Antibiotic resistance in enteric *Escherichia coli* and *Enterococcus* spp. isolated from ungulates at Marwell Zoo, England

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DECLARATION

I declare that this dissertation hereby submitted to the University of Pretoria for the degree of Master of Science (Animal/Human/Ecosystem Health) has not been previously submitted by me for the degree at this or any other University, that it is my own work in design and in execution, and that all material contained therein has been duly acknowledged.

Signed: 

Date: 07/11/2016

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I have learnt a huge amount from doing this project and hope to use this experience for future research in veterinary microbiology. I am very grateful to everyone for their support, patience and guidance.

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LIST OF ABBREVIATIONS

AMR	Antimicrobial Resistance
APHA	Animal and Plant Health Agency (United Kingdom)
BSAC	British Society for Antimicrobial Chemotherapy
ºC	Degrees Celsius
CBP	Clinical break point
CLSI	Clinical and Laboratory Standards Institute
DNA	Deoxyribonucleic acid
ECDC	European Centre for Disease Prevention and Control
ECOFF	Epidemiological cut off
EDTA	Ethylenediaminetetraacetic acid
EFSA	European Food Safety Authority
e.g.	For example
ESBL	Extended-spectrum beta-lactamase
et al.	And others
etc.	Et cetera
EU	European Union
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FAO	Food and Agriculture Organisation of the United Nations
g	Gram(s)
xg	Times gravity (relative centrifugal forces)
i.e.	That is
KOH	Potassium hydroxide
LAMP	Loop-Mediated Isothermal Amplification assay
MDR	Multi-drug resistance
MIC	Minimum inhibitory concentration
ml	Millilitre(s)
MLST	Multi-locus sequence typing
mm	Millimetre(s)
NaCl	Sodium chloride
NCTC	National Collection of Type Cultures
No.	Number
OIE	World Organisation for Animal Health
PCR	Polymerase Chain Reaction
rpm	Revolutions per minute
spp.	Species
µg	Microgram(s)
UK	United Kingdom
µl	Microlitre(s)
V	Volts
WHO	World Health Organisation
w/v	Weight per volume %

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SUMMARY

Antimicrobial resistance (AMR) has diminished the effectiveness of many antibiotics used to treat human and animal infectious diseases. AMR in bacteria is considered one of the most important emerging threats to animal and human health worldwide (Dobiasova et al. 2013). Understanding the dynamics of AMR in different populations is key to minimising the emergence of resistance to preserve the efficacy of existing and newly developed antimicrobial drugs (Holmes et al. 2016, Sundqvist et al. 2010).

This project investigated AMR in the commensal enteric microflora of animals at Marwell Zoo, United Kingdom. Although AMR has been studied in domesticated animals and free-ranging wildlife, there are few studies describing AMR in captive wild animals.

In this project, faecal samples were collected from 17 species of healthy ungulates weekly for three weeks which yielded a total of 39 *Escherichia coli* and 55 *Enterococcus* spp. isolates. Antibiotic sensitivity was investigated using agar disk diffusion methods.

The *E. coli* isolates were resistant to ampicillin (28.2%), streptomycin (17.9%), spectinomycin (17.9%), trimethoprim sulphamethoxazole (17.9%), neomycin (12.8%), doxycycline (12.8%), tetracycline (12.8%), amoxicillin-clavulanic acid (10.3%), cefotaxime (2.6%), cefpodoxime (2.6%), ceftazidime (2.6%) and amikacin (2.6%). All *E. coli* isolates were susceptible to apramycin, enrofloxacin, chloramphenicol and florfenicol and none tested positive for extended-spectrum beta-lactamase (ESBL) or AmpC activity using a disk diffusion screening kit. Seven out of 39 (18%) *E. coli* isolates were resistant to more than three antibiotic classes, the most common pattern of resistance was: penicillins, tetracyclines, aminoglycosides and sulphonamides. The *E. coli* isolates were further analysed using multi-locus sequence typing (MLST) which identified four pairs of identical sequence type (ST) isolates and 27 diverse strains.

The *Enterococcus* spp. isolates were resistant to cefpodoxime (95%), erythromycin (67%), tetracycline (33%), ciprofloxacin (40%), imipenem (11%), trimethoprim sulphamethoxazole (5%) and streptomycin (4%). All *Enterococcus* spp. isolates were susceptible to ampicillin, gentamicin, chloramphenicol and vancomycin.

This study identified multi-drug resistant phenotypes in the *E. coli* isolates that were similar to those commonly found in domestic ungulates. The resistance phenotypes were plotted on a map which did not show any significant spatial association between resistance traits in different species. Review of the medical records of individual animals showed previous use of penicillins, sulphonamides and tetracyclines in this population. Resistance to antibiotics that were rarely or never used may have been due to co-selection of resistance genes which were linked with other genes on the same genetic element (Acar & Moulin 2012).

INTRODUCTION

Even before man started using antibiotics, micro-organisms were evolving resistance mechanisms to withstand naturally produced antibiotic compounds produced by bacteria and fungi competing for resources (Holmes et al. 2016, Schwarz & Chaslus-Dancla 2001). The widespread use of man-made antibiotics has caused selection pressure towards resistant bacteria which has resulted in a much higher prevalence than would have naturally occurred (van den Bogaard & Stobberingh 2000). Bacterial resistance has quickly emerged following the use of every new antibiotic developed for clinical use (Hwang & Gums 2016, Schwarz & Chaslus-Dancla 2001).

Antimicrobial resistance (AMR) has diminished the effectiveness of many antibiotics used to treat human and animal infectious diseases. AMR in bacteria is considered one of the most important emerging threats to animal and human health worldwide (Dobiasova et al. 2013). Multi-drug resistant (MDR) infections were diagnosed in over 400 000 people resulting in 25 000 mortalities in Europe in 2007 (Hwang & Gums 2016). Long term persistence of resistance phenotypes in the absence of antibiotic exposure has reinforced the importance of minimising the emergence of resistance to preserve the efficacy of existing and newly developed antimicrobial drugs (Holmes et al. 2016, Sundqvist et al. 2010).

Bacteria acquire resistance genes either through mutation or through horizontal transfer from other bacteria (Radhouani et al. 2014). Horizontal transfer can occur between bacteria of the same or different species by incorporating free DNA from the environment (transformation), from bacteriophages (transduction) and directly between bacteria (conjugation) (Hwang & Gums 2016). Acquired antibiotic resistance has three basic mechanisms: reduced uptake (e.g. changes in membrane permeability and tetracycline efflux pump); inactivation (e.g. β-lactamase production inactivates penicillin) and alteration of the target binding sites (e.g. mutations of DNA gyrase prevents fluoroquinolones from binding (Cag et al. 2016). These mechanisms can create resistance to more than one antibiotic of the same class (cross-resistance) or multiple classes (multi-drug resistance) (Cag et al. 2016). Exposure to antibiotics favours bacteria with these capabilities and is an important driver of AMR. Because multiple genes coding for resistance can exist in the same genetic element, selection pressure directed towards one gene can cause incidental co-selection for the others. This means that the use of one antibiotic can select for bacteria resistant not only to that antibiotic but also to unrelated antibiotic classes (da Costa, Loureiro & Matos 2013). Decreasing AMR once established is not straightforward because removal of antibiotic exposure does not usually cause resistant bacteria to become susceptible (Acar & Moulin 2012).

Migration and travel of humans, animals and food have contributed to worldwide dissemination of resistance genes (da Costa, Loureiro & Matos 2013). Faeces can be a major source of resistance genes that can contaminate environment and water sources (Guenther, Ewers & Wieler 2011). Environmental contamination was blamed for the greater than expected prevalence of AMR bacteria isolated from wild animals and isolated human populations that had very low levels of exposure to antibiotics (Janatova et al. 2014, Jardine et al. 2012). Wild animals and birds are considered to be an important potential reservoir of bacteria carrying antibiotic resistance traits that can be transmitted to humans, animals and the environment (Guenther, Ewers & Wieler 2011).

Antibiotic use kills both pathogenic and normal commensal flora which can select for resistance in both (van den Bogaard & Stobberingh 2000, Witte 2000). Therefore commensal bacteria can be used as indicators of levels and types of background antimicrobial resistance because they reflect antibiotic selection pressure on the whole of the microflora population (van den Bogaard &

Stobberingh 2000). Understanding resistance phenotypes in commensal bacteria is important because they can be a reservoir of resistance genes for other pathogenic bacteria and the environment (Dobiasova et al. 2013, van den Bogaard & Stobberingh 2000, Witte 2000). Surveillance of the commensal bacteria from healthy subjects can be a useful way to detect and monitor resistance in a population.

Not only can AMR be increased by selection pressure from antibiotic use, but the latter can also can increase vulnerability to infectious diseases by inhibiting commensal microflora that normally have a protective function against invasion by exogenous micro-organisms (which may be resistant to antibiotics) (da Costa, Loureiro & Matos 2013).

Escherichia coli and *Enterococcus* spp. have been used in this project as indicator commensal species as they are very common enteric microflora of both humans and animals which can be easily isolated from faeces and reflect the levels of background AMR in a population (Baldy-Chudzik & Stosik 2007, Dobiasova et al. 2013, EFSA 2008). Both intestinal *E. coli* and *Enterococcus* spp. are good indicators because they quickly reflect any changes in the resistance profiles of intestinal bacteria caused by exposure to antibiotics (EFSA 2008, Sørum & Sunde 2001)Both species can be reservoirs of genes coding for resistance for pathogenic bacteria or even be opportunistic pathogens themselves (Radhouani et al. 2014).

The methods used to isolate *E. coli* and *Enterococcus* spp. were based on those recommended by European Food Safety Authority (EFSA) for surveillance of AMR in commensal bacteria in member European Union (EU) states (EFSA 2008). Classifying *E. coli* strains phylogenetically can assist in identification of pathways where resistance genes are passing between humans, animals and the environment (van den Bogaard & Stobberingh 2000). Multi-locus sequence typing (MLST) identifies strains of *E. coli* by comparing the sequences of seven housekeeping genes. MLST data can be used to study the phylogenetic relationships between different populations of bacteria and investigate their epidemiology.

Captive animals kept in zoos are usually subject to more intense contact with humans and other animals compared with their wild counterparts. The basic husbandry of zoo animals is similar to many farmed domestic species so it may be possible that their microflora would develop resistance profiles more in common with domestic than wild species

This project expands on current research because there are few studies that describe resistance phenotypes in bacteria from zoo animals. Marwell Zoo is an interesting context because it is a closed population with a known treatment history in a single location. This limits the number of variables when investigating the dynamics of antibiotic resistance

AIMS AND OBJECTIVES

This project investigated the types of antibiotic resistance in indicator commensal bacteria isolated from wild ungulates at Marwell Zoo. These cross-sectional data were used to describe the background AMR levels within the target population and explored possible drivers for AMR emergence such as previous treatment with antibiotics or exposure to possible sources of resistance genes.

The aims of this research project were to:

- Isolate commensal enteric *E. coli* and *Enterococcus* spp. from faecal samples collected from healthy animals
- Perform sensitivity testing on the isolates by means of antibiotic disk diffusion method

- Plot the resistance phenotypes on a map of the zoo
- Review the medical records of individual animals, including antibiotic consumption
- Perform MLST on *E. coli* isolates to investigate their clonality and what spatial associations exist between identical strains
- Compare the antibiotic resistance data with those of domestic and wild ungulates

We suspect that the resistance profiles of commensal bacteria of the target animals may be more similar to those of domestic ungulates than wild free-ranging ungulates.

LITERATURE REVIEW

AMR is the most important One Health issue globally, and responsible around 700 000 human deaths each year (O'Neill 2016). Increasing our understanding of the epidemiology of AMR is crucial to creating solutions for this problem. Solving AMR is being tackled on a global level via agreements between the World Health Organisation (WHO), the Food and Agriculture Organisation of the United Nations (FAO) and the World Organisation for Animal Health (OIE). This collaboration provides a framework for international co-operation and guidelines for national plans to minimise the emergence of AMR. Both surveillance of AMR prevalence and antibiotic consumption is necessary to monitor AMR. Harmonisation of surveillance methods is necessary to compare data from different sources so that it can be interpreted at an international level (Acar & Moulin 2013). Co-operation is needed across sectors involving policy-makers, public health bodies, agriculture, veterinary professionals, food safety regulators and laboratories (Acar & Moulin 2013).

The WHO has adopted a global action plan against AMR which has the following objectives:

- Education and training to improve awareness and understanding of AMR
- Surveillance and research to advance scientific knowledge
- Effective sanitation and hygiene to reduce infections
- Optimising how antimicrobials are used to treat humans and animals
- Increase investment in new medicines, diagnostic tests, vaccines and other treatments

(WHO 2015)

Apart from exposure to antibiotics, the main drivers of resistance are the environment in which animals live and the reservoirs they are exposed to (Radhouani et al. 2014). It has been shown that in the absence of antibiotic exposure, commensal *E. coli* isolated from free-ranging livestock and wild ungulates in a Spanish national park had similar resistance prevalences (Navarro-Gonzalez et al. 2013). The converse is also true because there are studies that showed that wildlife living in urban areas, or in close contact with human activities, had a higher prevalence of bacteria carrying AMR traits than those living more remotely (Jardine et al. 2012, Radhouani et al. 2014). In the study by Jardine et al. 2012, reduced susceptibility to at least one antibiotic was found in 17% of wild raccoons trapped in a rural site compared with 42% trapped at Toronto Zoo. Antibiotic resistance in bacteria isolated from wildlife is usually due to exposure to sources of AMR bacteria from human and domestic animals because wildlife are rarely treated with antibiotics (Guenther, Ewers & Wieler

2011). Because exposure to antibiotics and living near human activity are important drivers of AMR, we would expect the microflora of zoo animals to have resistance patterns more similar to farmed ungulates than their free-ranging wild counterparts. Sources of resistant bacteria for zoo animals would likely include contact with humans, with wildlife and via food and water (Ishihara et al. 2012, van den Bogaard & Stobberingh 2000).

Previous studies have shown a comparable prevalence of AMR in bacteria isolated from zoo animals and domestic livestock. A study at a Polish zoo showed that 64% of *E. coli* isolated from herbivores were resistant to at least one antibiotic and 24% resistant to three classes of antibiotic (Baldy-Chudzik & Stosik 2007). Ahmed et al. 2007 reported that 21% of Gram-negative bacteria isolated from zoo animals in Japan were resistant to two or more antibiotics. Borriello et al. 2014 reported that the average rate of multiple resistance (resistance to four or more antibiotics) was 54% in samples submitted to the Animal and Plant Health Agency, United Kingdom (APHA) from cattle, pigs, sheep, chickens and turkeys. Commensal *E. coli* isolated from healthy horses had a resistance rate of 13.4% in a study conducted on a livery (Schoster et al. 2012). The prevalence of AMR in domestic ungulates is variable and may be linked with the production system (i.e. intensive or extensive) and antibiotic use (EFSA and ECDC 2015). Likewise the AMR prevalences in zoos appear to be variable and could be influenced by factors such as frequency of antibiotic use and the living conditions of the animals.

Resistance phenotypes and patterns have also had some similarities in zoo and domestic animals. Recent surveillance data from the EU showed that coliform bacteria isolated from domestic ungulates (cattle and pigs) are most frequently resistant to ampicillin, sulphonamides and tetracyclines (EFSA and ECDC 2015). The highest levels of resistance in *E. coli* isolated from zoo animals in Poland were to sulphonamides, tetracyclines and cephalosporins (Baldy-Chudzik & Stosik 2007). Ahmed et al. 2007 reported the highest rates of resistance from *E. coli* at a zoo in Japan were against ampicillin, cephalothin, streptomycin, trimethoprim-sulfamethoxazole, kanamycin, tetracycline, nalidixic acid and ciprofloxacin. Another zoo in Japan had *E. coli* isolates that were most commonly resistant to tetracycline, streptomycin and ampicillin (Ishihara et al. 2012). The large variation in resistance profiles in bacteria isolated from zoo animals could be due to differences in antibiotic use and exposure to sources of resistance genes (Wang et al. 2012).

Treatment of captive wild animals is complicated by a lack of pharmacokinetic and pharmacodynamic data in these species. Few drugs are approved for wild species compared with domestic (Hunter & Isaza 2002). In addition there may be considerations such as lack of patient compliance and the stress of handling that limits choices regarding the method of administration and therapy duration (Hunter & Isaza 2002, Lees & Shojaee Aliabadi 2002). For example, catching and handling a wild animal daily for antibiotic injections would increase stress levels and risk of injury which could in turn compromise recovery (Ahmed & Kasraian 2002, Hunter & Isaza 2002). Therefore in the zoo context, where most animals have a high value, the benefits of every intervention needs to be weighed up against the possible risks. A combination of these factors may result in sub-optimal antibiotic concentrations and encourage the emergence of AMR bacteria (Papich 2014).

Ishihara and others demonstrated an association between antimicrobial administration and antimicrobial resistance in *E. coli* isolated from faeces collected from healthy zoo animals in Japan (Ishihara et al. 2012). The same study also found an association between amoxicillin use and the increased prevalence of resistance against multiple unrelated drug classes. This phenomenon has been documented in human and domestic animal studies and may be a result of co-selection of linked genes for multiple resistance traits on mobile genetic elements (Freitag et al. 2016). Thus the

use of one antibiotic can co-select for multiple resistance traits and result in MDR. Knowing the antibiotic treatment history of the subject animals at Marwell enable us to investigate for the presence of co-selection as part of our study.

Maintenance of genes coding for resistance in commensal microflora is a concern because resistance levels can remain high long after antibiotics have been withdrawn (Dobiasova et al. 2013). For example after avoparcin was banned for use in animals in Denmark, vancomycin (which is closely related to avoparcin) resistance in pigs did not decrease significantly in three years (van den Bogaard & Stobberingh 2000). Other longitudinal studies reported that resistance prevalence reduced back to pre-treatment levels within three days in cattle that received a single dose of florfenicol and in two months in horses that had received antibiotic treatment (Berge, Epperson & Pritchard 2005, Johns et al. 2012). Long term maintenance of resistance genes in the absence of antibiotic exposure depends on whether they confer any fitness cost to the bacteria hosting them (Holmes et al. 2016). Persistence of resistance genes can occur incidentally as a result of co-selection with other genes that code for characteristics that enable the bacteria to survive exposure to environmental toxins (e.g. heavy metals and disinfectants) (da Costa, Loureiro & Matos 2013, Williams et al. 2011). Other conditions where increased AMR has occurred independent of antibiotic use in coliform bacteria isolated from pigs include: increased stress, cold temperatures, hot temperatures, increased intestinal motility and young age (Sørum & Sunde 2001). Further research is needed to investigate what factors affect the persistence of AMR traits in commensal bacteria (Holmes et al. 2016).

Extended-spectrum β-lactamase (ESBL) production enable *E. coli's* with this trait to resist both cephalosporin and penicillin antibiotics, many of which are considered to be critically important in human medicine (Cavaco et al. 2008). The global emergence of ESBL-producing bacterial infections is considered to be a serious public health risk as it is associated with increased morbidity and mortality in humans (Liebana et al. 2013). ESBLs were first recorded in human hospitals in the late 1980's and have subsequently increased in prevalence and range to be detected in community settings and domestic and wild animals (Guenther, Ewers & Wieler 2011). Omnivorous wild birds and small mammals are considered some of the most important vectors of ESBL-producing *E. coli* because they can acquire AMR bacteria while scavenging on human waste and shed them in their faeces, contaminating food and water sources (Guenther, Ewers & Wieler 2011). Wild birds and small rodents could be a potential source of ESBL bacteria or genes in zoos. ESBL-producing *E. coli* have been isolated from zoo animals in Japan and the Czech Republic (Ahmed et al. 2007, Dobiasova et al. 2013). The use of β-lactam drugs is another significant risk factor for the emergence of ESBL-producing *E. coli* (Cavaco et al. 2008).

Animals colonised by resistant bacteria from a common source may share the same strain (Klimes et al. 2013). MLST can differentiate strains by comparing the sequences of seven house-keeping genes. Identification of identical (clone) isolates can reveal epidemiological relationships. Human studies have used this technique to track the transmission of *E. coli* ST 131 between family members and their pets (Mathers, Peirano & Pitout 2015). Maras in Copenhagen Zoo have been shown to share *Staphylococcus aureus* ST 130 with humans, sheep and cows in Denmark (Espinosa-Gongora et al. 2015). This project will use MLST to investigate clonality of *E. coli* isolates which may supply epidemiological information regarding the dissemination of AMR in the zoo.

There are many different methods for detecting AMR in commensal indicator bacteria however attempts have been made to harmonise techniques used to monitor AMR in *E. coli, Enterococcus faecalis* and *E. faecium* from food producing animals in the EU (EFSA 2008). These techniques were suitable for use in this project as they could be used to generate an overview of the resistance phenotypes present in the target animals. As recommended in the EFSA guidelines, the bacterial

growth media did not contain antibiotics which favoured the isolation of the dominant bacterial strains present in the sample (Borriello et al. 2014, EFSA 2008). Other studies such as (Dobiasova et al. 2013) used agar containing antibiotics but this technique tends to select for resistant isolates which may not be the dominant strains (EFSA 2008).

Because of differences in innate susceptibility, *E. faecium* should be differentiated from *E. faecalis* (EFSA 2008). Molecular methods (such as polymerase chain reaction (PCR)) have been shown to be more accurate than phenotypic and biochemical tests for identifying identify *E. faecium* and *E. faecalis*. Loop-mediated isothermal amplification of DNA (LAMP) is a recently developed alternative that can detect target DNA in a much shorter time than PCR. LAMP was used in this project to because it was much quicker and easier to use than conventional PCR.

AMR surveillance collects antibiotic sensitivity data from diseased humans, diseased food animals, healthy animals and meat (Acar & Moulin 2013). The first antibiotic sensitivity tests focussed on investigating the resistance phenotypes of pathogens from clinical cases in order to select effective treatments (Acar & Moulin 2013). Susceptibility in clinical cases is defined by a bacterial infection responding to therapeutic levels of antibiotics. A clinical breakpoint (CBP) is the concentration of antibiotic at which a bacteria that is not inhibited is likely to be associated with therapeutic failure (EFSA and ECDC 2014). Another measure of resistance is epidemiological cut off (ECOFF) which represents the concentration of antibiotic at which bacteria with acquired resistance can be differentiated from their susceptible counterparts (EFSA and ECDC 2014). It is possible that an isolate is microbiologically resistant, because it has acquired resistance genes, yet be clinically susceptible (EFSA 2008). By grouping the intermediate and resistant clinical categories as “non-susceptible”, this study used a similar standard to ECOFF (EFSA and ECDC 2015). Because the majority of isolates test sensitive or highly resistant to most antibiotics tested, breakpoint differences may not affect the overall result (Borriello et al. 2014, UK One Health Report 2015). Measuring AMR in commensal bacteria isolated from healthy animals is more representative of the effects of antibiotic use on background AMR levels in the general population compared with those isolated from clinical cases which may have been subject to greater antibiotic exposure (EFSA 2008).

METHODS AND MATERIALS

(See Appendix E for manufacturers' addresses)

Study Location

Marwell Zoo consists of 140 acres of parkland in rural Hampshire, England (co-ordinates 50° 59' 25.0404" N; 1° 17; 3.9444" W). It has been in existence since 1972 and is currently visited by 500 000 people a year. Since its inception, Marwell Zoo has focussed on breeding endangered ungulates and large cats.

Target Animals

This project sampled 17 species of ungulates living at Marwell Zoo in England. Table 1 details the species and numbers of animals in each group that was sampled. The subjects of this study lived in enclosures that usually had access to pasture (Figure 1). Each enclosure had a brick built house surrounded by concrete hard standing where food and water troughs were available. The enclosures contained mostly single species but there were mixed herds in some enclosures (nyala and waterbuck; addax 2 and dorcas 2; Arabian Oryx 2 and dorcas 1; Grevy's zebra and roan antelope). They were fed hay and concentrates according to their specific requirements.

Table 1 Species and number of animals sampled

Species	No. of animals in enclosure	**Species**	No. of animals in enclosure
Arabian Oryx 1 *Oryx leucoryx*	1	Grevy's zebra *Equus grevyi*	1
Arabian Oryx 2 *Oryx leucoryx*	5	Hartmann's mountain zebra *Equus zebra hartmannae*	4
Addax 1 *Addax nasomaculatus*	2	Nyala *Tragelaphus angasii*	8
Addax 2 *Addax nasomaculatus*	5	Przewalski's wild horse *Equus caballus przewalskii*	6
Anoa 1 *Bubalus depressicornis*	1	Roan antelope *Hippotragus equinus*	4
Anoa 2 *Bubalus depressicornis*	2	Sable antelope *Hippotragus niger*	6
Eastern bongo *Tragelaphus eurycerus isaaci*	4	Sitatunga *Tragelaphus spekii gratus*	5
Dwarf forest buffalo *Syncerus caffer nanus*	7	Somali Wild Ass *Equus africanus somaliensis*	4
Chapman's zebra *Equus quagga chapmani*	5	Warthog *Phacochoerus africanus*	1
Dorcas 1 (Nakheila gazelle) *Gazella dorcas osiris*	4	Common waterbuck *Kobus ellpsiprymnus ellipsiprymnus*	7
Dorcas 2 (Nakheila gazelle) *Gazella dorcas osiris*	4		

Figure 1 View of one of the enclosures at Marwell Zoo

Sample Collection

One universal container (30ml) of faeces was collected from each species on three occasions, a week apart in November 2015. In total 61 samples were collected from 19 locations and 17 species. There were two sites (Anoa 2 and Warthog) that were only sampled twice due to restricted access on the collection days. The location, species and date were recorded for each sample. Most enclosures had more than one animal living there so many of the samples will represent more than one individual. It was possible to differentiate faeces from each species when samples were collected from a mixed herd.

The samples were transported to the laboratory and processed on the same day. Surplus sample material was added to 100% glycerol and stored at -80^{o}C for possible use in future studies.

Bacterial isolation and identification

Escherichia coli

One gram of faeces was added to 10ml phosphate buffered saline and agitated using a vortex. A sterile swab was used to spread the suspension onto a MacConkey agar (Oxoid Ltd) plate which was incubated aerobically at 37^{o}C for 18-24 hours. One colony per sample with typical *E. coli* morphology (large pink colony) was selected from each plate and spread on a fresh MacConkey agar plate which was incubated at 37^{o}C for 18-24 hours. Conventional biochemical methods were used to identify *E. coli* isolates (Gram-staining, catalase, oxidase, indole production). A presumptive identification of *E. coli* was recorded if the isolate was catalase positive, oxidase negative, indole positive and resembled Gram-negative (pink or purple) rods microscopically..

Enterococcus spp.

One gram of faeces was added to 10ml of brain-heart-infusion broth (Oxoid Ltd) supplemented with 6.5% NaCl and agitated using a vortex. Incubation in salt broth (aerobically at 37^{o}C for 18-24 hours) followed by culture on Slanetz-Bartley agar (Oxoid Ltd). These media selected for the growth of *Enterococcus* spp by inhibiting the growth of other species. Following 18-24 hours incubation of the Slanetz-Bartley plates at 37^{o}C, a few colonies were transferred onto a fresh Slanetz-Bartley agar plate and incubated at 37^{o}C for a further 24-48 hours. Presumptive identification was based on growth in salt broth and on Slanetz-Bartley media, colony appearance and testing catalase and

oxidase negative. Identification of *Enterococcus faecalis* and *Enterococcus faecium* was performed using Loop-Mediated Isothermal Amplification assays (LAMP) run on a Genie®II (Optigene).

Any presumed *Enterococcus* isolates that could not be identified using LAMP were Lancefield typed following overnight growth on blood agar using the Oxoid™ Streptococcal Grouping Kit (Oxoid Ltd). Isolates that showed γ haemolysis (i.e. no haemolysis) and were positive reactors to D and or G antigen were classified as *Enterococcus* spp.

Identification of E. faecalis and E. faecium using loop-mediated isothermal amplification of DNA

The DNA template was prepared by adding one bacterial colony to 1ml KOH and heated to 100°C for 10 minutes then cooled and centrifuged at 13 000 rpm or approximately 17949 xg in an Eppendorf 5424 centrifuge (Eppendorf UK Ltd) for 5 minutes (Appendix C). Five microlitres of the supernatant was pipetted into wells in a preloaded strip containing Master Mix, buffer and primers (supplied by Optigene). The strip was centrifuged for 20 seconds at 6 000 rpm or approximately 2000 xg in a Prism™ Mini Centrifuge (Labnet International) and inserted into the Genie®II (Optigene) (Appendix C). The Genie®II measured the fluorescence emitted while the preparation was heated to 65°C. The fluorescence was plotted against temperature which determined whether DNA specific to *E. faecalis* and *E. faecium* was present.

Antibiotic sensitivity testing

Antibiotic sensitivity was tested using agar disk diffusion methods as described by the Clinical and Laboratory Standards Institute (CLSI) (CLSI M100-S22 2012).

Fresh cultures were prepared from the pure isolates by streaking onto Nutrient agar (Oxoid Ltd) and incubating at 37°C overnight. A sterile loop was used to transfer a few colonies to 2ml sterile distilled water which was agitated using a vortex. A spectrophotometer was used to standardise the inoculum to 0.5 McFarland standard. A sterile swab was dipped in the suspension and streaked onto a Mueller-Hinton agar (Oxoid Ltd) plate in three directions. Antibiotic disks (Table 5 and 6) were applied to the plates within 15 minutes and the plates were incubated at 37°C overnight. The diameters of the inhibition zones were measured from the underside of the plate using electronic callipers and recorded (Figures 2 and 3). Inhibition zones were interpreted using CLSI and European Committee on Antimicrobial Susceptibility Testing (EUCAST) reference values. *E. coli* NCTC 11560 (ATCC 10536) was used as a susceptible control and *E. coli* NCTC 11954 (ATCC 35218) was used as a resistant control (CLSI M100-S22 2012).

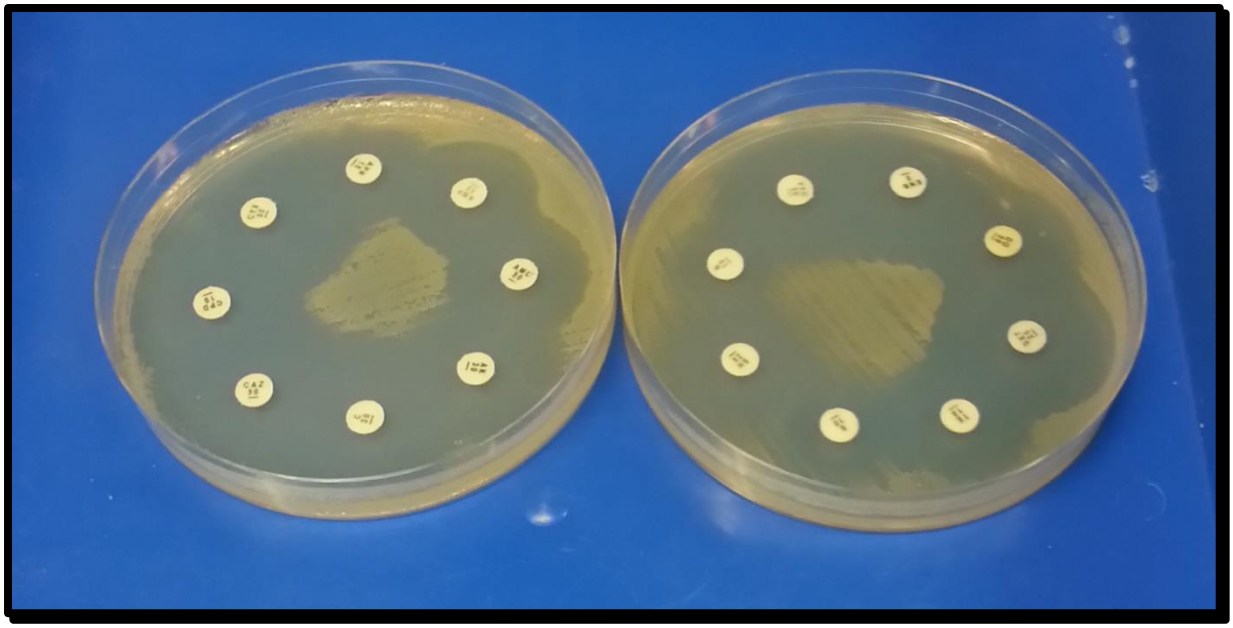

Figure 2 Photograph of an antibiotic sensitivity test showing how the antibiotic disks have inhibited growth of a susceptible isolate

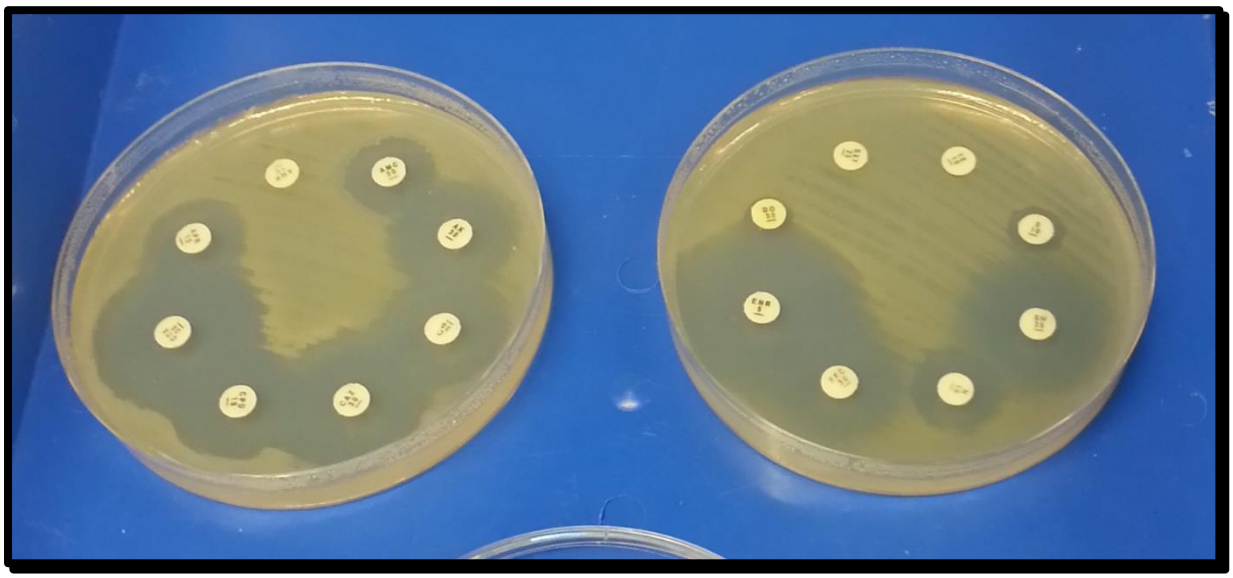

Figure 3 Photograph of an antibiotic sensitivity test on a resistant strain. Note the absence of zones of inhibition around some of the antibiotic disks

Further sensitivity testing was performed on the *E. coli* isolates in a similar manner using four antibiotic tablets which were the same size and shape as the antibiotic disks but consisted of compressed powder instead of antibiotic impregnated paper. These tablets contained combinations of cefotaxime, clavulanate and cloxacillin (cefotaxime 30µg, cefotaxime 30µg + clavulanate, cefotaxime 30µg + cloxacillin and cefotaxime + clavulanate + cloxacillin) (ESBL +AmpC screen ID kit, Rosco Diagnostica A/S). The interpretation of the test involved comparing the diameters of the zones of inhibition. If the zone of inhibition of the cefotaxime 30µg tablets was within 3mm of the other tablets then neither ESBL nor AmpC activity were present. If the difference was greater than 3mm then further comparisons were made and interpreted using the Rosco information leaflet (Appendix D).

All isolates were stored in broth supplemented with 15% glycerol at -80^{o}C.

Multilocus sequence typing (MLST)

PCR was performed on the *E. coli* isolates to amplify seven house-keeping genes as described in (Wirth et al. 2006) (Table 2). The oligonucleotide primers were supplied by Sigma Aldrich.

Table 2 Oligonucleotide primers used for E. coli MLST (Wirth et al. 2006)

	Locus size (bp)	Primer	Sequence (5' – 3')	Annealing temperature (T_a)
Adenylate kinase	adk (536)	adk-P1	ATTCTGCTTGGCGCTCCGGG	54°C
		adk-P2	CCGTCAACTTTCGCGTATTT	
Fumarate hydratase	fumC (469)	fumC-P1	TCACAGGTCGCCAGCGCTTC	54°C
		fumC-P2	GTACGCAGCGAAAAAGATTC	
DNA gyrase	gyrB (460)	gyrB-P1	TCGGCGACACGGATGACGGC	60°C
		gyrB-P2	ATCAGGCCTTCACGCGCATC	
Isocitrate/isopropylmalate dehydrogenase	Icd (518)	icd-P1	ATGGAAAGTAAAGTAGTTGTTCC GGCACA	54°C
		icd-P2	GGACGCAGCAGGATCTGTT	
Malate dehydrogenase	mdh (452)	mdh-P1	ATGAAAGTCGCAGTCCTCGGCGC TGCTGGCGG	60°C
		mdh-P2	TTAACGAACTCCTGCCCCAGAGC GATATCTTTCTT	
Adenylosuccinate dehydrogenase	purA (478)	purA-P1	CGCGCTGATGAAAGAGATGA	54°C
		purA-P2	CATACGGTAAGCCACGCAGA	
ATP/GTP binding motif	recA (510)	recA-P1	CGCATTCGCTTTACCCTGACC	58°C
		recAR-P2*	TCGTCGAAATCTACGGACCG GA	

* Alternative primer sequence sourced from MLST Database website at the University of Warwick (the one suggested by Wirth et al. did not work)

PCR Method:

A small loop of bacteria from an overnight plate culture was added to 200 µl pure water and agitated using a vortex. This was used as the DNA template. The positive control was *E. coli* NCTC 11560 and the negative control was sterile water. The reaction mix is shown in Table 3.

Table 3 Composition of PCR reaction mix

Reagent	µl
Water (Milli-Q® water, ThermoFisher)	9.75
Buffer (Promega Colorless GoTaq® Reaction Buffer M7805)	10
Upstream primer, 10µM	1
Downstream primer, 10µM	1
Nucleotides (Promega dNTP Mix U1511)	2
DNA Template	1
Taq polymerase (Promega GoTaq® G2 Polymerase M7805)	0.25

The PCR was run in a thermal cycler (SimpliAmp™, ThermoFisher) on the following program (Table 4):

Table 4 Thermal cycler program (sourced from MLST Database website*; (Wirth et al. 2006):

Number of cycles	Time	Temperature
1	2 minutes denaturation	95°C
30	1 minute denaturation	95°C
	1 minute annealing	T_a of specific primer
	2 minutes extension	72°C
1	5 minutes extension	72°C

*http://mlst.warwick.ac.uk/mlst/dbs/Ecoli/documents/primersColi_htm

Electrophoresis of the PCR products was performed on agarose gel at 100V for 45 minutes. The electrophoresis substrate consisted of 1% (w/v) agarose gel (Oxoid Ltd) supplemented with SYBR® Safe DNA Gel Stain (ThermoFisher) and immersed in Tris-EDTA buffer. The success of each PCR reaction was confirmed by the appearance of a single band for each sample on the gel when viewed under UV light.

The DNA products were purified using a DNA binding spin column system (QIAquick PCR Purification Kit, Qiagen). The purified PCR products were automatically sequenced (ABI 3730xl DNA Analyzer system, GATC Biotech) using the same primers as for the PCR reactions. Sequences were then compared with those on the MLST website and assigned a distinct allele number. This generated an allelic profile consisting of seven numbers per isolate. These seven numbers defined the sequence type (ST) which was compared with other isolates in a central database (http://mlst.warwick.ac.uk/mlst/dbs/Ecoli/; (Achtman, Velayudhan & Zhou). Strains with the same allelic profile or ST number are considered to be identical (clones).

Investigation of Medical Records at Marwell Zoo

Marwell Zoo stores information for individual animals including medical treatments on an internet based database program (©2015 International Species Information System ZIMS). The records were searched for any treatments administered to each target animal during their lifetime. It was not possible to search for treatments that were administered to animals before their arrival at Marwell zoo if they were imported.

RESULTS

Bacterial isolation and identification

In total 39 isolates were identified as *E. coli* and 59 as *Enterococcus* spp. from the 61 samples.

Of the *Enterococcus* spp., the LAMP identified 9 *E. faecalis*, 26 *E. faecium* and 7 mixed *E. faecalis/ faecium* isolates. Seventeen isolates were classified as *Enterococcus* spp. as they fulfilled the biochemical criteria for *Enterococcus* but were not identifiable as *E. faecalis* or *E. faecium* using LAMP.

Antibiotic sensitivity testing

Table 5 *E. coli* antibiotic sensitivity results

Antibiotic disks (Oxoid Ltd)	Zones of inhibition (mm)	No. of resistant isolates (n=39)
Ampicillin (10µg)	≥ 17[a]	11/39 (28.2%)
Amoxicillin-clavulanic acid (20/10µg)	≥ 18 [a]	4/39 (10.3%)
Cefotaxime (30µg)	≥ 26 [a]	1/39 (2.6%)
Cefpodoxime (10µg)	≥ 21 [a]	1/39 (2.6%)
Ceftazidime (30µg)	≥ 21 [a]	1/39 (2.6%)
Enrofloxacin (5µg)	≥ 23 [c]	0/39 (0%)
Chloramphenicol (30µg)	≥ 18 [a]	0/39 (0%)
Florfenicol (30µg)	≥ 19 [c]	0/39 (0%)
Doxycycline (30µg)	≥ 14 [a]	5/39 (12.8%)
Tetracycline (10µg)	≥ 15 [a]	5/39 (12.8%)
Amikacin (30µg)	≥ 17 [a]	1/39 (2.6%)
Apramycin (15µg)	≥ 15 [c]	0/39 (0%)
Neomycin (10µg)	≥ 15 [b]	5/39 (12.8%)
Spectinomycin (25µg)	≥ 17 [a]	7/39 (17.9%)
Streptomycin (10µg)	≥ 15 [a]	7/39 (17.9%)
Trimethoprim sulphamethoxazole (25µg)	≥ 16 [a]	7/39 (17.9%)

[a] CLSI M100-S23 (M02-A11): "Disc diffusion supplemental tables" Performance standards for antimicrobial susceptibility testing

[b] CLSI: VET01S 3rd Edition

[c] Oxoid package leaflet for Oxoid Antimicrobial Susceptibility Test Discs TSMX7764

E. coli isolates were most frequently resistant to ampicillin (28.2%), streptomycin (17.9%), spectinomycin (17.9%), trimethoprim sulphamethoxazole (17.9%), neomycin (12.8%), doxycycline (12.8%) and tetracycline (12.8%) (Table 5). Lower rates of resistance were found for amoxicillin-clavulanic acid (10.3%), cefotaxime (2.6%), cefpodoxime (2.6%), ceftazidime (2.6%) and amikacin (2.6%) (Table 5). There were no isolates resistant to apramycin, enrofloxacin, chloramphenicol or florfenicol.

Fifty-four per cent of *E. coli* isolates were susceptible to the entire antibiotic panel, 13% were resistant to one antibiotic and the remaining 33% were resistant to more than one antibiotic.

None of the *E. coli* isolates tested positive for ESBL or AmpC activity using the screening kit.

Table 6 *Enterococcus* spp. antibiotic sensitivity results

Antibiotic disks (Oxoid Ltd)	Zones of inhibition (mm)	No. of resistant isolates (n=55)
Ampicillin (10µg)	≥ 17[a]	0/55 (0%)
Cefpodoxime (10µg)	≥ 13[e]	51/55 (95%)
Ciprofloxacin (5µg)	≥ 21[a]	22/55 (40%)
Streptomycin (300µg)	≥ 10[a]	2/55 (4%)
Erythromycin (15µg)	≥ 23[a]	37/55 (67%)
Gentamicin (200µg)	≥ 10[a]	0/55 (0%)
Tetracycline (10µg)	≥ 19[a]	18/55 (33%)
Chloramphenicol (30µg)	≥ 18[a]	0/55 (0%)
Imipenem (10µg)	≥ 21[bd]	6/55 (11%)
Vancomycin (30µg)	≥ 17[a]	0/55 (0%)
Trimethoprim sulphamethoxazole (25µg)	≥ 21[bc]	3/55 (5%)

[a] (CLSI M100-S25 2015)

[b] (EUCAST version 5.16b)

[c] Not determined in *E. faecalis* (one of the three resistant isolates was *E. faecalis)*

[d] Not determined in *E. faecium* (four of the six resistant isolates were *E. faecium)*

[e] No reference values exist in CLSI and EUCAST because *Enterococcus* spp. are intrinsically resistant to cephalosporins

Ninety-five per cent of the *Enterococcus* isolates were resistant to cefpodoxime, 67% to erythromycin, 33% to tetracycline and 40% to ciprofloxacin (Table 6). No isolates were resistant to ampicillin, gentamicin, chloramphenicol and vancomycin. Very few were resistant to trimethoprim sulphamethoxazole (5%), imipenem (11%) and streptomycin (4%) (Table 6).

Medical history report

The most common antibiotics used on the animals in this study were amoxicillin, trimethoprim sulphadiazine and tetracyclines (oxytetracycline and doxycycline). Other antibiotics that were used very rarely included dihydrostreptomycin (combined with penicillin), enrofloxacin, lincomycin, amikacin, ceftiofur and neomycin. In the 12 months prior to sampling, only 13 individuals were treated with antibiotics. Trimethoprim sulphadiazine and doxycycline were used in these treatments (Table 7). The reasons given for antibiotic treatment were varied and included infected wounds, hoof abscesses, conjunctivitis, facial swelling, perinatal complications and prophylactically at the time of castration. No antibiotics were administered during the three weeks when sampling took place.

Table 7 Species sampled, resistant phenotypes and treatment history

Species	**Date of collection**	**Resistance phenotype *E. coli***	**Resistance phenotype *Enterococcus* spp.**	***Enterococcus* identified as**	**Antibiotic Treatment History in last year**
Arabian Oryx 1	16/11/2015	AMP, SH	CFP, TET, ERY	Spp.	None
	23/11/2015		CFP	Spp.	
	30/11/2015		CIP, CFP, ERY, IMI	Spp.	

Arabian Oryx 2	16/11/2015	AMP, NEO, SH	CFP	Mixed	None
	23/11/2015	SUSCEPTIBLE	CFP	*E. faecium*	
	30/11/2015		CIP, CFP, TET, ERY	Spp.	
Addax 1	16/11/2015	SUSCEPTIBLE			None
	23/11/2015	NEO, SH			
	30/11/2015				
Addax 2	16/11/2015	AMP	CIP, CFP, ERY	*E. faecium*	None
	23/11/2015	SUSCEPTIBLE			
	30/11/2015		CIP, CFP, ERY	Spp.	
Anoa 1	16/11/2015	CTX	CFP, ERY	Mixed	None
	23/11/2015	SUSCEPTIBLE	CFP, TET	*E. faecalis*	
		SH			
	30/11/2015	SUSCEPTIBLE			
Anoa 2	16/11/2015	SUSCEPTIBLE	CFP, TET	Spp	None
	23/11/2015	NO SAMPLE			
	30/11/2015		CFP	*E. faecium*	
Eastern bongo	16/11/2015	SUSCEPTIBLE	CFP	Spp.	None
	23/11/2015		CFP, ERY	*E. faecium*	
	30/11/2015	SUSCEPTIBLE	CIP, CFP, ERY, IMI	Spp.	
Dwarf forest buffalo	16/11/2015		CFP	*E. faecalis*	None
	23/11/2015	SUSCEPTIBLE	CIP, CFP, TET, ERY	*E. faecium*	
	30/11/2015		CFP, TET, ERY	*E. faecium*	
Chapman's zebra	16/11/2015	AMOX, TPS	CIP, CFP, TPS, ERY	Spp.	TPS 05/11/2015; 15/10/2015
	23/11/2015	SH, TPS	CFP	*E. faecium*	
			CIP, CFP, ERY	Spp.	
	30/11/2015	SUSCEPTIBLE	CIP, CFP, ERY	Spp.	
Dorcas 1	16/11/2015		CFP	Spp.	DOX 24/03/2015
	23/11/2015		CIP, CFP, ERY	*E. faecium*	
	30/11/2015		CFP, ERY	*E. faecium*	
Dorcas 2	16/11/2015	SUSCEPTIBLE			None
	23/11/2015	AMP, CFP, CAZ, NEO, SH, S	CFP, ERY	*E. faecalis*	
	30/11/2015		SUSCEPTIBLE	Spp.	
Grevy's zebra	16/11/2015	AK, SH	CIP, CFP, TET, ERY	Spp.	TPS 03/03/3015
	23/11/2015		CFP	*E. faecium*	

	30/11/2015	SUSCEPTIBLE	CIP, CFP, TET, ERY	*E. faecium*	
Hartmann's mountain zebra	16/11/2015	AMP, DOX, S, TET, TPS	CFP	*E. faecium*	TPS 12/04/2015; 23/11/2014
		AMOX, AMP, DOX, S, TET, TPS	CFP	*E. faecium*	
	23/11/2015		CIP, CFP, TPS, ERY	*E. faecium*	
	30/11/2015	SUSCEPTIBLE	CIP, CFP, TET, ERY	*E. faecalis*	
Nyala	16/11/2015	SUSCEPTIBLE	TPS, ERY	*E. faecalis*	TPS 24/4/2015
	23/11/2015	SUSCEPTIBLE	CFP, TET, ERY	*E. faecalis*	
	30/11/2015	SUSCEPTIBLE	TET, ERY	*E. faecalis*	
Przewalski's wild horse	16/11/2015				TPS 24/08/2015
	23/11/2015		CIP, CFP, ERY	*E. faecium*	
	30/11/2015		CIP, CFP, ERY	*E. faecium*	
Roan antelope	16/11/2015		CIP, CFP, ERY	Mixed	None
	23/11/2015		CIP, CFP, TET, ERY	*E. faecium*	
	30/11/2015		CIP, CFP	*E. faecalis*	
Sable antelope	16/11/2015	SUSCEPTIBLE	CIP, CFP, ERY	*E. faecium*	None
	23/11/2015		CIP, CFP, ERY	*E. faecium*	
	30/11/2015	SUSCEPTIBLE	CFP	Spp.	
Sitatunga	16/11/2015		CFP, ERY	*E. faecium*	TPS 02/02/2015
	23/11/2015	SUSCEPTIBLE	S, CFP, TET, ERY	Mixed	
		AMP, SH	CFP, ERY	Spp.	
	30/11/2015	AMP	S, CFP, TET, ERY	*E. faecium*	
Somali Wild Ass	16/11/2015	AMOX, AMP, DOX, S, TET, TPS	CFP, TET	*E. faecium*	TPS 31/01/2015; 26/02/2015; 19/10/2015 DOX 31/10/2015; 24/11/2015
	23/11/2015				
	30/11/2015	AMOX, AMP, DOX, S, TET, TPS	CIP, CFP, TET, ERY	Mixed	
Warthog	16/11/2015	AMP, DOX, NEO, S, TET, TPS	CFP, TET, ERY	*E. faecium*	None
	23/11/2015	SUSCEPTIBLE			
	30/11/2015	No Sample			
Common waterbuck	16/11/2015	NEO	ERY	*E. faecalis*	None
	23/11/2015		CFP	Mixed	
	30/11/2015	SUSCEPTIBLE	CIP, CFP, TET, ERY	Spp.	

Key:

AK	amikacin	IMI	imipenem
AMOX	amoxicillin-clavulanic acid	NEO	neomycin
AMP	ampicillin	S	streptomycin
CFP	cefpodoxime	SH	spectinomycin
CIP	ciprofloxacin	TET	tetracycline
CTX	cefotaxime	TPS	trimethoprim-sulpha
CAZ	ceftazidime		Both isolates from the same sample
DOX	doxycycline		Resistant to three or more classes of antibiotic
ENR	enrofloxacin	Mixed	Tested positive for both *E. faecalis* and *E. faecium*
ERY	erythromycin	Spp.	*Enterococcus* spp. other than *E. faecalis* and *E. faecium*

More details of treatment administered to individuals during their lifetime and resistance phenotypes of the group are available in Appendix F

Multilocus sequence typing

MLST identified four pairs of *E. coli* isolates that were identical out of 35 strains (i.e. 27 unique strains isolated) (Table 8). Two pairs were from the same species and two were from different species and locations.

Table 8 Identical *E. coli* isolates and resistance phenotypes

Species and location	Date collected	Resistance phenotype	Species and location	Date collected	Resistance phenotype
Dorcas gazelle 2 PW6a	16/11/2015	Susceptible	Dorcas gazelle 2 PW6a	23/11/2015	Ampicillin, cephalosporins, chloramphenicol, neomycin, streptomycin
Somali Wild Ass JA20	30/11/2015	Doxycycline, streptomycin, tetracycline, trimethoprim sulphamethoxazole	Hartmanns Zebra ZR20	30/11/2015	Susceptible
Nyala PW4	23/11/2015	Susceptible	Nyala PW4	30/11/2015	Susceptible
Anoa 1 LIT3a	16/11/2015	Susceptible	Warthog ZR10	16/11/2015	Doxycycline, streptomycin, tetracycline, trimethoprim sulphamethoxazole

Only five isolates had reference ST strains which have previously been isolated from a variety of sources and locations (Table 9).

Table 9 Isolates with ST numbers

Zoo isolates		ST reference isolates		
Species	ST number	Species	Location	Pathology
Addax 1	2014	Horse	Hungary	Soft tissue infection
Bongo	6118	?	?	?
Sable	2715	Wallaby	Australia	None
Sitatunga	1204	Bovine	Egypt	Diarrhoea
Sitatunga	5500	?	?	?

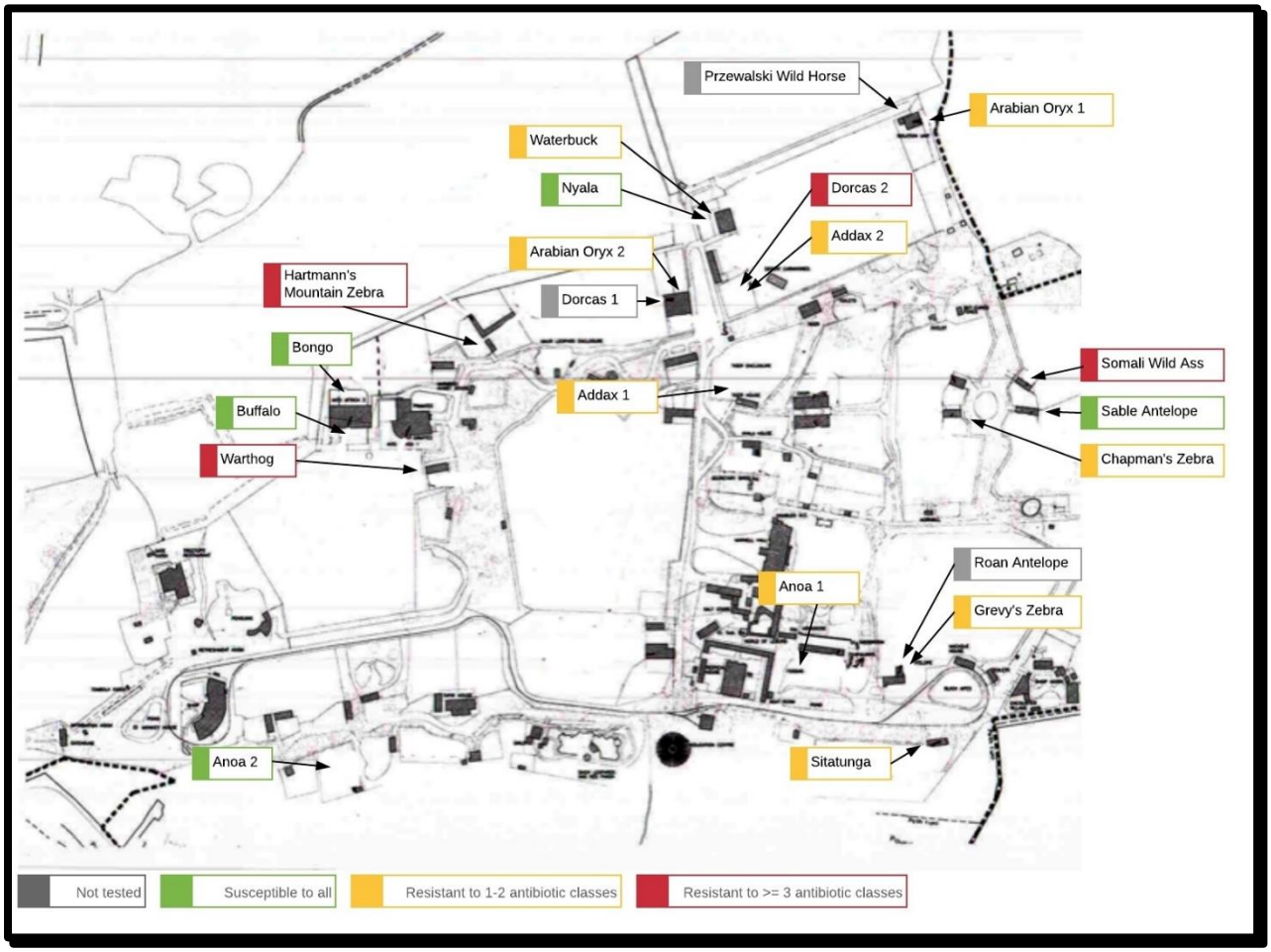

Figure 4 Map of zoo showing geographical locations and resistance phenotypes of *E. coli*

DISCUSSION

The objective of this study was intended to generate an overview of the types of resistant phenotypes present in commensal bacteria isolated from the faeces of healthy animals at Marwell Zoo. Pooled faecal samples were used in order to increase the probability of all individuals being represented in a sample. Contamination of the samples with extraneous environmental microflora was minimised by collecting samples from the concrete hard standing which is normally cleaned daily. Sampling each group three times improved the chances for each individual in the group being sampled. No antibiotics were administered to the target animals during the three weeks in which they were sampled.

Similar to many other surveillance studies in the United Kingdom (UK) and Europe, this study did not use media supplemented with antibiotics which favoured the isolation of the dominant bacterial strains present in the sample but did not select specifically for antibiotic resistant isolates (Borriello et al. 2014, EFSA 2008).

The LAMP techniques used in this project have been internally validated at the University of Surrey by using the LAMP method concurrently with conventional (biochemical and morphological) identification protocols. There were a few instances of mixed *E. faecium* and *E. faecalis* being identified by LAMP within an isolate which would indicate that the cultures tested were not pure in all instances. Ideally this should have been rectified by purifying the culture to obtain a single isolate. This was not done which affected the interpretation of two results where the mixed isolate showed resistance to imipenem. *E. faecium* is considered to be intrinsically resistant to carbapenems whereas *E. faecalis* is not, so interpretation for this antibiotic required knowing which species was being tested (Magiorakos et al. 2012). LAMP identification was only possible for *E. faecium* and *E. faecalis*, any other enterococci could only be identified up to genus level using the methods described.

To check whether similar resistance phenotypes occurred throughout the commensal microflora, four samples were cultured twice and antibiograms performed on the isolates. Most of these isolates expressed different resistance phenotypes which showed that the samples contained bacteria that were heterogenous in terms of resistance characteristics (Table 7). Schoster et al. 2012 had similar findings where some *E. coli* isolates from the same sample of horse faeces tested susceptible and some resistant. Testing more than one isolate from each sample may therefore have improved the odds of detecting resistance in these samples (Duse et al. 2016).

The *E. coli* isolates were tested against a panel of antibiotics similar to those used by the APHA for surveillance and antibiotic sensitivity testing of clinical isolates. This panel included a range of antibiotics that represented classes of antibiotics both relevant to veterinary and human medicine. The APHA panel was revised for *Enterococcus* spp. to include antibiotics with published reference values and imipenem and vancomycin were added to represent classes of antibiotics that appear on the WHO critically important drug list (Appendix A)(WHO 2012). Bacteria resistant to these critically important antibiotics (such as ESBL producing *E. coli* and vancomycin-resistant enterococci) are important indicators for monitoring the transfer of bacterial resistance between humans, domestic animals, wildlife and the environment (Radhouani et al. 2014). As there were no isolates with these indicator phenotypes it showed that the target animals were probably not colonised by these bacteria at the time of sampling.

Disk diffusion was the best option for the time and resources available for this study and allowed the screening of a larger number of samples than would have been possible using micro-dilution

methods. Micro-dilution methods are recommended in favour of disk diffusion for antibiotic sensitivity testing for *Enterococcus* spp. by the European Food Safety Authority (EFSA) because ECOFF values have not been defined for many of the antibiotics using the disk diffusion method (EFSA 2008). Minimum inhibitory concentrations (MIC) provide semi-quantitative information regarding the degree of resistance of an isolate whereas disk diffusion only defines whether it is resistant or susceptible. In some cases where there was no CLSI reference available for the antibiotic and bacterial species combination, an alternative source was used. This has been an issue with other studies (Borriello et al. 2014).

The most common antibiotics administered at the zoo were amoxicillin, trimethoprim sulphadiazine and tetracyclines (oxytetracycline and doxycycline). As expected, resistance of the *E. coli* isolates to these classes of antibiotics was relatively high (ampicillin 28.2%, trimethoprim sulphamethoxazole 17.9% and tetracycline 12.8%) compared with antibiotics that were administered less often. Streptomycin, spectinomycin and neomycin were rarely used yet resistance rates were quite high (17.9%) which may have been due to co-selection for resistance to aminoglycosides. The most common antibiotics that *E. coli* isolates were resistant to in this study are the same as those reported in AMR bacteria isolated from pigs and cattle in Europe (ampicillin, tetracycline, streptomycin, and sulphonamide) (EFSA and ECDC 2015) (Table 10).

Direct comparisons between studies should be interpreted cautiously because different methods and breakpoints may generate different conclusions. However if one looks at the basic trends of this study compared with data from similar human, domestic and wildlife studies it appears that our zoo population has produced a resistance profile that looks roughly like that of farmed cattle. This could be due to similar husbandry and antibiotic use in both contexts. Wild free-ranging ungulates had a much lower prevalence of AMR.

Table 10 Comparison of resistance rates of commensal *E. coli* isolated from humans and animals in UK and Europe

Data source	(Vinué et al. 2008)	UK VARSS 2013 (Borriello et al. 2014)	EUSR 2013 (EFSA and ECDC 2015)	Marwell Zoo	(Navarro-Gonzalez et al. 2013)
Species	Human	Pigs	Cattle	Zoo Ungulates	Wild Ungulates
Location	Spain	UK	Europe	UK	Spain
Method	CLSI	BSAC	EFSA	CLSI	EUCAST
Ampicillin	35	31	13.9	28.2	2.3-4.8
Cefotaxime	0	0.6	1.2	2.6	0-1.6
Sulphonamide	40.2/ 28 [tps]	52	20.2	17.9[tps]	1.1-6.3
Tetracycline	31	67	23.2	14.3	2.3-7.9
Chloramphenicol	7	22	8	0	0
Aminoglycoside	35 [s]; 2.13[g]	37[s]; 3[g]	17.6[s]; 2 [g]	17.9[s]	2.3-4.8[s]; 0 [g]
Fluoroquinolones	0 [c]	1.3[c]	5[c]	0[e]	0-7.1[c]
Imipenem	0	NT	NT	NT	0

[c] ciprofloxacin; [e] enrofloxacin; [g] gentamicin; [s]streptomycin; [tps] trimethoprim-sulfamethoxazole

NT = Not Tested

Although there are several definitions that are used to describe MDR, the European Centre for Disease Prevention and Control defines MDR as a bacteria being non-susceptible to at least one drug

in more than three antimicrobial classes that the species would normally be susceptible to (Magiorakos et al. 2012, Mathers, Peirano & Pitout 2015). In this study 6/39 (15%) of *E. coli* fulfilled that criterium as being MDR and the most common pattern of resistance included penicillins, sulphonamides, aminoglycosides and tetracyclines (Table 7). The prevalence and pattern of *E. coli* MDR appears similar to isolates cultured from cattle in Europe of which 20.8% qualified as being MDR using similar criteria and the most common MDR pattern was streptomycin, sulphonamides and tetracyclines (EFSA and ECDC 2015).

Mobile genetic elements carrying genes coding for resistance to multiple antibiotics (most commonly ampicillin, streptomycin, sulphonamides and tetracycline) may be responsible for these resistance combinations (de Jong et al. 2013). This could be why some of the animals in this study had bacteria resistant to antibiotics they had never been treated with. These genes can be maintained without any selection pressure from antibiotics, provided they do not reduce the fitness of the bacteria hosting them (Williams et al. 2011).

There was no evidence of ESBL production in the *E. coli* isolates. Although this is an unusual finding, the methods used were not as sensitive as other studies that supplemented their media with cephalosporins to select for ESBL-producing *E. coli* (Dobiasova et al. 2013). So the presence of ESBL producing *E. coli* cannot be ruled out without further effort to isolate them. ESBL-producing *E. coli* have emerged from human health settings and spread very rapidly to communities, wildlife, companion and food-producing animals (Wu et al. 2013). Although there is less selection pressure on this target population for ESBL-producing *E. coli*, there is still a chance they could have acquired this type of resistance from reservoirs such as humans and free-living wildlife (e.g. wild birds) (Guenther, Ewers & Wieler 2011, Wu et al. 2013). It would be evidence of good biosecurity measures if this population had not yet acquired this type of resistance (Guenther, Ewers & Wieler 2011).

Resistance to third and fourth generation cephalosporins and fluoroquinolones is of particular interest in AMR surveillance because they are very important second line antibiotics for treating resistant bacterial infections. There were low rates of cephalosporin resistance and no fluoroquinolone resistance amongst the *E. coli* isolates. Resistance to fluoroquinolones is facilitated either by plasmid or chromosomal genes (EFSA and ECDC 2015).

Chloramphenicol resistance is commonly found in the microflora from humans and animals despite it being rarely used in humans and banned in food producing animals (Table 10) (EFSA and ECDC 2015). This may be due to co-selection with associated resistance genes against other antimicrobials (EFSA 2008). Florfenicol is a similar compound to chloramphenicol which is used in animals (EFSA 2008). None of the *E. coli* isolates were resistant to chloramphenicol or florfenicol.

Enterococcus spp. are intrinsically resistant to many of the antibiotics that are commonly used in humans and animals. Susceptibility *in vitro* to cephalosporins, aminoglycosides and trimethoprim-sulphamethoxazole does not always predict efficacy *in vivo* in enterococcal infections so antibiotic susceptibility results may be misleading (CLSI M100-S25 2015). Efficacy of aminoglycosides used in combination with other drugs can be predicted by testing them using a high-level aminoglycoside screening test (CLSI M100-S25 2015, EFSA 2008). Detecting resistance over and above intrinsic levels was achieved in this study by using antibiotic disks with higher concentrations of streptomycin and gentamicin than would normally be used in antibiotic sensitivity tests. Only 2/55 (3.6%) of the *Enterococcus* isolates were resistant to streptomycin or gentamicin using this method. Resistance to cefpodoxime was extremely high (95%) which is expected because enterococci have reduced susceptibility to β-lactam antibiotics including cephalosporins (EFSA 2008).

Apart from cefpodoxime, the most common antibiotics that the *Enterococcus* spp. isolates were resistant to were ciprofloxacin (40%), erythromycin (67%) and tetracycline (33%). This pattern is similar to another study on wild rabbits, which found the most common antibiotics that enterococci isolates were resistant to were ciprofloxacin (14.1%), erythromycin (20.3%) and tetracycline (29.7%) (Silva et al. 2010). Normally *Enterococcus* spp. are considered to be good indicators of the effect that antibiotics have on the intestinal microflora of animals but in this study it did not show high resistance levels to the antibiotics most frequently used which included penicillins, tetracyclines and sulphonamides (EFSA 2008). This may be because antibiotics were rarely used and did not exert enough selection pressure to change the resistance phenotype at a herd level. Increased resistance to antibiotics that were rarely used may have been due to co-selection of these genes with others that code for resistance against antibiotics or environmental toxins (da Costa, Loureiro & Matos 2013, Williams et al. 2011).

MDR in the enterococci isolates was difficult to assess because none of the isolates fulfilled the criteria suggested by (Magiorakos et al. 2012) which defines MDR as resistance to three or more of the following antibiotic classes: streptomycin (high level), gentamicin (high level), imipenem (not in *E. faecium*), ciprofloxacin, vancomycin, ampicillin and tetracycline (Appendix B).

Vancomycin is a critically important drug for treating enterococcal infections which is why vancomycin resistance is regularly monitored by surveillance (EFSA 2008, WHO 2012). Historically avoparcin feed supplementation led to an increased prevalence of resistance to vancomycin in food-producing animals (van den Bogaard & Stobberingh 2000). There were no isolates resistant to vancomycin in this study and surveillance of livestock shows low levels of less than 1.6%.

Plotting the AMR phenotypes on a map of the zoo did not reveal any significant “clustering” of resistance. The absence of spatial association between resistance phenotypes suggests a good use of biosecurity to prevent the cross-contamination between animals in different enclosures and the humans that care for them. Wildlife such as birds or rodents could potentially transfer bacteria between enclosures but there was no evidence of this.

MLST was used to investigate the clonality of the *E. coli* isolates using the methods described by (Wirth et al. 2006). One of the primers (recA-P2) did not work and was replaced by an alternative primer from the MLST Database website at the University of Warwick. Of the four pairs of identical isolates, two were from unrelated species that were not living near each other. Feed could be a reservoir for one pair of clones as they were isolated from Hartmann’s zebra and Somali wild ass which are fed a similar diet. No obvious connections were found between the anoa and warthog as they were fed different diets. In both cases human sources were unlikely as the enclosures were serviced by different staff.

The isolates with ST numbers corresponded with bacteria previously isolated on three different continents which demonstrates the dissemination of bacteria globally. Of the four pairs of identical isolates, one pair had similar resistance phenotypes and three did not. Bacteria of the same ST would normally be expected to have similar resistance phenotypes because they are very similar genetically. However MLST does not take into account the DNA on mobile genetic elements. Different resistance phenotypes in identical bacteria could be caused by the addition of mobile genetic elements containing different genes coding for resistance (Freitag et al. 2016).

Zoo animals can be a reservoir of resistant bacteria however, the risk to visitors is very small because they do not have direct contact with the animals. Zoo staff could be subjected to colonisation with resistant bacteria from zoo animals and vice versa so personal protective wear is used to prevent the transfer of pathogens.

CONCLUSIONS

This project's main objective was to create an overview of the resistance phenotypes of commensal bacteria isolated from the faeces of ungulates living at Marwell Zoo. When the results were compared with the results of other studies, many similarities were found with domestic ungulates which are kept under similar conditions. Many acquired resistance types were not found (ESBL-production, vancomycin resistance, chloramphenicol resistance) which can either be because the target population was not exposed (e.g. traits not prevalent in local wildlife, environmental contamination absent, good biosecurity) or the traits were not maintained (antibiotic use too low to provide sufficient selection pressure, fitness cost to bacteria). Co-selection was a possible explanation for resistance against antibiotic classes that are not administered to this population. Horizontal transmission of mobile genetic elements may be a significant part of the epidemiology of antibiotic resistance.

There are some areas where this project could be expanded to investigate the results further. Mobile genetic elements are an integral part of the epidemiology of antibiotic resistance so it would be worthwhile investigating the isolates from this study using molecular techniques or conjugation assays. Since most resistance phenotypes have become ubiquitous, it was unusual that some resistance types were absent in this study. Further investigation using tests with higher sensitivity could improve the certainty of this finding (e.g. PCR detection of resistance genes in faecal samples or using antibiotic enriched media for the initial culture).

The data from this project can be used as a baseline to monitor the prevalence and phenotypes of antibiotic resistant bacteria, measure the effects of any interventions and guide future policies to minimise the development of AMR at Marwell Zoo. Periodic surveillance could be undertaken to monitor AMR in this population, to evaluate biosecurity and monitor the effects of antibiotic use. Monitoring AMR in indicator bacterial species in each herd could reveal how resistance genes are transferred around the zoo which could guide improvements in biosecurity measures (Radhouani et al. 2014). Where differences in prevalence exist they can be investigated to understand the mechanisms behind them.

Although there were good records of antibiotic use, no conclusions could be made as to how this affected AMR in treated individuals. By sampling the faeces from the entire group, the effects of antibiotic use were diluted by the members of the group who were not treated. Future investigations could investigate resistance phenotypes of individuals and identify any associations with antibiotic treatment. Collection of faecal samples from individuals could take place concurrently with other procedures or molecular methods could be used link faeces collected off the ground with the individuals that produced them.

Since other studies from zoos have shown the presence of resistance types that are not present in the population at Marwell it might be worth sampling imported animals while in quarantine to make sure they don't introduce new resistance phenotypes. Resistance in commensal bacteria can transfer to pathogenic bacteria which can lead to treatment failure. Since zoo animals are valuable both in monetary and conservation terms, protecting them from AMR is worth consideration.

APPENDICES

Appendix A

Table 11 WHO critically important antimicrobials list (WHO 2012)

Antibiotic class	Example
Aminoglycosides	Amikacin, gentamicin, streptomycin
Ansamycins	Rifampin
Carbapenems	Imipenem, meropenem
Cephalosporins, 3rd generation	Cefotaxime, cefpodoxime, ceftazidime
Cephalosporins, 4th generation	Cefepime, cefpirome, cefoselis
Lipopeptides	Daptomycin
glycopeptides	Teicoplanin, vancomycin
Macrolides	Azithromycin, erythromycin
Oxazolidinones	Linezolid
Penicillins, aminopenicillins	Ampicillin, amoxicillin clavulanate
Penicillins, natural	Penicillin G, penicillin V
Quinolones	Nalidixic acid, ciprofloxacin, enrofloxacin
Streptogramins	Quinupristin/ dalfopristin
Drugs used solely to treat tuberculosis	Cycloserine, ethambutol, ethionamide

Appendix B

Table 12 Antibiotic categories used to define multi-drug resistance (Magiorakos et al. 2012)

E. coli	Tested	*Enterococcus* spp	Tested
Aminoglycosides	Yes	Aminoglycosides (except streptomycin)	Yes
Anti-MRSA cephalosporins	No	Streptomycin	Yes
Antipseudomonal penicillins + ß-lactamase inhibitors	No	Carbapenems (*E. faecium*)	Yes
Carbapenems	No	Fluoroquinolones	Yes
Non-extended spectrum cephalosporins; 1st and 2nd generation cephalosporins	No	Glycopeptides	Yes
Extended-spectrum cephalosporins; 3rd and 4th generation cephalosporins	Yes	Glycylcyclines	No
Cephamycins	No	Lipopeptides	No
Fluoroquinolones	Yes	Oxazolidinones	No
Folate pathway inhibitors	Yes	Penicillins	Yes
Glycylcyclines	No	Streptogramins	No
Monobactams	No	Tetracycline	Yes
Penicillins	Yes		
Penicillins + ß-lactamase inhibitors	Yes		
Phenicols	Yes		
Phosphonic acids	No		
Polymyxins	No		
Tetracyclines	Yes		

Appendix C

Formula for converting rpm to xg: $g = (1.118 \times 10^{-5}) RS^2$ (Anonymous)

g = gravity

R = radius of centrifuge arm

S = speed in rpm

Table 13 Centrifuge forces defined as revolutions per minute (rpm) and relative centrifugal forces (xg)

Centrifuge	R	S	xg
Eppendorf	9.5cm	13 000	17949
Prism™ Mini Centrifuge	Depends on position of the tube in the strip (Figure 5)	6 000	~2 000 (Prism Mini Centrifuge User Manual*)

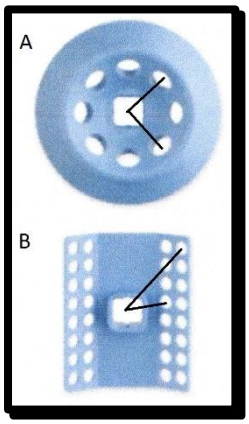

Figure 5 Comparison of rotors to spin individual tubes (A) vs strips of tubes (B).

All the tubes in (A) will have the same radius and therefore be subject to the same relative centrifugal force. The relative centrifugal force will differ in (B) depending on the position of the tube in the strip which affects the radius. Source: Prism Mini Centrifuge User Manual*

* http://northamerica.labnetinternational.com/sites/www.labnetinternational.com/files/product-documents/Prism%20Mini-C1801.pdf, accessed on 28/01/2017

Appendix D

ESBL + AmpC Screen Kit instructions for interpretation

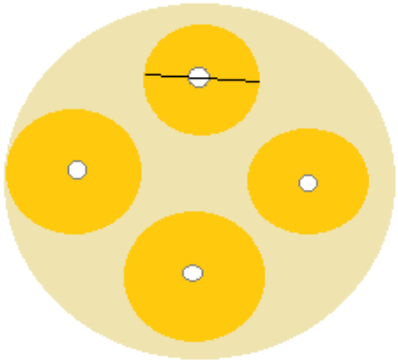

Figure 6 Drawing of an agar plate following incubation showing inhibition zones around each tablet. Diameter measurement shown of one inhibition zone.

Procedure for reading results (use Table 14):

1. After incubation compare the diameter of zone of inhibition around the cefoxatime 30µg and the inhibition zones around each of the other three tablets. If all zones are within 3mm of each other, then neither ESBL nor AmpC activity are present.
2. If B-A and/or D-C ≥ 5mm then sample is ESBL positive. Otherwise test is negative for ESBL activity.
3. If isolate negative for ESBL activity (in 1) then if C-A and/or D-B ≥ 5mm then AmpC positive. Otherwise negative for ESBL and AmpC
4. If isolate positive for ESBL activity (in 1) then if D-B ≥ 5mm and/ or B-A < 5mm then test is positive for AmpC (and for ESBL) activity.

Table 14 Interpretation of results

		A	B	C
ESBL only	B	≥ 5mm	-	-
	D	-	<5mm	≥ 5mm
AmpC only	C	≥ 5mm	-	-
	D	-	≥ 5mm	<5mm
ESBL + AmpC	B	<5mm (AmpC)	-	-
	D	-	≥ 5mm (AmpC)	≥ 5mm (ESBL)

A = cefotaxime; B = cefotaxime + clavulanate; C= cefotaxime + cloxacillin; D= cefotaxime + clavulanate + cloxacillin; "-" means the difference is irrelevant to the mechanism

Appendix E

Table 15 Manufacturers' details

Name	Address
GATC-Biotech AG	European Custom Sequencing Centre, Gottfried-Hagen-Strasse 20, 51105 Cologne, Germany
Eppendorf UK Ltd	Eppendorf House, Gateway 1000 Whittle Way, Arlington Business Park, Stevenage, SG1 2FP, United Kingdom
Labnet International, Inc	31 Mayfield Ave., Edison, NJ 08837, United States of America
Optigene Limited	Unit 5 Blatchfield Road, Horsham, West Sussex, RH13 5QR, United Kingdom
Oxoid Limited	Wade Road, Basingstoke, Hampshire, RG24 8PW, United Kingdom
Promega Corporation	2800 Woods Hollow Road, Madison, WI 53711-5399, United States of America
Qiagen Ltd UK	Skelton House, Lloyd Street North, Manchester, M15 6SH, United Kingdom
Rosco Diagnostica	Taastrupgaardsvej 30, DK-2630 Taastrup, Denmark
Sigma Aldrich Company Ltd.	The Old Brickyard, New Road, Gillingham, Dorset, SP8 4XT, United Kingdom
Thermofisher Microbiology	Wade Road, Basingstoke, Hampshire, RG24 8PW, United Kingdom

Appendix F

Table 15 Details of treatment administered to individuals during their lifetime and resistance phenotypes of the group

Species/ location	Resistance phenotypes		Details of individuals in group and antibiotic treatment during lifetime					
	E. coli	*Enterococcus* spp.	ID	M/F	DOB	Date	Antibiotic treatment	Reason
Arabian Oryx 1	AMP, SH	CFP, TET, ERY CFP; CIP, CFP, ERY, IMI;	8533	M	19/02/2004	26/11/2009	amoxicillin LA im	broken horn tip
Arabian Oryx 2	AMP, NEO, SH; SUSCEPTIBLE	CFP; CFP; CIP, CFP, TET, ERY	9282	F	25/03/2006	-		
			9283	F	06/06/2006			
			9325	F	3/11/2011	?	oxytetracycline	
			9486	F	7/8/2014	-		
			9421	F	29/03/2009	07/07/2014	TPS po sid 7d	
Addax 1	SUSCEPTIBLE; NEO, SH	No isolates	8012	M	22/07/2003	30/07/2013	TPS po sid 5d	lame
						23/08/2013	TPS po sid 3d	
			9425	M	17/03/2014	29/12/2015	amoxicillin po	
Addax 2	AMP; SUSCEPTIBLE	CIP, CFP, ERY; CIP, CFP, ERY	5058	F	21/06/2000	?	TPS, amoxicillin	
			5512	F	14/06/2001	-		
			6293	F	20/04/2003			
			6836	F	14/07/2004	-		
			9412	F	13/02/2014	21/02/2014	amoxicillin LA sc	neonatal issues
						23/02/2014	amoxicillin LA sc	neonatal issues
						24/02/2014	penicillin and streptomycin sc	neonatal issues
						25/02/2014	penicillin and streptomycin sc	neonatal issues
Anoa 1	CTX; SUSCEPTIBLE	CFP, ERY; CFP, TET	7984	M	09/08/2000	06/02/2008	oxytetracycline	lethargic and pyrexic

	SH; SUSCEPTIBLE					08/02/2008	oxytetracycline	lethargic and pyrexic
Anoa 2	SUSCEPTIBLE	CFP, TET; CFP	9287	M	01/07/2010			
			7985	F	02/11/1998	-		
Eastern bongo	SUSCEPTIBLE; SUSCEPTIBLE	CFP; CFP, ERY; CIP, CFP, ERY, IMI	8983	M	04/09/2006	18/12/2013	oxytetracycline LA im	
						23/12/2013	TPS po sid 10d	
			7371	F	24/02/2006	23/01/2013	amoxicillin LA im	lame
						25/01/2013	TPS po sid 18d	
			9524	F	26/12/2010	-		
			9522	F	08/02/2011			
Dwarf forest buffalo	SUSCEPTIBLE	CFP; CIP, CFP, TET, ERY; CFP, TET, ERY	6169	M	23/08/1999			
			1472	F	21/07/1990	14/06/2011	TPS sid 3d	
			3596	F	30/12/1996	20/02/2000	amoxicillin inj sid x5d	
			3638	F	26/03/1997			
			3960	F	11/02/1998			
			5616	F	29/08/2001	-		
			6835	F	13/07/2004	-		
Chapman's zebra	AMOX, TPS; SH, TPS; SUSCEPTIBLE	CIP, CFP, TPS, ERY; CFP; CIP, CFP, ERY; CIP, CFP, ERY	6908	M	16/06/19??	7/12/2012	TPS sid 10d	
						24/11/2012	TPS sid 8d	
						6/3/2012	TPS sid 6d	
						16/2/2011	TPS sid 5d	
			3976	F	11/03/1998	05/11/2015	TPS sid 10d	
						15/10/2015	TPS bid 6d	
						11/08/2014	TPS sid 10d	
						30/06/2014	TPS sid 10d	
						12/09/2007	TPS sid 14d	Hoof abscess
						12/09/2007	Penicillin im	Hoof abscess
						24/03/1998	TPS im sid 7d	

			5691	F	2/11/2001	25/04/2002	Amoxicillin LA im	
			6505	F	05/09/2003	-		
			8500	F	13/07/2009	24/02/2012	Enrofloxacin	
Dorcas 1 (Nakheila gazelle)	No isolates	CFP; CIP, CFP, ERY; CFP, ERY	9080	M	20/10/2004	17/04/2013	doxycycline po bid 10d	
						29/04/2013	amoxicillin LA	
						14/06/2013	amoxicillin LA	
						02/07/2013	oxytetracycline LA	abscess
						25/07/2013	ceftiofur inj sc sid 5d	
						12/05/2014	oxytetracycline LA	swelling
						16/05/2014	doxycycline po sid 5d	
						16/07/2014	doxycycline po sid 7d	
			9202	M	29/02/2012	24/03/2015	doxycycline po bid 7d	
			9204	M	04/03/2012	16/07/2012	oxytetracycline LA	abscess
			9372	M	08/09/2013	-		
Dorcas 2	SUSCEPTIBLE; AMP, CFP, CAZ, NEO, SH, S	CFP, ERY; SUSCEPTIBLE	8252	F	08/11/2008	19/12/2009	amoxicillin LA	leg fracture
						17/2/2010	amoxicillin LA	horn stick injury
						31/3/2010	amoxicillin LA	lame
			8384	F	07/05/2009			
			8644	F	22/04/2010			
			9371	F	20/09/2013			
Grevy's zebra	AK, SH; SUSCEPTIBLE	CIP, CFP, TET, ERY; CFP; CIP, CFP, TET, ERY	9462	M	17/07/1995	03/03/3015	TPS po sid 5d	
Hartmann's mountain zebra	AMP, DOX, S, TET, TPS; AMOX, AMP, DOX, S, TET, TPS; SUSCEPTIBLE	CFP; CFP; CIP, CFP, TPS, ERY	9221	M	14/07/2007	12/04/2015	TPS po sid 7d	
			8594	F	16/06/2006	25/09/2013	TPS sid 7d	
						06/10/2013	TPS sid 5d	
						14/10/2013	TPS sid 5d	

						06/08/2014	TPS sid 5d	
						23/11/2014	TPS sid 4d	
			8014	F	08/08/2006	28/07/2012	TPS sid 5d	
						19/08/2013	TPS sid 3d	
						27/08/2013	TPS sid 5d	
			9568	F	13/07/2008			
Nyala	SUSCEPTIBLE; SUSCEPTIBLE; SUSCEPTIBLE	TPS, ERY; CFP, TET, ERY; TET, ERY	8800	M	03/08/2010	?	amox	
			8832	M	19/08/2010	-		
			6086	F	03/09/2002	01/06/2011	doxycycline sid inj x5d	lame
						09/06/2011	amoxicillin la inj	wound
			6108	F	14/09/2002	13/11/2013	oxytetracycline LA im	
						25/3/2014	oxytetracycline LA im	
						24/4/2015	TPS sid 21d	
			7403	F	12/04/2006	5/12/2013	doxycycline po bid x5d	
			7547	F	08/09/2006	19/07/2008	lincocin inj sid 7d	swollen jaw
						18/08/2008	amoxicillin la inj	wound
						05/12/2011	enrofloxacin sid 5d	conjunctivitis
						15/07/2013	TPS sid 10d	
						06/08/2013	doxycycline bid 12d	eye infection
						17/09/2013	oxytetracycline LA im	eye infection
						12/11/2015	inj with la antibiotics	corneal ulcer
			7784	F	29/05/2007	05/02/2013	oxytetracycline LA im	
						10/02/2013	oxytetracycline LA im	
						18/02/2013	oxytetracycline LA im	
			8730	F	19/07/2010	28/10/2013	TPS po sid 10d	
						24/8/2015	Enrofloxacin	
Przewalski's wild horse	No isolates	CIP, CFP, ERY; CIP, CFP, ERY	7895	M	07/04/1994	22/12/2011	TPS 5d	castration and hoof trim

						24/8/2015	TPS 10d	
			528	F		-		
			529	F	11/07/1986	18/07/2010	enrofloxacin	corneal ulcer
						19/07/2010	Oxytetracycline LA subconjunctival	corneal ulcer
						21/07/2010	Oxytetracycline LA subconjunctival	corneal ulcer
						21/07/2010	enrofloxacin sid 6d inj/ oral	corneal ulcer/ enucleation
						31/07/2010	enrofloxacin sid 14d	corneal ulcer/ enucleation
						25/07/2012	TPS sid x 3d	
			4721	F	31/08/1999	27/09/2000	Oxytetracycline subconjunctival	
			5018	F	26/05/2000	-		
			8157	F	13/07/2008	-		
Roan antelope	No isolates	CIP, CFP, ERY; CIP, CFP, TET, ERY; CIP, CFP	8403	F	22/05/2009	30/09/2011	oxytetracycline LA im	peritonitis from horn stick injury
			9495	M	18/03/2013			
			8511	F	28/07/2009			
			8799	F	22/07/2010			
Sable antelope	SUSCEPTIBLE; SUSCEPTIBLE	CIP, CFP, ERY; CIP, CFP, ERY; CFP	3271	F	23/05/1996	2/11/2002	TPS sid 10d	
						12/11/2002	oxytetracycline subconjunctivally	
						2/8/2012	amoxicillin LA im	horn removal
			3822	F	21/07/1997	08/09/2000	amoxicillin LA im	
			7821	F	08/06/2007	-		
			8523	F	12/08/2009	-		
			8666	F	22/05/2010	8/10/2013	oxytetracycline inj	
			8706	F	25/06/2010	-		
Sitatunga	SUSCEPTIBLE;	CFP, ERY;	7136	F	19/06/2005	31/12/2007	TPS bid 15d	

	AMP, SH; AMP	S, CFP, TET, ERY; CFP, ERY; S, CFP, TET, ERY				1/4/2013	doxycycline sid 5d	
						2/2/2015	TPS po sid 5d	
			7385	F	02/04/2006	31/12/2007	TPS bid 15d	
						7/5/2010	doxycycline po bid x 5d	
						12/5/2010	oxytetracycline LA im	lame/ purulent vulval discharge
						15/5/2010	doxycycline po bid x 2d	lame/ purulent vulval discharge
						10/8/2010	doxycycline po bid x 6d	
			7951	F	30/09/2007	31/12/2007	TPS bid 15d	
						1/10/2007	amoxicillin	neonatal
			8011	F	17/02/2008	-		
			8293	F	11/02/2009	12/02/2009	amoxicillin im	
Somali Wild Ass	AMOX, AMP, DOX, S, TET, TPS; AMOX, AMP, DOX, S, TET, TPS	CFP, TET; CIP, CFP, TET, ERY	2809	F	18/08/1989	05/06/1995	neomycin	
						28/12/1999	amoxicillin LA	
						31/12/1999	amoxicillin LA	
						14/06/2000	TPS bid 5d	
						07/04/2014	TPS sid 10d	
						20/10/2014	TPS sid 10d	
						31/1/2015	TPS sid 10d	
						26/2/2015	TPS sid 62d	
			4615	F	08/10/1997	26/08/1999	TPS po bid 5d	
						14/06/2000	TPS bid 5d	
						19/07/2009	amoxicillin LA	hoof abscess
						20/07/2009	TPS bid 5d	hoof abscess
						24/07/2009	amoxicillin LA	
						09/05/2011	TPS sid 5d	
						18/05/2011	TPS sid 10d	

			7928	F	06/09/2007	19/10/2015	TPS sid 10d	
						31/10/2015	doxycycline bid	
						24/11/2015	doxycycline po bid 21d	
			7931	F	25/09/2007	20/11/2013	TPS sid 5d	
						8/7/2013	TPS sid 6d	
Warthog	AMP, DOX, NEO, S, TET, TPS; SUSCEPTIBLE	CFP, TET, ERY	9653	F	09/04/2010	-		
Common waterbuck	NEO; SUSCEPTIBLE	ERY; CFP; CIP, CFP, TET, ERY	8582	M	17/11/2009	16/03/2011	amoxicillin LA	castration
			8621	M	05/02/2010	15/03/2011	amoxicillin LA	castration
			4677	F	03/08/1999	23/01/2000	TPS sid 7d	
						31/01/2000	amoxicillin LA inj eod x2 4d	
						26/07/2013	TPS sid 5d	swollen nose and lip
			4772	F	08/10/1999	23/01/2000	TPS sid 7d	
						31/01/2000	amoxicillin LA inj eod x2 4d	
			5731	F	17/01/2002	-		
			6082	F	29/08/2002	-		
			7189	F	10/08/2005	-		

DOB date of birth; M male; F female; AMP ampicillin; AMOX amoxicillin clavulanic acid; AK amikacin; CAZ ceftazidime; CFP cefpodoxime; CIP ciprofloxacin; CTX cefotaxime; DOX doxycycline; ENR enrofloxacin, ERY erythromycin; IMI imipenem; NEO neomycin, S streptomycin, SH spectinomycin; TET tetracycline; TPS trimethoprim sulpha

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Animal Ethics Committee

PROJECT TITLE	**nvestigation of antimicrobial resistance in Escherichia coli and Enterococcus spp. isolated from zoo animals**
PROJECT NUMBER	**V059-15**
RESEARCHER/PRINCIPAL INVESTIGATOR	**Dr. M-A Frank**

STUDENT NUMBER (where applicable)	**UP_99115931**
DISSERTATION/THESIS SUBMITTED FOR	**MSc**

ANIMAL SPECIES	4 Somali Wild Ass (Equus africanus somalicus) 5 Chapman's Zebra (Equus burchelli chapmanni) 7 Przewalski's Wild Horse (Equus caballus przewalskii) 6 Grevy's Zebra (Equus grevyi) Upload flow charts or any related data for the experimental design here: Upload 4 Hartmann's Mountain Zebra (Equus zebra hartmannae) 1 Warthog (Phacochoerus africanus)	9 Dorcas Gazelle (Gazella Dorcas) 9 Nyala (Tragelaphus angasii) 2 Bongo (Tragelaphus eurycerus isaaci) 6 Sitatunga (Tragelaphus spekii gratus) 7 Addax (Addax nasomaculatus) 4 Roan Antelope (Hippotragus equinus) 7 Sable Antelope (Hippotragus niger) 25 Scimitar-horned Oryx (Oryx dammah) 7 Arabian Oryx (Oryx leucoryx) 10 Common Waterbuck (Kobus ellipsiprymnus) 7 Dwarf Forest Buffalo (Syncerus caffer nanus) 4 Lowland Anoa (Bubalus depressicornis)
NUMBER OF ANIMALS	**To be reported**	**To be reported**
Approval period to use animals for research/testing purposes		**September 2015 – September 2016**
SUPERVISOR	**Prof. M van Vuuren**	

KINDLY NOTE:

Should there be a change in the species or number of animal/s required, or the experimental procedure/s - please submit an amendment form to the UP Animal Ethics Committee for approval before commencing with the experiment

APPROVED	Date	9 September 2015
CHAIRMAN: UP Animal Ethics Committee	Signature	

Certificate of Approval by the Animal Ethics Committee, University of Pretoria

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