	-
	W21	S	R	I	I	I	I	S	S	S	I	I	S	-	+
	W22	S	R	S	R	I	S	S	S	S	I	I	S	-	+
	W23	S	S	I	I	I	S	S	S	S	I	I	S	NT	NT
	W24	S	S	S	I	I	S	S	S	S	I	I	S	NT	NT
	W25	S	R	S	I	I	S	S	S	S	I	I	S	-	+
	W26	S	R	S	I	R	I	S	S	S	I	I	I	-	+
	W27	S	R	S	I	I	S	S	S	S	I	I	S	-	+
	W28	S	R	S	I	I	S	S	S	S	I	I	S	-	+
	W29	S	R	S	I	I	S	S	S	S	I	I	S	-	+
	W30	S	R	S	S	I	I	S	S	S	I	I	S	-	+
PET PIG FAECES	**DP1**	S	R	S	S	S	S	S	S	S	I	I	S	-	+
	DP2	S	R	S	I	I	S	S	S	S	I	I	S	-	+
	DP3	S	R	S	I	S	S	S	S	S	S	I	S	-	+
	DP4	S	R	I	I	I	S	S	S	S	I	R	S	-	+
	DP5	S	S	I	R	I	I	S	S	S	I	I	S	-	-
	DP6	R	R	I	R	I	S	S	S	S	I	I	S	-	+
	DP7	S	R	S	R	I	S	S	S	S	I	R	S	-	-
	DP8	S	S	S	I	I	S	S	S	S	I	I	S	NT	-
	DP9	S	R	I	I	I	S	S	S	S	I	R	S	-	-
	DP10	S	R	I	I	I	S	S	S	S	I	I	S	-	-
	DP11	S	R	I	R	I	S	S	S	S	I	I	S	-	+
	DP12	S	R	S	I	I	S	S	S	S	I	R	S	-	-
	DP13	S	R	S	S	I	S	S	S	S	I	I	S	-	-
	DP14	S	R	I	R	I	S	S	S	S	I	R	S	-	-
	DP15	S	R	S	I	S	S	S	S	S	I	I	S	-	-
	DP16	S	R	I	I	I	S	S	S	S	I	I	S	-	-

	DP17	S	S	S	R	I	S	S	S	S	I	I	S	-	-
	DP18	S	R	S	I	S	S	S	S	S	I	I	S	-	+
	DP19	S	R	I	R	I	S	S	S	S	I	I	S	-	+
	DP20	S	R	S	I	S	S	S	S	S	I	R	S	-	+
	DP21	S	R	S	I	S	S	S	S	S	I	I	S	-	-
	DP22	S	R	I	I	I	S	S	S	S	I	I	S	-	+
	DP23	S	S	S	R	R	S	S	S	S	S	I	S	-	-
	DP24	S	R	S	R	I	S	S	S	S	I	R	S	-	+
	DP25	S	S	S	I	S	S	S	S	S	I	I	S	NT	NT
	DP26	S	R	S	I	I	S	S	S	S	I	I	S	-	-
	DP27	S	R	S	I	S	S	S	S	S	I	I	S	-	-
	DP28	S	S	S	I	I	I	S	S	S	I	I	S	NT	NT
	DP29	S	R	S	I	I	S	S	S	S	I	I	S	-	+
	DP30	S	R	I	I	S	S	S	I	S	I	I	S	-	+
FARM PIG FAECES	**FP1**	S	S	S	I	S	S	S	S	S	I	I	S	NT	-
	FP2	S	S	S	I	I	S	S	S	S	I	I	S	NT	NT
	FP3	S	S	S	I	S	S	S	S	S	I	I	S	NT	NT
	FP4	S	S	S	I	I	S	S	S	S	I	I	S	NT	NT
	FP5	R	R	S	R	I	S	S	S	S	I	I	S	+	+
	FP6	R	R	I	R	I	S	S	S	S	R	I	S	+	+
	FP7	S	S	S	I	I	I	S	S	S	I	I	S	NT	NT
	FP8	S	R	S	I	I	S	S	S	S	I	I	S	-	-
	FP9	S	S	S	I	S	S	S	S	S	S	S	S	NT	NT
	FP10	S	S	S	I	I	S	S	S	S	I	I	S	NT	NT
	FP11	S	S	S	I	S	S	S	S	S	I	I	S	NT	NT
	FP12	S	S	S	S	I	I	S	S	S	I	I	S	NT	NT
	FP13	S	S	S	S	I	S	S	S	S	I	I	S	NT	NT
	FP14	S	R	S	S	S	S	S	S	S	I	I	S	-	-

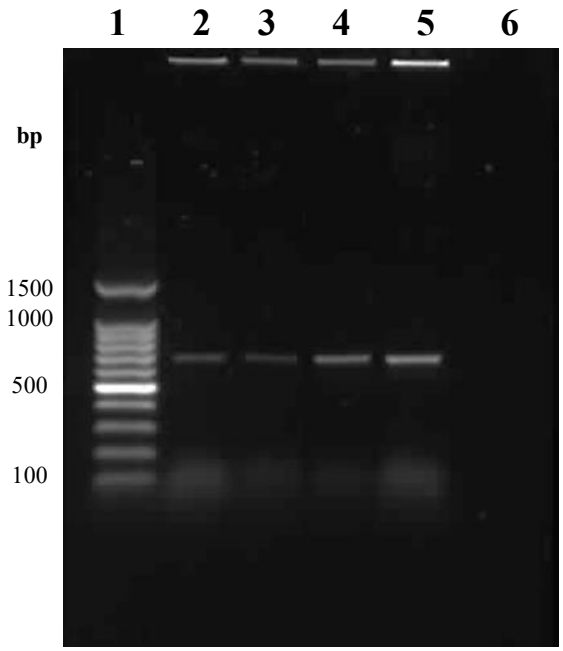
	FP15	S	R	S	I	I	S	S	S	S	I	I	S	-	-
	FP16	S	S	S	I	I	S	S	S	S	I	I	S	NT	NT
	FP17	S	S	S	I	I	S	S	S	S	I	I	S	NT	NT
	FP18	S	S	S	I	I	S	S	S	S	I	I	S	NT	NT
	FP19	S	R	S	I	S	S	S	S	S	I	I	S	-	-
	FP20	S	S	S	I	I	S	S	S	S	I	I	S	NT	NT
	FP21	S	R	I	I	I	S	S	S	S	I	I	S	-	-
	FP22	S	R	I	R	I	S	S	S	S	I	I	S	-	-
	FP23	S	S	I	I	I	I	S	S	S	I	I	S	NT	NT
	FP24	S	R	S	I	I	I	S	S	S	I	I	S	-	-
	FP25	S	S	S	S	S	S	S	S	S	I	I	S	NT	NT
	FP26	S	S	I	I	R	S	S	S	S	I	R	S	-	-
	FP27	S	R	S	R	I	S	S	I	I	I	I	S	-	+
	FP28	S	R	S	I	I	S	S	S	S	I	I	S	-	+
	FP29	R	R	S	I	R	S	S	R	R	I	I	S	+	+
	FP30	S	R	S	R	I	S	S	S	I	I	I	S	-	+

[a] + denotes the expected amplification product (1080 bp) for the *bla*$_{TEM}$ gene was obtained

[b] + denotes a change in colour of the nitrocefin/biomass mixture from yellow to red after 30 minutes incubation at 30°C in the dark

NT Not tested

Note: one isolate from each animal group presenting susceptibility to all β-lactam antibiotics was tested using nitrocefin as a negatiative control: all 5 were negative (no formation of red colour)



APPENDIX FIGURE A1. Representative 1% agarose gel image depicting results of PCR based amplification of the *gad*A gene (680 bp), from farm pig *Escherichia coli.* *E. coli* ATCC 8739 served as a positive control and sterile nuclease free water as a no template control

1 - 100 bp Molecular Weight Marker; **2-4** - FP1-3 (farm pig); **5** - *E. coli* ATCC 8739;
6 - Nuclease free water