MASSEY UNIVERSITY – INSTITUTE OF VETERINARY, ANIMAL AND BIOMEDICAL SCIENCES

Antimicrobial Resistance in Young New Zealand Horses

A thesis submitted in fulfilment of the requirements for the degree of a Master of Veterinary Science

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Abstract

The emergence of bacteria that are resistant to antibiotics used in horses has been reported worldwide, including in Australia, the USA and United Kingdom. There is a lack of published comparative scientific information on the New Zealand equine population. However, recent individual cases of multi-drug resistant (MDR) bacterial infections reported by veterinarians have raised concerns about the situation in New Zealand. The use of antimicrobials coupled with less than ideal prescription practice in the horse industry may have led to inadvertent selection for MDR bacteria. An initial perspective on antimicrobial resistance in NZ is gleaned through a retrospective description of laboratory submissions in the form of a database analysis from 2004 to 2013/2014. In neonates (foals less than three weeks of age), the presence of MDR bacteria was identified in 37.5% (24/64) of foals; although 81.6% (102/125) of bacteria cultured from foals included in the study were sensitive to either penicillin or gentamicin. Of the respiratory samples from horses three-years-old and younger, the most commonly cultured bacteria were *Staphylococcus* species accounting for 40.1% (310/774) of isolates. These bacteria were sensitive to penicillin, ceftiofur and gentamicin for > 90% of isolates. Of all respiratory equine submissions, MDR bacteria were recovered from 39.2% (93/237) of horses. Using multiple correspondence analysis, MDR was associated with submissions from 2009-2014 and two-year-old horses from the Waikato region.

These two population groups were targeted specifically for examination due to the potentially severe consequences of bacterial disease in neonates (**Chapter 3**), and the anecdotal experience of high clinical use of antimicrobials in young horses, especially in the treatment of clinically apparent respiratory disease (**Chapter 4**). Multi-drug resistance was assessed as part of this work, and is presented in the systematic literature review (**Chapter 2**), and in both descriptive studies (**Chapters 3** and **4**). The results of the two retrospective descriptive studies presented show that there is a substantial proportion of submissions from young horses in New Zealand that grow multi-resistant bacterial isolates, and that there is decreased efficacy of commercially available antimicrobials in this country. The antimicrobial resistance reported in this study has potential clinical implications, and reflects the first step in a multifactorial approach to improve and maintain horse and human health.

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And of course, thank you to my mum!

Abbreviations

ACVIM American college of veterinary internal medicine
AMR Antimicrobial resistance
CC Clonal complex
CI Confidence interval
CIA Critically important antimicrobial
ESBL Extended-spectrum beta-lactamase
OR Odds ratio
MCA Multiple correspondence analysis
MDR Multi-drug resistance
MIC Minimum inhibitory concentration
MLST Multi-locus sequence typing
MPI Ministry for primary industries
MRCoNS Methicillin resistant coagulase-negative *Staphylococcus* species
MRS Methicillin resistant *Staphylococcus* species
MRSA Methicillin resistant *Staphylococcus aureus*
NZ New Zealand
OIE World organiation for animal health (*office international des epizooties*)
PCR Polymerase-chain reaction
PFGE Pulse-field gel electrophoresis
PMQR Plasmid-mediated quinolone resistance
PRISMA Preferred reporting items for systematic reviews and meta-analysis
SVARM Swedish veterinary antimicrobial resistance monitoring
TMPS Trimethoprim-sulfonamide combination (i.e. trimethoprim-sulfamethoxazole, trimethoprim-sulphadiazine)
VRE Vancomycin resistant *Enterococcus*
WHO World health organisation

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1 Introduction

1.1 Overview

Along with the expansion of the global industrial community, the pressures that drive antimicrobial resistance (AMR) also increase (Levy and Marshall 2004). This is attributable to both human and animal use, and the responsibilities fall to all parties involved (Prescott 2014). Veterinary use of antimicrobials, especially antibiotics, has been subject to recent scrutiny across the world (de Jong *et al.* 2013; Liebana *et al.* 2013). Efforts have been made to curtail the use and promote the responsible stewardship of antimicrobial agents in veterinary practice (Teale and Moulin 2012; Prescott 2014). Equine practitioners have moved to improve prescribing practices (Bowen 2013), and this includes the provision of standards that aim to guide equine clinicians to make informed choices about the use of antimicrobials (Anonymous 2012a).

Antimicrobial (specifically antibacterial or antibiotic) resistance occurs for a number of reasons that are intrinsically associated with cellular biology (e.g. bacterial production of antibiotic break-down enzymes, altered drug transport mechanisms, and alteration of drug targets in the bacteria) (Levy and Marshall 2004). The reporting of resistance, and multi-resistance, quickly followed the clinical use of antimicrobials in humans, especially within hospital-based populations (Barber 1961). Community associated multi-resistant bacterial infections are an increasing concern in human healthcare, and where enteric bacteria are concerned, pose a veterinary public health issue as well (Levy and Marshall 2004; Lazarus *et al.* 2014).

Antimicrobial use has the potential to select for AMR pathogenic and commensal bacterial flora (Maddox *et al.* 2011). The bacteria contained within the microbiota of an animal's skin and mucosa (including the gastrointestinal tract) provide as a reservoir for horizontally-transmissible AMR genetics (Dunowska *et al.* 2006; Ahmed *et al.* 2012b; Damborg *et al.* 2012; Johns *et al.* 2012). Therefore, it is important that antimicrobial resistance is monitored and good stewardship principles are advocated and maintained (Prescott 2014). An example of monitoring resistance in veterinary species at a national level is the Swedish Veterinary Antimicrobial Resistance Monitoring (SVARM) program in Sweden (Bengtsson *et al.* 2012). The generation of guidelines for appropriate treatment of bacterial infections in animals, and specifically with respect to the findings of this thesis, horses, is an essential part of that stewardship (Morley *et al.* 2005).

1.2 Motivation for this study: a clinical perspective

Omphalitis, omphalophlebitis and septic arthritis are relatively common sequelae to neonatal sepsis in foals (Palmer 2014). The routine treatment of foals with broad spectrum antimicrobials is common (Wohlfender *et al.* 2009), and potentially lifesaving for the compromised neonate (Palmer 2014). Potential risks associated with unnecessary antimicrobial treatment are highlighted in the case described below, where two multi-drug resistant (MDR) bacteria were cultured from an animal bred on a farm that had previous cases of MDR infection (Herdan *et al.* 2012).

The first sick foal presented to the author during her equine internship at Massey University Veterinary Teaching Hospital, a one-day-old Thoroughbred colt, was admitted for evaluation in the spring of 2013. The foal showed signs of systemic sepsis (Palmer 2014) and neonatal maladjustment syndrome (Bernard 2003), and been delivered by a dam that required assistance during foaling. Broad-spectrum parenteral antimicrobials (sodium penicillin and gentamicin sulphate) were administered and intensive supportive care commenced. The foal showed clinical improvement over the following days. After a week of hospitalisation, the foal developed ultrasonographic and physical signs of omphalitis (Reimer 1993), and corresponding increasing concentrations of serum amyloid-A (an acute phase protein used as a marker of inflammation or infection). The umbilical vessels and urachus were monitored ultrasonographically over the succeeding days. Antimicrobial treatment was also changed to ceftiofur, as the probable infection had developed in the face of broad-spectrum antimicrobial therapy. Ten days after admission into the hospital, the colt had developed effusion in a tarsocrural (hock) joint and was taken to surgery for umbilical resection and joint lavage. There was no external injury to the hock and therefore it was most likely sepsis of haematogenous origin, although no sample was cultured for bacterial growth. The joint was treated with a high concentration of intra-articular gentamicin sulphate at the time of lavage. An aseptic sample from the umbilicus was submitted for both aerobic and anaerobic culture and sensitivity; two bacterial isolates were cultured as a result. These were an *Escherichia coli* that was resistant to 5 of 6 tested antimicrobials, and an *Enterococcus* species that was resistant to 6 of 6 tested antimicrobials (including enrofloxacin and ceftiofur). Subsequent culture and sensitivity revealed that the *Enterococcus* isolate was sensitive to vancomycin; however this was not used in the treatment of the foal. Without the use of an effective antimicrobial, and in the face of pre-surgical infection with two MDR bacteria, the foal

improved and was discharged from hospital. He continued to do well and was weaned along with his cohort of foals in the autumn of 2014.

Whilst reviewing the published literature on the treatment of septic foals, it became clear that there was regional variation in bacterial susceptibility patterns (Marsh and Palmer 2001; Wilson 2001; Clark *et al.* 2008; Russell *et al.* 2008). There was no comparable information regarding the situation in NZ, and reading the literature for this case showed a deficit in published NZ data. Increasing AMR is suspected on NZ farms, and it was known to the author that previous animals on the farm had been infected with MDR bacteria; although published information was limited to a case-series involving one of these horses (Herdan *et al.* 2012). I believe it is one of the fundamental tenants of the profession to not only look after the animals we treat during our working day, but also to preserve our ability to treat future patients. Therefore, the overarching aim of this thesis is assimilate data that may begin to address this deficit and to help the NZ equine industry to develop stewardship protocols to guide veterinary clinicians in the rational use of antimicrobials.

1.3 A brief review of antimicrobial resistance in horse populations

Antimicrobial (more specifically, antibiotic) susceptibility of bacterial isolates from horses (*ex vivo* or *in vitro*) has been reported in scientific literature since the 1960's (Moreno *et al.* 1968). Additionally, using antimicrobial susceptibility patterns as part of a rational approach to the treatment of horses has been advocated for more than 35 years (Knight and Heitala 1979). This chapter aims to be a brief review of the literature pertaining to antimicrobial (antibacterial) sensitivity and resistance of bacteria cultured from horses. Knight and Heitala (1979) were the first to quantify both pathogenic and non-pathogenic bacteria (isolated from horses) by the frequency of culture, and antimicrobial sensitivities using the Kirby-Bauer disk diffusion method (Bauer *et al.* 1966). In this chapter, and throughout the thesis, "sensitivity" and "resistance" will be used interchangeably in a reciprocal fashion, where appropriate or best fits the situation; "antimicrobial" and "antibiotic" are interchanged in a similar manner, although the more appropriate term is "antimicrobial".

1.3.1 General results of equine culture and sensitivity

There has historically been an emphasis on sensitivity profiles of bacteria from specific anatomic locations, such as the uterus (Misra 1971; Davis *et al.* 2013), or eye (Whitley *et*

al. 1983; Keller and Hendrix 2005), or musculoskeletal system (Snyder *et al.* 1987; Moore *et al.* 1992). Foals as a specifc subpopulation have also been the subject to research, as both bacterial culture and sensitivity results are of clinical importance (Marsh and Palmer 2001; Russell *et al.* 2008; Theelen *et al.* 2014a; Theelen *et al.* 2014b). Comparisons between antimicrobial sensitivities from different geographic locations are difficult, as standard limits change with time and method of assessment (Hodgson *et al.* 2008). Within the studies done of clinical isolates in equine populations, both MIC (minimum inhibitory concentration) (Wilson 2001; Theelen *et al.* 2014a) and Kirby-Bauer disk diffusion methods (Clark *et al.* 2008; Russell *et al.* 2008) have been used.

A summary of selected antimicrobial sensitivity patterns of bacteria cultured from horses is outlined in **Table 1.1.** However, even within the confines of similar methodology and geographic location (Canada), direct comparisons between the studies published by Giguère *et al.* (2013), Clark *et al.* (2008) and Prescott *et al.* (1984**)** should be made with caution. This is due to probable differences in testing methodology such as laboratory operational protocols (Hodgson *et al.* 2008), and potential laboratory errors (Feary *et al.* 2005), or epidemiologic factors such as geographic location or source population (Marsh and Palmer 2001; Clark *et al.* 2008).

Table 1.1 Selected published antimicrobial sensitivities of bacteria cultured from horses

Author (Year)	Region (Study period)	Bacteria	Antimicrobial Sensitivities (%)	Study; denominator (n)
Prescott *et al.* (1984)	Canada	*S. aureus*	96% sensitivity to TMPS; 100% sensitivity to cephalothin	Clinical; 48 isolates
		S .zooepidemicus	27% sensitivity to TMPS; 100% sensitivity to cephalothin	Clinical; 144 isolates
		E coli	83% sensitivity to TMPS; 77% sensitivity to cephalothin	Clinical; 47 isolates
Wilson (2001)	California, U.S.A. (1998)	*S. aureus*	30% sensitivity to penicillin G; 69% sensitivity to ceftiofur; 55% sensitivity to TMPS; 48% sensitivity to gentamicin	Clinical; 33 isolates
		S. zooepidemicus	100% sensitivity to penicillin G, ceftiofur, TMPS; 7% sensitivity to gentamicin	Clinical; 14 isolates
		E. coli	94% sensitivity to ceftiofur; 60% sensitivity to TMPS; 86% sensitivity to gentamicin	Clinical; 74 isolates
		Salmonella	100% sensitivity to ceftiofur; 73% sensitivity to tetracycline; 34% sensitivity to TMPS	Clinical; 18 isolates
Clark *et al.* (2008)	Western Canada (1998-2003)	*S. aureus*	97% sensitivity to ceftiofur; 100% sensitivity to gentamicin and TMPS; 97% sensitivity to tetracycline and enrofloxacin; 55% sensitivity to penicillin	Clinical; 36 isolates
		S. zooepidemicus	99% sensitivity to ceftiofur; >90% sensitivity to enrofloxacin and penicillin; 85% sensitivity to gentamicin; 55% sensitivity to TMPS	Clinical; 221 isolates
		E. coli	94% sensitivity to ceftiofur; 91% sensitivity to enrofloxacin; 80% sensitivity to gentamicin; 62% sensitivity to TMPS	Clinical; 82 isolates
		Enterococcus spp	29% sensitivity to ceftiofur; 46% sensitivity to enrofloxacin; 86% sensitivity to penicillin; 68% sensitivity to TMPS	Clinical; 28 isolates
Goncagul and Intas (2013)	Germany (2006-2008)	*Streptococcuc* (β-haemolytic)	100% sensitivity to penicillin, ceftiofur and TMPS; 85% sensitivity to gentamicin; 82% sensitivity to tetracycline	Uterine; 51 isolates
		E. coli	100% sensitivity to ceftiofur, TMPS, and tetracycline	Uterine; 73 isolates
Giguère *et al.* (2013)	Canada (2005-2012)	*S. aureus*	57% sensitivity to ceftiofur; >80% sensitivity to TMPS	Unknown; 221 isolates
		S. zooepidemicus	>99% sensitivity to ceftiofur; TMPS	Unknown; 758 isolates
		E. coli	92% sensitivity to ceftiofur; 53% sensitivity to TMPS	Unknown; 362 isolates
		Salmonella	<25% sensitivity to TMPS	Unknown; 185 isolates

Abbreviations: *S.aureus: Staphylococcus aureus S.zooepidemicus*: *Streptococcus equi* ssp. *zooepidemicus E coli: Escherichia coli Salmonella Salmonella enterica* (various serovars); TMPS: trimethoprim-sulfonamide antimcrobial

In Clark *et al.* (2008), the predominant culture results reported were *Streptococcus equi* ssp. *zooepidemicus* and *E. coli* from which sensitivities were reported. Susceptibility to ceftiofur was found for >90% of isolates of both species of bacteria, and for between 50 and 70% of isolates for TMPS and tetracycline; 95% of *S. zooepidemicus* isolates were sensitive to penicillin (Clark *et al.* 2008). This study described a larger number of participants/isolates than the previous study by Prescott *et al.* (1984) and was conducted 20 years later, however no convincing evidence of increased AMR was presented, although it was suspected.

In a conference proceedings that relied upon limited clinical sensitivity information, Wilson (2001) outlined important principles of antimicrobial use, including the necessity of bacterial disease to be involved and that the antimicrobial agent used must be effective. These findings were based on the retrospective knowledge of the results of antimicrobial culture and sensitivity testing, and from this rational empirical recommendations were made (Wilson 2001). For the treatment of pneumonia in horses, the recommended first choice antibiotic was penicillin, ceftiofur or trimethoprim-sulfonamide (TMPS); and for pleuropneumonia penicillin/ampicillin plus gentamicin with or without metronidazole. For the treatment of a septic foals, amikacin plus ampicillin were recommended (Wilson 2001). These recommendations were made on the basis of likely causes of sepsis, and region-specific clinical knowledge of treatment success and culture results. This rationalisation for treatment contrasts with a long-term (30-years) study where both culture and sensitivity results were used to make recommendations for empirical treatment of foals based on described bacterial causes of sepsis [Theelen *et al.* (2014a) and Theelen *et al.* (2014b)], which suggested a beta-lactam and aminoglycoside combination, or more explicitly ampicillin and amikacin respectively. Changes in sensitivity patterns were also recognised over the studied time-period, with both increases and decreases in susceptibility of certain isolates to antimicrobials noted (Theelen *et al.* 2014a).

The sensitivity results reported by Goncagul and Intas (2013), from a European horse population, were higher (i.e. the percentage of bacterial isolates that were sensitive was higher) than those seen in North American horses (Clark *et al.* 2008). Therefore sensitivity results are likely to vary with time and place.The authors in Goncagul and Intas (2013) made recommendations for for veterinarins to continue to monitor culture

and sensitivity results. Additionally, a statistically significant difference in sensitivity patterns has been found between horse, cattle and companion animal isolates to various antimicrobial agents (Prescott *et al.* 1984), indicating variation in AMR among species. The extent of this inter-species variation is not within the scope of this thesis.

Mercer (1979) described the potential effects of MDR bacterial infections of *Salmonella* in horses. A number of strains of bacteria isolated from food animals were resistant to more than one antimicrobial. In this study it was noted that 91% of *Salmonella* had "transferable factors" (if they were resistant to at least one antimicrobial) (Mercer 1979), demonstrating at least the presence of clinically relevant AMR (and likely MDR) in veterinary species. With molecular advances in microbiology, transferrable resistance factors have been described (Levy and Marshall 2004). Some of these include integrons, plasmids and other mobile genetic elements that contribute to the horizontal transfer of AMR (or MDR) that have subsequently been investigated in equine Enterobacteriaceae isolates (Seiffert *et al.* 2013; Ewers *et al.* 2014a; Ewers *et al.* 2014b; Schmiedel *et al.* 2014).

Multi-drug resistance is most commonly described phenotypically as resistance to either three-or more classes of antimicrobials (Beard 2010), or four or more classes (Johns *et al.* 2012). It is likely that the incidence of MDR bacterial infections will become more common in the future (Beard 2010; Herdan *et al.* 2012). The systematic literature review in **Chapter 2** aims to more completely address the published literature on equine multi-resistant bacterial infections than will be done in this Chapter (**Chapter 1**). The review aims to describe the way information has been collected and presented on the subject of multi-resistant bacterial infections in horses.

1.3.2 Antimicrobial use and stewardship

Antimicrobial stewardship is an increasingly important aspect of the management of antimicrobial use in the veterinary community. A consensus statement made by the American College of Veterinary Internal Medicine (ACVIM) by Morley *et al.* (2005) advocates the use of guidelines in practice, and the development of practice-based protocols. This also includes the categories of antimicrobials selected, and the practice of reserving secondary antimicrobials for use only after culture and sensitivity results necessitate such use (Morley *et al.* 2005). Antibiotics that should be reserved for secondary or tertiary use are those listed as "critically important" by the WHO and OIE

(Orand 2012). Critically important antimicrobials (CIA) are those that are used to either treat serious human infections that have limited therapeutic options, or are used to treat zoonotic infections in people or may select for resistance genes in zoonotic (or transmissible) infections (Anonymous 2012b, 2014b). This list (as of 2012) includes high priority CIAs that meet additional criteria such as 3rd and 4th generation cephalosporins (including ceftiofur), fluroquinolones (e.g. enrofloxacin and marbofloxacin), glycopeptides (e.g. vancomycin), and macrolides (e.g. azithromycin and erythromycin) (Anonymous 2012b). Included in the list of CIAs were penicillin G, gentamicin and rifampicin; oxytetracyline and tetracycline, potentiated sulfonamides (TMPS) were listed as highly important (Anonymous 2012b) placing most antibiotics apparently used in NZ horses (Anonymous 2013) into these two categories.

The responsible use of antimicrobials has been raised in the context of equine veterinary treatment by Hollis and Wilkins (2009), where antimicrobial selection was listed as a "current controversy". Recommendation for neonatal use included advocating the use of broad spectrum antimicrobials in critically ill foals (Corley and Hollis 2009), although not necessarily those of extended-spectrum (e.g. 3rd or 4th generation cephalosporins), and for treatment to be related to regional antimicrobial sensitivities (Hollis and Wilkins 2009). Other areas of conflicting ideologies include their use in enterocolitis and for perioperative prophylaxis (Hollis and Wilkins 2009). These authors also advocate the prudent use of antimicrobials, including the reservation of certain antimicrobials (3rd and 4th generation cephalosporins, enrofloxacin, clavulanaic acid, azithromycin, doxycycline and chloramphenicol) for use based on culture and sensitivity AMR (Hollis and Wilkins 2009); and is therefore in line with the recommendations made by Morley *et al.* (2005).

By comparison, recommendations regarding antimicrobial use in equine reproduction have been made based on limited evidence (LeBlanc 2009). While reproductive management is an area of considerable study, it is acknowledged that most antimicrobial use guidelines are based on clinical experience (LeBlanc 2009). In this paper both antibiotic and antifungal recommendations were made based on expected sensitivities (LeBlanc 2009), and not apparently on evidence.

More recently, the expectations of antimicrobial stewardship among veterinarians and also veterinary researchers have increased (Teale and Moulin 2012; Bowen 2013). Bowen (2013) reinforcing the central tenants of the 'Protect M.E.' programme instituted by the

British Equine Veterinary Association in 2012 (Anonymous 2012a), as well as restrictions around the publication of use of protected antimicrobials (Orand 2012; Bowen 2013). In Hughes *et al.* (2013), British equine veterinarians were surveyed on their current use of antimicrobials. Clinical case scenarios were used as a proxy for the prediction of behaviour of veterinarians, and to determine factors associated with antimicrobial prescription (Hughes *et al.* 2013). An interesting conclusion of the study was that incorrect dosing and selection of antimicrobials is likely to occur frequently (Hughes *et al.* 2013). Trimethoprim-sulfonamide combinations were the most commonly prescribed antimicrobial, accounting for approximately half of all choices in scenarios. This is likely to be a true reflection of clinical practice due to the ease of administration and perceived broad-spectrum action of the antimicrobial (Hughes *et al.* 2013).

Although little is known of prescribing and clinical use of antimicrobials by equine veterinarian in NZ, a technical paper from the NZ Ministry of Primary Industries (MPI), collated antimicrobial sales data from 2009-2011 (Anonymous 2013). An increase in the sales of 3rd and 4th generation cephalosporins was noted (especially long-acting formulations); including an 118% increase in large animal product sales was seen. Equine veterinarianss reported an increased use of marbofloxacin (although this product is not licenced for horses, therefore no direct figures were available); and a 9% decrease in equine virginiamycin sales was noted (Anonymous 2013). On a per-kilogram basis, oral formulations and sulfonamides made up the majority of sales, in a combined category of horses and sheep (Anonymous 2013). In the author's experience, this is most likely attributable to practicality of administration, as TMPS (a potentiated sulfonamide) is one of the most likely to be acceptable to the equine clients who are unwilling to give intra-muscular injections (or pay for a daily veterinary visit to administer intravenous medication).

1.3.3 Selected research into equine bacterial resistance

Reports of experimental and observational studies into AMR in horses have largely focussed on enteric bacteria, especially *E coli* (Dunowska *et al.* 2006; Ahmed *et al.* 2010; Maddox *et al.* 2011; Johns *et al.* 2012; Maddox *et al.* 2012a; Maddox *et al.* 2012b). An observational study reported by Dunowska *et al.* (2006) follwed faecal *E coli* susceptibility subsequent to at least three days of antibiotic treatment, with untreated and non-hospitalised horses acting as controls. Both hospitalisation and antimicrobial use were associated with increased faecal bacterial AMR (Dunowska *et al.* 2006),

corroborating previous studies' findings in both human and other veterinary populations. Equine *E coli* in England has also been examined, initially in Ahmed *et al.* (2010), where PCR (polymerase chain reaction) was used for the identification of resistant genetic elements from 138 horses. This was a mixed population of livery (community) and hospitalised horses where a significant ($p<0.001$) difference between the proportion of horses culturing an isolate resistant to at least one antimicrobial was found. MDR in isolates from hospitalised animals was statistically significantly greater than the rate of MDR from the community horses. Isolates from resistant-horses were selected for further evaluation, including description of MDR; this was defined as AMR to four-or-more antimicrobials. These MDR isolates were then typed, and multiple resistance genes were found. Horses were hypothesised to be both source and recipient of AMR gene transference with humans/other animals (Ahmed *et al.* 2010).

A slightly different focus was taken in the paper by Maddox *et al.* (2011), where a longitudinal study was performed to assess AMR in hospitalised horses in the United Kingdom. This study found MDR in 47.7% of *E coli* samples, with 27.3% extended-spectrum beta-lactamase producing. Epidemiologic investigation using a multivariate model showed that resistance odds ratio (OR) increased, overall, with time in hospital (4 days maximum), and previous antibiotic administration in the past 7-days. These were especially true for quinolone (ciprofloxacin) resistance. Hospital use of TMPS was also implicated. Another longitudinal study was performed on horses after receiving antimicrobial therapy in Johns *et al.* (2012). As well as assessing MDR, unlike Maddox *et al.* (2011), extended-spectrum beta-lactamase production (ESBL) was also assessed in Johns *et al.* (2012). A small proportion of horses studied produced ESBL enzymes (3.1% total), and 6 isolates (from 2 horses) were positive for beta-lactamase (AmpC) production. MDR was present in 13% of all samples, and there was an increased OR of AMR carriage noted for at least two-weeks after antimicrobial treatment in horses (Johns *et al.* 2012). Both Maddox *et al.* (2011) and Johns *et al.* (2012), defined MDR as resistance to at least four antimicrobial classes.

Extended-spectrum beta-lactamase production has also been assessed by Dierikx *et al.* (2012) and Schmiedel *et al.* (2014), with both studies including horses among other species. Dierikx *et al.* (2012) found the types and distribution of ESBL genes were similar to other Dutch studies of human and poultry. Whereas in Schmiedel *et al.* (2014), while

the distribution of beta-lactamase genes was found to be similar between companion animals and people, there were some differences. Humans cultured a predominance of *bla*$_{\text{CTX-M-15}}$ type beta-lactamase Enterobacteriaceae, and animals cultured *bla*$_{\text{CTX-M-1}}$. Horses were one of the few species to culture *bla*$_{\text{CTX-M-2}}$, and this was absent in human samples (Schmiedel *et al.* 2014). The result never-the-less indicates transmission of resistant genetics between humans and animals is likely to occur.

Multi-drug resistant *Salmonella* was described as early as the 1970's in horse populations in North America (Mercer 1979). More recently it has been described in a United States veterinary teaching hospital outbreak (Ward *et al.* 2005), and in Indian equine populations (Singh *et al.* 2009). The outbreak of MDR *Salmonella* in hospitalised horses in Ward *et al.* (2005) consisted of 33 cases of *Salmonella typhimurium*; two different phage types were present although one was more common among case isolates (phage type DT193 compared with DT208) and all isolates showed resistance to ceftiofur, TMPS & gentamicin (Ward *et al.* 2005). An interesting hypothesis posed by the authors was that the infection may have persisted by the use of ceftiofur in affected horses (Ward *et al.* 2005). Salmonellosis in horses poses both zoonotic and welfare issues, and in India >75% of *Salmonella* isolates tested by Singh *et al.* (2009) were MDR; MDR was also found in isolates not normally pathogenic to horses (Singh *et al.* 2009). The level of MDR is likely to be associated with less stringent regulations around antimicrobial use than many western countries, in both human and veterinary medicine, and further emphasises the need for appropriate use in many areas of health and production (Levy and Marshall 2004; Prescott 2014).

A clinical case report from the United States, identified methicillin resistant *Staphylococcus aureus* (MRSA) in a horse with an infection subsequent to a laceration repair (Hartmann *et al.* 1997). This was the first published clinical case of MRSA infection in horses. Equine MRSA carriage rates are not known in NZ, however some work has been done in Australia (Axon *et al.* 2011). Prevalence was not investigated, however MRSA strains cultured from both clinical and screening submission found that strain CC8 (probably horse-adapted human-strain) was found most often, although the authors indicated that further studies were required (Axon *et al.* 2011). In a paper by Van den Eede *et al.* (2013b) MRSA was identified in horse/caretaker pairs and carriage rates or 1-2% were noted in both.The two horse/caretaker pairs which cultured MRSA each

shared the same *spa*-type, and the isolates shared identical antibiograms (Van den Eede *et al.* 2013b). No strong associations could be determined in this study as carriage rates were too low; veterinary care exposure was a factor that was identified as a potential risk for MRSA carriage (Van den Eede *et al.* 2013b). In contrast Mallardo *et al.* (2013) examined all methicillin-resistant Staphylococci (MRS), and respective prevalence was noted to be different in different classes of Italian horses; MRS was found in 68/191 of all horses, although MRSA carriage was 0.5% (1/191). Nasal samples were used (Mallardo *et al.* 2013), and harness racing horses were reported at having over 50% of horses positive for a *mec*A containing isolate. A recent Austrian study of MRSA in multiple companion species, found that the most common equine isolate was ST398, a large-animal associated *spa* type (Loncaric *et al.* 2014).

1.4 Summary of objectives

This thesis aims to begin addressing the current deficits in the understanding of AMR in NZ equine populations. This broad aim will be addressed through a systematic review of literature, and two analytic chapters presented within this thesis.

The objectives of this thesis are as follows:

1. To conduct a systematic review of the reporting of multi-drug resistance in equine populations.
2. To describe bacterial culture results and antimicrobial susceptibility patterns of clinical isolates submitted from foals in NZ.
3. To describe bacterial culture results and antimicrobial susceptibility patterns of respiratory isolates submitted from young horses in NZ.

To date, there are limited systemic reviews of AMR in veterinary species, especially horses. The systemic review in **Chapter 2** will synthesise the current global status of MDR in horses, and highlight the importance of MDR surveillance in veterinary populations. In **Chapter 3**, bacterial culture and antimicrobial sensitivity data from laboratory submissions from foals will be investigated. The chapter will address not only national deficits, but also investigates a non-hospital-based population of equine neonates. The study described in **Chapter 4** will use data from young NZ horses, specifically looking at the patterns of sensitivity to commonly used antimicrobials and multi-drug resistance. This will therefore provide rationale for selection of appropriate antimicrobial therapy if bacterial respiratory disease is suspected. Using multiple correspondence analysis to describe MDR isolates, the association between MDR and horse signalment data will be assessed. The studies reported in **Chapters 3** and **4** were conducted to identify AMR patterns of bacteria isolated from NZ horses. The subsequent findings may provide information for the development of guidelines for the prudent use of antimicrobials in this country. The general discussion in **Chapter 5** will summarise the overarching findings of the thesis, including the limitiations, and suggest directions for further research into AMR and MDR in equine and human health.

2. Systematic review: how is multi-drug resistance reported in equine populations?

2.1 Abstract

This systematic review assesses the way that multi-drug resistance (MDR) has been reported in horses over a ten-year period from 2004-2014, using Web of Science and Scopus databases. After duplicates were removed from search results, 164 publications were evaluated first by abstract and then by full-text analysis. From these, 75 peer-reviewed original publications were included for analysis and review. Multi-drug resistance is studied in a number of ways, and these include both clinical and non-clinical isolates, and by AMR phenotype or genotype. Equine research focussed on both public health and clinical perspectives, with a large proportion of studies relating to methicillin-resistant staphylococci (41/75; 55%) which is important both in human and animal health. This review identifies a need for more studies to be done worldwide, as there is a distinct bias toward European equine populations, while other areas of the world (such as Asia, Africa, Australasia and the Middle East) have few studies published, which may mean there is under-reporting of equine MDR bacteria.

2.2 Introduction

Antimicrobial resistance is of holistic and global importance, and this significance has been brought into focus by the emergence and propagation of MDR bacteria over the last two decades (Levy and Marshall 2004; Wieler *et al.* 2011). Multi-drug resistance has been recognised in equine populations as a therapeutic concern (Beard 2010) as well as a biosecurity and public health concern (Weese 2014). While systematic reviews of AMR exist in human healthcare (Hoffmann *et al.* 2011) and veterinary medicine (Burow *et al.* 2014), there is a scarcity of them published in equine medicine.

Relevant published literary reviews that include animals have focussed on the public health concerns of multi-resistant bacteria with specific emphasis on methicillin-resistant staphylococci (Loeffler and Lloyd 2010; Cuny *et al.* 2013), and extended-spectrum β-lactamase producing Enterobacteriaceae (Wieler *et al.* 2011; Ewers *et al.* 2012). A number of narrative (or non-systematic) reviews of methicillin-resistant staphylococci in veterinary species have been published in the last five years (Loeffler and Lloyd 2010; Weese and van Duijkeren 2010; Doyle *et al.* 2012; Petinaki and Spiliopoulou 2012; Cuny *et al.* 2013). Conclusions from these suggest that clinical infection by methicillin-resistant

Staphylococcus aureus (MRSA) in horses is commonly associated with human clonal type CC8 [HA-MRSA (Loeffler and Lloyd 2010; Petinaki and Spiliopoulou 2012), and CMRSA-5/USA500 (Doyle *et al.* 2012)]. Of increased importance in continental Europe is the food-animal associated strain CC398/ST398 (Cuny *et al.* 2013), which has increased in apparent prevalence in equine populations in the last ten years to be the predominant strain reported in continental European equine studies (Petinaki and Spiliopoulou 2012; Vincze *et al.* 2014), although horse-adapted human strains currently continue to predominate in North America (Weese 2004; Van Balen *et al.* 2014).

The epidemiology of MDR and extended-spectrum beta-lactamase producing Enterobacteriaceae in equine veterinary medicine has changed over the last thirty years (Beard 2010). Multi-drug resistant *Salmonella* infections have been reported in equine hospital outbreaks in North America since the 1980s (Ikeda and Hirsch 1985), and were suspected earlier (Knight and Heitala 1979). Although the prevalence of MDR *Salmonella* in the United States equine community has been reported at low levels (<5% MDR isolates) (Dargatz and Traub-Dargatz 2004), it is potentially much higher in other populations (e.g. India) (Singh *et al.* 2007b; Singh *et al.* 2009). The emergence of other Enterobacteriaceae with multi-resistant or ESBL genes in horses is also of concern (Ewers *et al.* 2012).

This systematic review was performed within the framework of the preferred reporting items for systematic reviews and meta-analysis (PRISMA), without the inclusion of quantitative analysis (Moher *et al.* 2009). The aims for conducting this review were to identify MDR reported in equine populations, document and synthesise the studies and to document any changes in reporting over the past decade, including some of the epidemiological factors associated with MDR bacteria. While this review is not intended to be a comprehensive analysis of all reports of types of antimicrobial resistant bacteria in horses, the results and findings gained through the systematic review process are intended to be used to aid in the furthering of focussed scientific investigation in this area.

2.3 Methodology

A research librarian was consulted in the process of constructing adequate search terms for this systematic review. The initial searches for publications was done using Web of Science® (Thomson Reuters, United States of America) and Scopus® (Elsevier, The Netherlands) search engines. A search of Web of Science was conducted on October 30th 2014 using the search Boolean: *(equine OR horse OR foal OR equus OR stallion OR mare) AND (Antimicrobial OR antibiotic) AND (suscept* OR sensit* OR resist*) AND (multi-drug* OR MDR OR multidrug* OR multi drug OR ESBL OR MRS* OR mecA OR AMPC).* This search was repeated with slight modifications on Scopus searching abstract, title and keywords using the Boolean: *(equine OR horse OR foal OR mare OR stallion) AND (antimicrob* OR antibiotic*) AND (resistan*) AND (mdr OR multi-drug OR "multi drug" OR multidrug OR esbl OR mrsa OR "methicillin resistant" OR methicillin-resistant).*

Primary studies, and reports were included in this review. Selection of papers for inclusion was based initially upon screening of abstract, then on full-text reading for relevancy. Publication dates included were those between January 1, 2004 and the date of the respective search in 2014. Inclusion of papers was also determined by accessibility; the full text was required to be available in English and available in the Massey University library, or under subscription, or open access. Multiple publications referring to the same study were included and assessed together, where appropriate, and where more than one study was described in a paper.

Publications excluded were review or tutorial articles, publications with no new or unique information regarding AMR, or if they did not contain assessment of horse isolates with respect to MDR. Opinion and editorial articles in peer-reviewed journals, and lay-publications were also excluded on the basis of non-primary research and quality, respectively.

Studies and publications for descriptive analysis used in this review were retrieved by database search, and subsequently screened and reviewed in accordance with the PRISMA recommendations (Moher *et al.* 2009).

Studies were scored based on the criteria shown in **Table 2.1**, and the sum (out of a maximum of eight) was used as a relative quality score. This scoring system was

modified from a more detailed scoring system used for the purpose of meta-analysis (Sanchez et al. 2007). For example a study may recieve a score of 7, using **Table 2.1** below. This score was calculated using the sum of the three criteria [i.e. it was published in 2010 (grade 2 = scored 2), and was a longitudinal cohort study with molecular typing of resistant bacteria (grade 3 = scored 3), and involved 148 animals (grade 2 = scored 2), for a total of 7]. Publication date was included for evaluation of recency of the information; study design and results were included for evaluation of information regarding MDR bacteria; study size was included for evaluation of quantitative importance of study with respect to larger populations, and a grade of zero included in this category to allow studies that had tested horses or horse-products for MDR bacteria to be included in review. Study design was not explicitly scored, however studies that assessed relative risk of MDR carriage (e.g. odds ratios) or assessed microbial genetic elements in detail (with phylogenetic or pathogenic as well as AMR genetics) received the highest score for type of study. Impact factor of journal was not included in scoring.

Table 2.1 Quality criteria for grading of studies and publications

	Grade 0	Grade 1	Grade 2	Grade 3
Publication date	N/A	2004-2007	2008-2011	2012-2014
Type of study	N/A	Retrospective or database report; case report or case series of 10-or-less horses.	Research-Observational study without epidemiologic information; presence/screening with limited molecular typing.	Research-observational study with epidemiologic information; other microbiologic study with molecular typing
Study size*	No horses cultured MDR bacteria in study	1-100 horses in observational study; or cultured MDR bacteria from horses	>100 horses in observational study; or cultured MDR bacteria from horses	N/A

*Number of horses included in observational studies, or number MDR horse isolates in other published studies

2.4 Results

Of the results returned from the database search approach, 164 abstracts and records were screened from which 99 available full-text publications were assessed. Subsequently, 24 studies were excluded after assessment and in accordance with the exclusion criteria outlined above, the most important of these was study relevance. However, 12 studies not included for full-text evaluations were relevant studies not available in English; 5 studies that were potentially relevant were not included as only the abstract was available. Of the full-text studies considered as potentially relevant, 75 studies were subsequently reviewed (**Figure 2.1**).

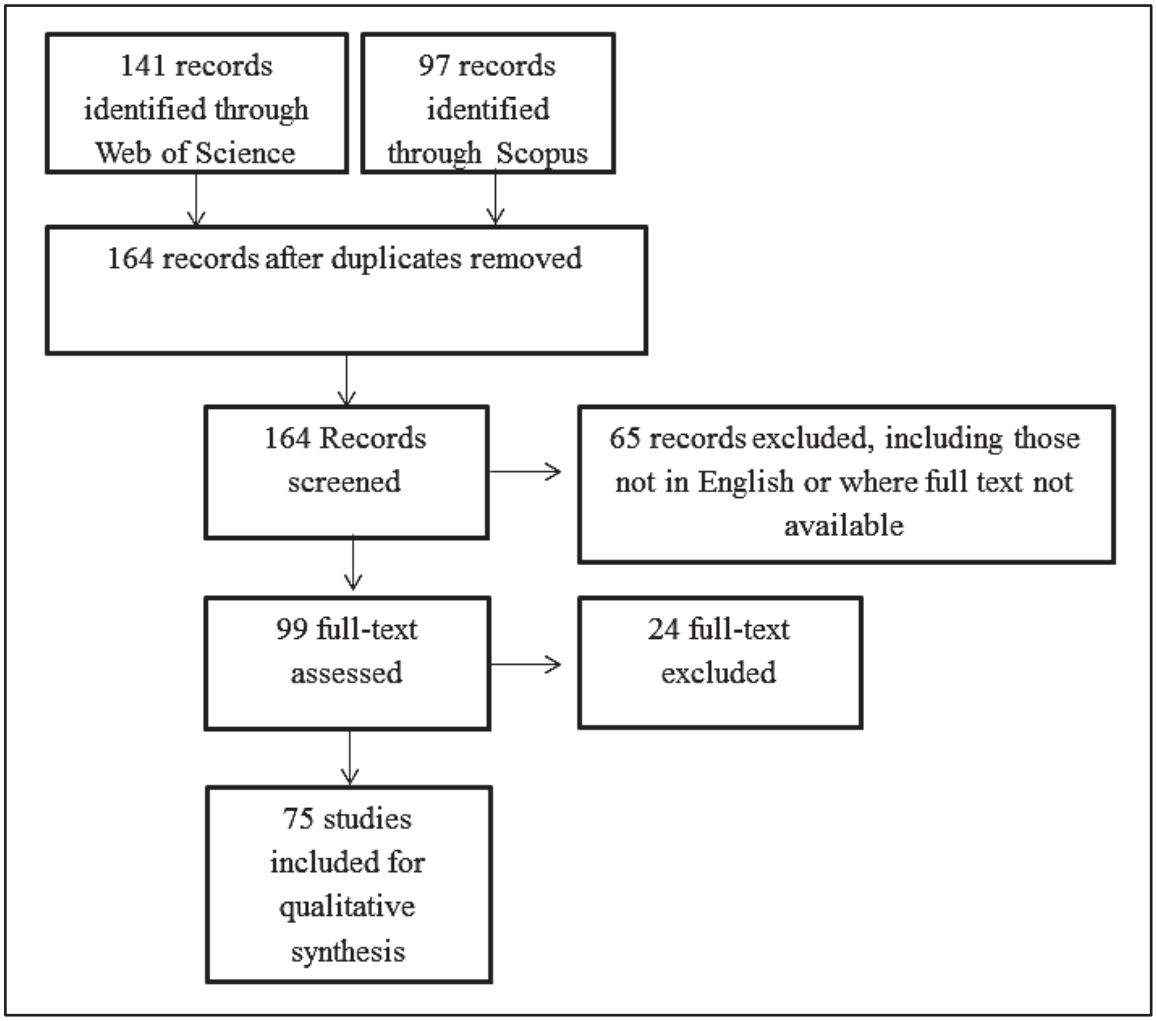

Figure 2.1 **Flowchart to determine studies of equine MDR included for qualitative assessment by systematic review following PRISMA guidelines (Moher et al. 2009)**

A graphic depiction of the geographic origin of each study is seen in **Figure 2.2**. This demonstrates the predominance of studies originating from Europe, and to a lesser extent

from North America. In total, 57.3% (43/75) of included studies originated from Europe between 2004 and 2014. The number of studies that include MDR bacteria of equine origin by time period is shown in **Figure 2.3**, where 41/77 (53%) studies include MDR or methicillin resistant *Staphylococcus*, and 31/77 (40%) studies included MDR or ESBL-producing bacteria.

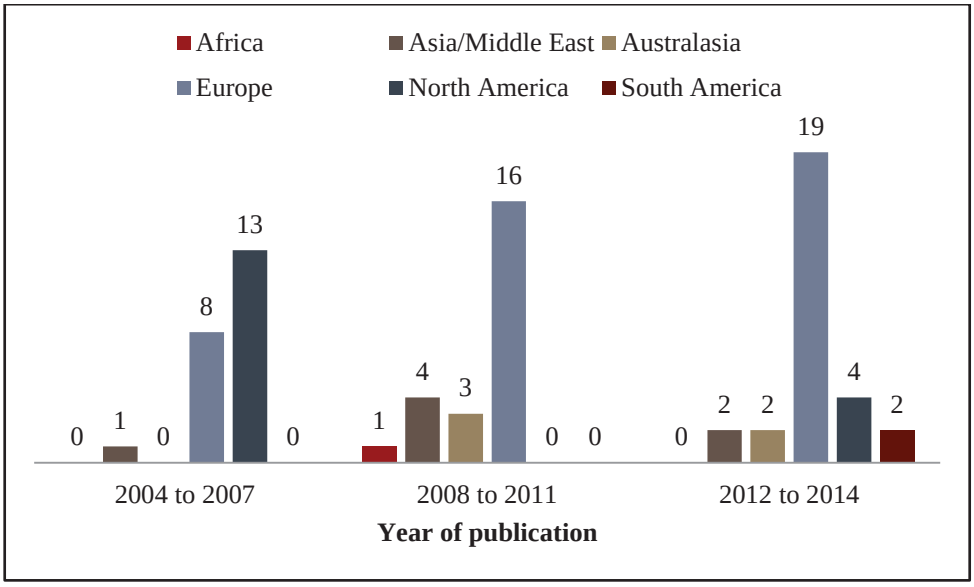

Figure 2.2 Number of equine MDR studies by geographical region 2004-2014**.**
Years of publication are 2004-2007 inclusive, 2008-2011 inclusive and 2012-October 30th 2014; 75 studies are represented in this figure.

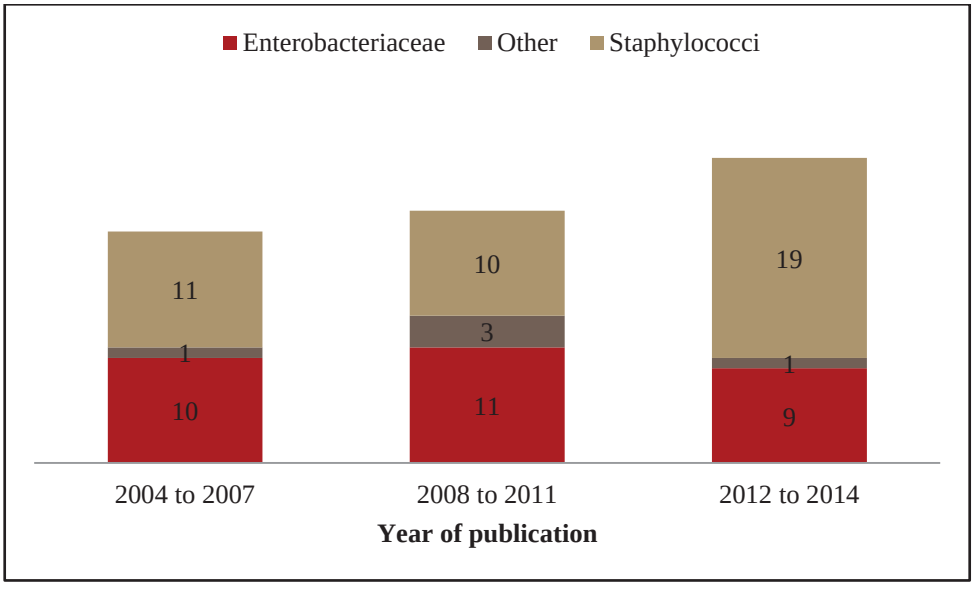

Figure 2.3 Number of equine MDR studies by bacterial type over the time-period of the systematic review 2004-2014**.**
Studies are stratified by publication year bracket, and by bacterial species of focus. Group 'other' includes enterococci, mixed species and anerobic bacteria. Years of publication are 2004-2007 inclusive, 2008-2011 inclusive and 2012-October 30th 2014; 75 studies are represented in this figure.

The quality of studies included in this review was assessed using the three criteria as described in **Table 2.1**, and a graphic depiction of the graded studies is seen in **Figure 2.4**. The majority of the studies scored ≥5 (81%; 61/75); there was also a generally increasing trend over time towards studies of higher quality, with 59% (17/29) of studies published January 2012- November 2014 scoring 7-or-higher.

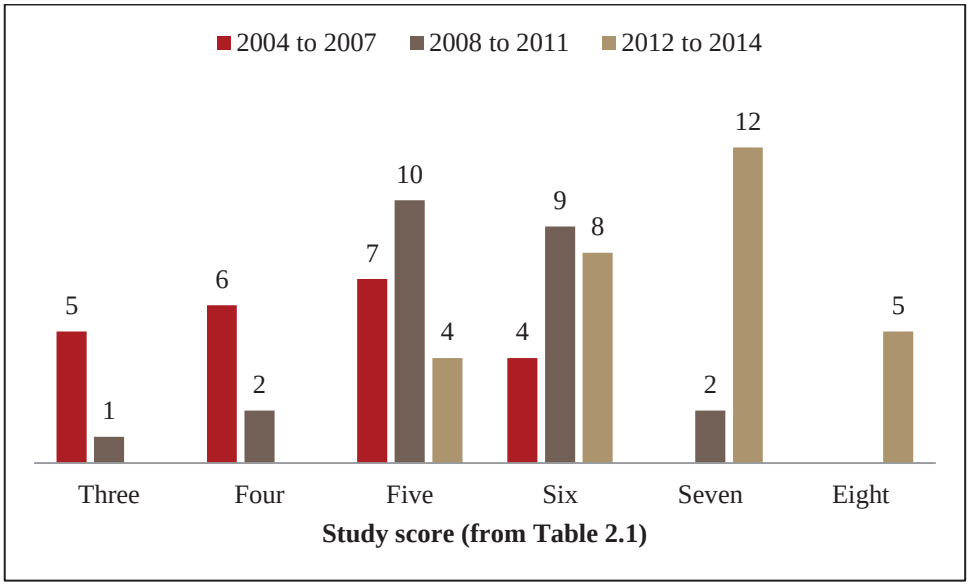

Figure 2.4 Equine MDR study score distribution over years 2004-2014**.**
Publications are divided into three year-brackets 2004-2007 inclusive, 2008-2011 inclusive and 2012-October 30th 2014. Score for studies are out of a maximum of 8; 75 studies are represented in this figure.

A summary of the studies included in this review is found in **Table 2.2**, where studies are grouped by geographical region (continent) and within region by year of publication and bacterial group of interest (staphylococci, Enterobacteriaceae, other).

Most studies assessed molecular MDR (73%; 55/75), and 25% (19/75) of defined MDR by a described phenotype; some studies were described either by both phenotype and genotype, or by neither explicitly. These studies included a longitudinal observational study, where an MDR phenotype was observed in 62.1% (341/549) of all commensal *E coli* isolates over the seven-day course of the study on 48 horses in England (Williams *et al.* 2013). By comparison, in Schmiedel *et al.* (2014) MDR of Enterobacteriaceae was defined by genotype [including beta-lactamase (ESBL) and plasmid-mediated quinolone resistance (PMQR)]. Of 100 horse isolates in this study, 17 had genetic MDR with an ESBL gene, a PMQR gene, and one other resistance gene (i.e. at least three genes)

(Schmiedel *et al.* 2014). Of the studies that defined phenotypic MDR, the number of AMR required for a bacterial isolate to be classed as "MDR" ranged from two (Zhao *et al.* 2007) to five (Singh 2009a) antimicrobial classes.

Where appropriate, numbers in the form of proportions are given for isolates or horses in studies in **Table 2.2**. Some of these illustrate the study population prevalence of certain MDR bacterial traits [e.g. MRSA and ESBL in the two studies in Maddox *et al.* (2012a), or MRSA-ST398 in Van den Eede *et al.* (2009)]. Other proportions represent equine isolates in relation to the whole study isolate population such as reported in two European studies identifying ESBL genetics in *E coli* (dogs, cats and horses) and *Klebsiella* (multiple animals, inclusing companion animals, farm animals and humans) (Ewers *et al.* 2014a; Ewers *et al.* 2014b).

Multi-locus sequence typing (MLST), if described in a publication, is reported in **Table 2.2**; if phylogenetic relatedness or a dendrogram was performed on bacterial isolates using pulse-field gel electrophoresis (PFGE), and reported in the table. Also reported in the table is specific resistance genetics such as *mec*A [e.g. MRS in Mallardo *et al.* (2013) or in Moodley and Guardabassi (2009)], and $bla_{\text{CTX-M-type}}$ [e.g. in Schmiedel *et al.* (2014)]. Preservation of different conventions for describing housekeeping genetic lineage of MRSA was done to remain consistent with the author of the described publication's intention; such as clonal complex (CC) (Axon *et al.* 2011) or PFGE/USA (Van Balen *et al.* 2014).

Table 2.2 Studies reporting equine bacterial multi-drug resistance 2004-2014

Region	Country	MDR bacteria	MDR characterisation	Other information	Reference	Score
Europe	Germany	Staphylococci	MRSA (n=16 horses) -CC398 (n=14/16) -CC8 (n=2/16)	Case-control study assessing risk factors across multiple species in vet hospitals, MRSA risk associated with increased veterinary staff, previous antimicrobial use, and surgical site infection.	(Vincze *et al.* 2014)	7
	Lithuania	Staphylococci	MRSA (n=0/120 horses)	No horses positive. Pigs only species to carry MRSA in study, all were ST398	(Ruzauskas *et al.* 2014)	5
	Austria	Staphylococci	MRSA (n=78 isolates) -ST398 (n=65/78; specifically 62/65 ST398-t011-IVa) MDR phenotype	Not all isolates MDR, 9/78 MRSA resistant to fewer than three antimicrobials.	(Loncaric *et al.* 2014)	8
	Germany	Enterobacteriaceae	ESBL, AmpC, PMQR (n=98/100 isolates with one or more resistant gene). $bla_{\text{CTX-M-1}}$ (n=37/100) $bla_{\text{CTX-M-15}}$ (n=38/100)	Clinical samples with resistance to 3rd/4th generation cephalosporin. Multiple resistant genes (PMQR+ESBL+/-another) (n=17/100)	(Schmiedel *et al.* 2014)	8
	Europe: Multiple	Enterobacteriaceae	ESBL (n=131 horses isolates) -D-ST648-CTX-M-type (n=6/131)	*E. coli* isolates all ESBL producers, n=131 horses/1152 all animals. Specifically looking for D-ST648-CTX-M-type	(Ewers *et al.* 2014a)	7
	Europe: Multiple	Enterobacteriaceae	ESBL (n=5/160 horse isolates) -equine isolate type not identified individually	*Klebsiella* spp, of all animal species isolates ESBL (n=89/1519) 85% *bla*CTX-M-15	(Ewers *et al.* 2014b)	7

	Country	MDR bacteria	MDR characterisation	Other information	Reference	Score
	Portugal	Staphylococci	MRS (n=14 isolates) SCC*mec* type: -III (n=8/14) *dru* type: -dt11a (n=8/14) MRSA (n=2/50horses) -not typed	Biocide of MDR isolates from horses; n=2/14 MRS isolates carried genes that reduced efficacy of biocidal agents; n=4/14 MDR (three or more antimicrobials); 50 horses screened for MRSA, 14 MRS isolates retrieved.	(Couto *et al.* 2013)	6
	Italy	Staphylococci	MRS (n=68/184) -*mecA* presence MRSA (n=2/184) -not typed	Significant difference between riding school horses MRS (n=12/60), and breeding horse or harness racing horse MRS (n=24/60 & 32/60 respectively)	(Mallardo *et al.* 2013)	7
	Switzerland	Staphylococci	MRCoNS (n=7/43) *mecA* (n=7/7) -IV (n=2/7) -V (n=2/7) -not typed (n=3/7) bla_Z (n=6/7)	Coagulase-negative staphylococci assessed in multiple animal species, with multiple bacterial species cultured.	(Kern and Perreten 2013)	7
	Belgium	Staphylococci	MRSA (equine n=2/166 horses) -CC398 (n=2/2) *spa* types t011, t23330	Screening for carriage by horse-caretaker/human couples. Both human caretakers of horses carried matching CC-*spa* type.	(Van den Eede *et al*. 2013b)	7
	United Kingdom	Enterobacteriaceae	MDR phenotype (n= 341/549 isolates) -day 0 (n=76/216) -day 7 (n=265/333)	Longitudinal study of MDR resistant *E. coli* to four or more antimicrobials. Increased OR of AMR *E coli* acute musculoskeletal disease (3.2), medical gastrointestinal disease (7.4).	(Williams *et al*. 2013)	7
	Sweden	Staphylococci	MRSA (n=12horses) -ST398 *spa*t011 (n=11/12) -ST8 *spa*t064 (n=1/12)	Clinically infected horses (outbreak associated n=10 horses) Screening 2007: MRSA 1/300 horses Screening 2008: MRSA 1/14 horses All isolates typed.	(Bergstrom *et al*. 2012)	6

	Country	MDR bacteria	MDR characterisation	Other information	Reference	Score
	Poland	Staphylococci	MRCoNS (n=12/42 horses carried *mec*A isolate; n=17/87 *Staphylococcus* isolates carried *mec*A)	Screening of isolates from equine nasal swabs. Ceftoxin resistance present in 5/17 *mec*A isolates, the remainder (12/17) were ceftoxin sensitive.	(Karakulska *et al.* 2012)	6
	United Kingdom	Staphylococci	MRSA (n=4/678 horses) -*mec*A, *fema*, *nuc* genes -SCC *mec*IV -*spa* t064 (n=2/4 isolates) -*spa* t451 (n=1/4 isolates) -*spa* t032 (n=1/4 isolates) MRCoNS (n=202/678 horses)	Screening study of community horses. Total culture results 29 *S.aureus* cultured (4/29 MRSA); 227 coagulase-negative *S.*(202/227 MRCoNS). Risk factors not assessed for MRSA carriage as number affected too low.	(Maddox *et al.* 2012a)	8
	Belgium	Staphylococci	MRSA (n=2 horses/64 all animal MRSA) -ST398 (n=2/2)	Of all animal isolates assessed, 18/64 MRSA-ST398. No known virulence found associated with ST398 isolates.	(Jamrozy *et al.* 2012)	6
	United Kingdom	Enterobacteriaceae	ESBL (n=17 isolates/70 horses) -bla_{SHV} (n=4/17 isolates) -$bla_{CTX\text{-}M/TEM}$ (n=12/17 isolates)	Longitudinal study of horses (hospitalised n=56 and community n=14); risk factors for transient increased shedding AMR isolates included antimicrobial treatment; hospitalisation correlated with increased length of time shedding AMR isolates.	(Johns *et al.* 2012)	7
	United Kingdom	Enterobacteriaceae	ESBL phenotype (n=42 isolates/650 horses) MDR phenotype (n=233/650 horses)	Cross-sectional prevalence study, n=650 faecal samples from horses. Prevalence estimate from proportion adjusted for clustering; MDR defined as resistance to 4+ antimicrobial classes .	(Maddox *et al.* 2012a; Maddox *et al.* 2012b)	8

	Country	MDR bacteria	MDR characterisation	Other information	Reference	Score
	Netherlands	Enterobacteriaceae	ESBL (n=12 equine ESBL isolates/65 all ESBL isolates) -$bla_{CTX-M-1}$ (n=3/12) -$bla_{CTX-M-1}{}^{+}{}_{TEM-1}$ (n=5/12) -$bla_{CTX-M-1}{}^{+}{}_{TEM-1}{}^{+}{}_{CTX-M-14}$ (n=1/12) -$bla_{CTX-M-14}$ (n=1/12) -$bla_{CTX-M-2}{}^{+}{}_{TEM-1}$ (n=1/12) -bla_{TEM-1} (n=1/12)	2007-2009 ESBL and AmpC producing isolates. Of all isolates 65/2700 isolates ESBL phenotype. Horse isolates 11/12 *E. coli*; 1/12 *Enterobacter cloacae*.	(Dierikx *et al.* 2012)	8
	United Kingdom	Enterobacteriaceae	MDR by phenotype (n=16/24 isolates) -DT104 n=5/16	Equine *Salmonella* isolates (n=24 isolates from horses); 21/24 *Salmonella* Typhimurium, 2/24 *Salmonella* Enteritidis, 1/24 *Salmonella* (serovar unknown). MDR defined as to AMR 4+ classes.	(Ahmed *et al.* 2012a)	7
	Czech Republic	Enterobacteriaceae	ESBL (n=12 isolates/37 equine samples) -$bla_{CTX-M-1}$ (n=12/12)	*E coli* community samples 2007-2008. Samples from horses (n=37), ESBL from other sources: environmental samples (n=17/50), insect/fly samples (n=33/179), human rectal swabs (n=1/12).	(Dolejska *et al.* 2011)	6
	Ireland	Enterobacteriaceae	ESBL (number horse isolates not defined) -$bla_{CTX-M-2}$ (n=8 isolates all species) AmpC-type - bla_{CMY-2} (n=18 isolates all species) -4 MDR horse isolates used in conjugation experiments and showed transference of MDR genotype.	*E coli* from hospitalised animals, multiple species (n=44 MDR equine isolates/74 MDR isolates from all species). MDR defined as AMR to 3+ classes of antimicrobial resistance. Multiple individual resistance genes identified for isolates.	(Karczmarczyk *et al.* 2011)	5

Country	MDR bacteria	MDR characterisation	Other information	Reference	Score
United Kingdom	Enterobacteriaceae	ESBL (n=131/457 isolates) and MDR (n=225/457 isolates) by phenotype.	Longitudinal study (7+ days) of *E coli* from hospitalised horses (n=103 horses). Risk factors (significant in multivariate model) for AMR were location within hospital and reason for admission.	(Maddox *et al.* 2010; Maddox *et al.* 2011)	6
Switzerland	*Acinetobacter baumanii*	AMR-genes and MDR phenotype (n=4 equine isolates). -2+ *bla*-type genes (n=4/4)	All animal isolates (n=19) (carbapenem resistance: n=3/19 animal isolates; MLST-human types: ST12, ST15)	(Endimiani *et al.* 2011)	5
Finland	Staphylococci	MRSA -ST398 t011	Human cases following horse outbreak.	(Salmenlinna *et al.* 2010)	4
Switzerland	Staphylococci	MRSA (n=83/378 horses)	Multi-species hospital-admission screening (n=378 horses/557 all animals)	(Panchaud *et al.* 2010)	6
France	Staphylococci	MRS (n=58 isolates) MRSA (n=3/58 isolates) -CC8 t064	Post-mortem coagulase-positive Staphylococci from horses; AMR penicillin-G (62.7%)	(Haenni *et al.* 2010)	5
Ireland	Staphylococci	MRSA (n=29 isolates) -CC22 (n=1/29) -CC8 *spa* t064 (n=17/29) -CC5 *spa* t002 (n=3/29)	Multiple species MRSA, CC22 shared with dogs and humans; CC8 shared with humans; CC5 shared with humans and dogs.	(Abbott *et al.* 2010)	5
Germany	Enterobacteriaceae	ESBL phenotype (n=50 horse isolates/177 all animal). -O25:H4-ST131 $bla_{CTX\text{-}M\text{-}15}$ ($n \geq 1/50$)	Investigation of human MDR strain in animals. Overall 87% genetic similarity to European human strains.	(Ewers *et al.* 2010)	5
Ireland	Enterobacteriaceae	Phenotypic MDR (n=63/412 isolates)	*E coli* isolated cultured from horses (hospitalised and non-hospitalised) (n=29 horses).	(Bryan *et al.* 2010)	5

	Country	MDR bacteria	MDR characterisation	Other information	Reference	Score
	United Kingdom	Enterobacteriaceae	Phenotypic MDR (hospitalised n=106/219 isolates; non-hospitalised n=9/77 isolates) Phenotypic ESBL (n=17/296 isolates)	Resistant (AMR 1+) *E coli* isolates from hospitalised (n=219 isolates) and non-hospitalised (n=77 isolates) horses. Conjugation experiments done on 35 MDR isolates (n=8/35). Significant difference between MDR in hospitalised and non-hospitalised horses.	(Ahmed *et al.* 2010)	6
	Denmark	Staphylococci	MRCoNS (n=32/39 horses) -*mec*A	Screening survey of horses (n=39 horses), humans (n=32 humans), and environment (n=76 environmental samples). PGFE patterns indistinguishable between groups.	(Moodley and Guardabassi 2009)	5
	Europe: multiple countries	Staphylococci	MRS (n=14/110 horses) MRSA (n=12/110 horses) -ST398 (n=3/12) -SCC *mec*IVa (n=12/12) MDR phenotype (n=10/12 MRSA isolates)	Screening survey of horses (n=110 horses). Multiple resistance phenotypes	(Van den Eede *et al.* 2009)	7
	Germany	Staphylococci	MRSP (n=4 MRSP horse isolates/46 all MRSP isolates) -*mec*A (n=4/4 isolates) MRSA (n=3 horse MRSA isolates/126 horse SA isolates)	Clinical samples from multiple species (n=16103 samples). MRSA typing not performed.	(Ruscher *et al.* 2009)	6
	United Kingdom	Staphylococci	MRSA (n=2 horses) -ST398	Case report of first clinical case of ST398 in U.K animals, other horse as part of MRSA screening.	(Loeffler *et al.* 2009)	4

	Country	MDR bacteria	MDR characterisation	Other information	Reference	Score
	United Kingdom	Staphylococci	MRSA (n=13 horse isolates) -CC1 (n=4/13) -CC873(n=3/13) -CC8 (n=2/13) -CC771 (n=2/13) -CC22 (n=1/13) -other (n=1/13)	Epidemiologic linkage between MRSA from different animals. Association between horse isolates and human types (58% horse isolates in human clusters). No CC398 isolated from animals.	(Sung *et al*. 2008)	6
	Netherlands	Enterobacteriaceae	Genetic resistance -Integrons (n=26/61 isolates; 2-gene n=9/26 islates) -DT104 (n=12/26)	*Salmonella* Typhimurium (n=61 equine isolates). Genetic transference of resistance to *E coli* in conjugation experiments.	(Vo *et al.* 2007a)	5
	Netherlands	Enterobacteriaceae	ESBL phenotype/ceftiofur resistance (n=7/1347 isolates) -$bla_{\text{CTX-M-1}}$	*E coli* and *Klebsiella* (n=13447 horse isolates/1582 isolates). Genetic transference of *bla* in conjugation experiments.	(Vo *et al.* 2007b)	5
	Denmark	Staphylococci	MRCoNS (n=51/90 horses) -*mec*A	Screening of hospitalised (n=48 horses) and non-hospitalised (n=42 horses) animals. Significant difference between two groups. *MecA* rates higher in horses compared to other species.	(Bagcigil *et al.* 2007)	5
	United Kingdom	Staphylococci	MRSA (n=6 equine MRSA isolates/561 all animal MRSA isolates)	Laboratory summary 2003-2006, equine isolates from clinical cases. No further typing done.	(Rich and Roberts 2006)	3
	Switzerland	Staphylococci	MRS (n=21/75 equine Staphylococcal isolates) -*mec*A	Screening and risk assessment in hospital with colic surgery (n=12 horses with surgery; n=7 other elective surgery).	(Schnellmann *et al*. 2006)	5

	Country	MDR bacteria	MDR characterisation	Other information	Reference	Score
	Netherlands	Staphylococci	MRS (n=175 equine isolates; n=45MRS-culturing horses) -*mec*A -MDR (n=4 isolates/175 MRS isolates) No MRSA	Horses and humans in prevalence study (n=200 horses; n=42 humans) MRSA cultured from a human (veterinarian).	(Busscher *et al.* 2006)	6
	Ireland	Staphylococci	MRSA (n=8 horse isolates) -PFGE-typing MDR (n=8/8)	Clinical isolates; large genetic disparity between horse isolates and other animal MRSA. Horse isolates displayed a higher degree of AMR.	(O'Mahony *et al.* 2005)	4
	Ireland	*Acinetobacter baumanii*	MDR integron (n=1 horse)	Clinical case (n=1 horse), MDR phenotype and genotype experimental horizontal gene transfer.	(Abbott *et al.* 2005)	4
North America	Canada	Staphylococci	MRS -*mec*A MRSP (n=1 horse isolate) -dt11a	Reporting of MRS from horses 2011-2012, including *Staphylococcus delphini* (n=13 horses). Laboratory submissions retrospectively used; ceftoxin not able to be used as a screen for methicillin resistance (and *mec*A gene presence).	(Stull *et al.* 2014)	5
	USA	Staphylococci	MRSA (n=7/120 horses; 8 isolates) -SCC*mec*IV SCC*mec*V -PFGE USA500 (n=4/8 isolates) -PFGE USA800 (n=2/8) -PFGE USA300 (n=1/8) -PFGE USA100 (n=1/8)	Circulation of equine MRSA, and environmental surfaces that are in predominant contact with humans or horses. SCCmec type IV (90% all sources); USA500 (62% all sources). Molecular typing supported constant introduction and clearing of PFGE-types throughout the year.	(Van Balen *et al.* 2014)	7

Country	MDR bacteria	MDR characterisation	Other information	Reference	Score
USA	Enterobacteriaceae	None	Animals screened for potentially zoonotic diseases, (n=2 horses); no *Salmonella* or *E coli* isolated from horses.	(Roug *et al*. 2013)	5
USA	Staphylococci	MRSA (n=0/49 horses)	Screening of horses and environment from three different farms, all horses negative.	(Peterson *et al.* 2012)	6
Canada	Staphylococci	MRSA (n=69/3372 horses) -CA-MRSA	Case-controlled study for MRSA carriage risk factors (n=69 cases; n=276 controls). Risk factors for MRSA carriage were highest if a horse had previouslycultured MRSA; other factors include if other horses on farm positive, a NICU or non-surgical admission, or antimicrobial administration in past 30-days.	(Weese and Lefebvre 2007)	6
USA	Enterobacteriaceae	ESBL and AmpC -bla_{CMY-2} (n=102/125 tested ceftiofur resistance isolates)	Ceftiofur resistance in *Salmonella* 1999-2003 (n=249/1258 diagnostic equine isolates); ceftiofur resistance increased from 4% in 1999 to 19% in 2003.	(Frye and Fedorka-Cray 2007)	6
USA	Enterobacteriaceae	MDR phenotype (n=2/10 horse isolates)	*Salmonella enterica* (serovars Typhimurium, Heidelberg, Dublin, Newport, Derby, Cholerasuis) 2002-2003 equine isolates (n=10). MDR 2+ antimicrobials.	(Zhao *et al*. 2007)	4
Canada	Staphylococci	MRSA (n=1 horse case)	Case of MRSA in a foal, human-associated colonisation (n=10/103 humans in contact) and skin infections in some ICU staff (n=3 humans). Strain CA-MRDSA-5, MDR.	(Weese *et al*. 2006)	5
Canada	Enterobacteriaceae	None	*Salmonella* Newport (n=3 horses/119 all animals) Bovine isolates MDR (n=35/70 isolates)	(Poppe *et al.* 2006)	3

	Country	MDR bacteria	MDR characterisation	Other information	Reference	Score
	USA; Canada	Staphylococci	MRSA (n=38/120 horses horses on two farms) -CMRSA-5 (farm one) -CMRSA-5, H6 (farm two)	Attempted eradication of MRSA on two horse farms. Biosecurity and hygiene effective controls.	(Weese and Rousseau 2005)	5
	Canada	Staphylococci	MRSA (n=46/972 horses) -CMRSA-5 (n=46/46) -AMR 2+ antimicrobials (n=46)	Prevalence study in targeted (n=581 horses previous MRSA exposure) and non-targeted (n=391 horses) populations. Size of farm postulated as increased risk factor for MRSA exposure.	(Weese *et al.* 2005)	6
	Canada	Staphylococci	MDR *Staphylococcus* and *Enterococcus* (n=15 horses) MRSA (n=2 horses)	Clinical treatment of MDR bacterial infections with vancomycin. Reported as largely successful, although morbidity associated with disease still caused	(Orsini *et al.* 2005)	3
	USA	Enterobacteriaceae	MDR (n=60 equine cases) B-lactamase -$bla_{\text{TEM-1b}}$ -$bla_{\text{SHV-12}}$ -bla_{CMY}	*Salmonella* Newport 2003-2004 outbreak associated MDR infections (AMR 8 antimicrobials).	(Rankin *et al.* 2005)	4
	USA	Enterobacteriaceae	MDR	*Salmonella* Typhimurium (n=29 horses/4333 all animals). Overall animals 81% MDR.	(Wedel *et al.* 2005)	4
	USA	Enterobacteriaceae	MDR (n=33 horses) -DT193 (n=18/24 isolates) -DT208 (n=3/24 isolates) -not performed (n=3/24 isolates) -PGFE identical (n=15/33 isolates) -$bla_{\text{CMY-2}}$	*Salmonella* Typhimurium 1999-2000 hospital outbreak (n=33 equine cases). All 33 isolates MDR (8 antimicrobial classes).	(Ward *et al.* 2005)	4

	Country	MDR bacteria	MDR characterisation	Other information	Reference	Score
	Canada	Staphylococci	MRSA -CMRSA-5 SCC*mec*IV	Unpublished reports.	(Weese 2004)	3
	USA	Enterobacteriaceae	MDR	Nosocomial *Salmonella* infections (95 isolates, not determined to be nosocomial), MDR not tabulated although resistance >45% to amoxycillin-clavulanic acid, ampicillin, ceftifur, cephalothin, chloramphenicol, kanamycin, streptomycin, sulfamethoxzone, tetracycline.	(Dargatz and Traub-Dargatz 2004)	3
Other	Brazil	Staphylococci	MRS -*mec*A	Army-based animal isolates *Staphylocccus sciuri* (n=6 equine isolates; n=210 rodent sample isolates). All samples analysed together, and oxacillin resistance showed good correlation with *mec*A presence.	(Calazans-Silva *et al*. 2014)	6
	Korea	Staphylococci	MRCoNS (n= 29/125 staphylococcal isolates) -*mec*A	MRS presence (n=29/195 horses) None MRSA.	(Lee *et al.* 2014)	7
	Australia	Staphylococci	MRSA (n= 1 horse) -ST8 *spa* t064	Outbreak of MRSA in a veterinary hospital (n=1 equine/5 all clinical isolates). One human MRSA isolate same PFGE pattern as 4/5 clinical case isolates.	(Allen *et al.* 2013)	6
	Turkey	Staphylococci	MRCoNS (n=101/209 horses; 123 isolates) -*mec*A MDR (n=5/123 isolates)	MRS survey of environment, humans and horses (n=209 horses). No MRSA isolated.	(Aslantas *et al.* 2012)	7
	Brazil	Staphylococci	MRS (n=0/21 horses)	Community sampling, no MRS isolated from horses.	(Aquino *et al.* 2012)	6
	NZ	Enterococci	MDR phenotype (n=3 equine cases)	MDR *Enterococcus* case series isolates speciated but no further molecular analysis done.	(Herdan *et al.* 2012)	5

	Country	MDR bacteria	MDR characterisation	Other information	Reference	Score
	Australia	Staphylococci	MRSA (n=88 isolates) -CC8 ST612 SCC*mec*IVa (n=24/36 typed-isolates)	MRSA presence in horses. Screening (n=8/216 horses), clinical cases (n=80 cases).	(Axon *et al.* 2011)	7
	Tunisia	Enterobacteriaceae	ESBL (n=0/1 horses)	*E coli* in food samples (n=1 equine/79 samples). Of all samples ESBL/AmpC production from 10/79 all samples.	(Slama *et al.* 2010)	3
	Australia	Enterobacteriaceae	ESBL (n=5 equine isolates) -bla_{CMY-7} (n=3/5) -not determined (n=2/5)	MDR *E coli* from clinical isolates (n=4 horses/55 all animals).	(Gibson *et al.* 2010)	5
	India	Enterobacteriaceae	MDR phenotype (n=84/111 isolates)	*Salmonella* (multiple serovars) 51 different resistotypes.	(Singh *et al.* 2009)	6
	India	Enterobacteriaceae	MDR phenotype and thermotolerance (n=9/10 isolates). -*E coli* (n=38/41 MDR) -*Enterobacter* spp (n=14/16 MDR) -*Klebsiella pneumonia* (n=8/9 MDR) -*Proteus* spp (n=3/3 MDR) -*Pseudomonas* spp (n=3/3 MDR)	Multiple species (*E coli*, *Enterobacter* spp, *Klebsiella pneumonia*, *Proteus* spp, *Pseudomonas* spp) thermotolerance (n=10/138 isolates pasteurisation resistant)	(Singh 2009b)	5
	Japan	Enterobacteriaceae	DT104 (n=16 isolates) -MDR phenotype (n=7/16 isolates)	*Salmonella* Typhimurium from horses.	(Niwa *et al.* 2009)	6

	Country	MDR bacteria	MDR characterisation	Other information	Reference	Score
	India	Enterococci	VRE phenotype (n=214/267 isolates) MDR phenotype (n=265/267 isolates)	*Enterococcus* isolates from clinical and non-clinical isolates. VRE more present in sick/clinical isolates c.f. healthy horses (p-value 0.04). AMR to 19 antimicrobials (n=13/267 isolates). MDR defined as AMR 5+ antimicrobials.	(Singh 2009a)	6
	Australia	Mixed	MDR phenotype (n=41/116 isolates) - Gram-positive (n=32/41 isolates)	Multiple bacterial species isolated from septic foals (n=116 isolates), 32/41 MDR definition AMR 3+ antimicrobials. Survival to discharge from hospital 78% MDR foals (n=32/41 foals).	(Russell *et al.* 2008)	5
	India	Enterobacteriaceae	MDR phenotype (n=42/646 horses) -plasmid	MDR *Salmonella* from equids (n=646 horses/872 all equids); comparisons between high and low income farms. MDR defined as AMR to 3+ antimicrobials. 50 serovar types isolated from all equids, (n=37/50 servoars contained plasmids).	(Singh *et al.* 2007a)	5

Abbreviations:

Bacteria- *E coli*: *Escherichia coli*. Staph: *Staphylococcus*. ESBL Extended-spectrum beta-lactamase MRCoNS Methicillin resistant coagulase-negative *Staphylococcus* species; MRS Methicillin resistant *Staphylococcus* species; MRSA Methicillin resistant *Staphylococcus aureus*; VRE Vancomycin resistant Enterococcus; AMR Antimicrobial resistance;

Other- CI Confidence interval; OR Odds ratio; MCA Multiple correspondence analysis ; MDR Multi-drug resistance; MIC Minimum inhibitory concentration; PCR Polymerase-chain reaction;TMPS Trimethoprim-sulfonamide combination (i.e. trimethoprim-sulfamethoxazole, trimethoprim-sulphadiazine); Housekeeping genetic typing: MLST (CC/ST), single locus (spa), phage type (DT). PFGE: pulse-field gel electrophoresis. Resistance factor typing: ESBL (*bla*-type), *mec*A.

Region “Other” includes Asia, Africa, South America, Australasia, and the Middle East.

2.5 Discussion

Multi-drug resistance is loosely defined as resistance of a bacterial isolate to more than one class of antimicrobial agent (Dargatz *et al.* 2000). Often authors of studies will further redefine MDR as being resistant to at least three (Beard 2010; Herdan *et al.* 2012), or four (Ahmed *et al.* 2010; Maddox *et al.* 2012a), antimicrobial agents or classes. This is a phenotypic definition, however specific genetically defined resistance mechanisms are also associated with a MDR phenotype and these include the expression of ESBL enzymes (Ewers *et al.* 2012) and the presence of a *mec*A gene (Van Balen *et al.* 2014).

Type of ESBL in bacteria from horses may be more similar to those types described in human and companion animals ($bla_{\text{CTX-M-15}}$/ST131 compared to food animals; $bla_{\text{CTX-M-1}}$/ST648) (Ewers *et al.* 2014a), however food animal-type ESBL genes are also found in equine populations (Dierikx *et al.* 2012). Public health implications of this are potentially large, especially if transmission to humans or between animals can be demonstrated in anything other than a theoretical context. Methicillin-resistant *Staphylococcus* has been largely assessed based upon the genetic presence of an altered beta-lactam binding protein (*mec*A) (Busscher *et al.* 2006; Axon *et al.* 2011; Maddox *et al.* 2012a; Kern and Perreten 2013; Mallardo *et al.* 2013; Stull *et al.* 2014; Van Balen *et al.* 2014).

Based upon the current analysis, reporting of MDR has an apparent bias toward European populations. The studies included in this review of antimicrobial resistance in horses showed overall high levels of reporting in Western Europe; this trend is also reflected in the presence of published reviews of MDR pathogens in veterinary species from the region (Loeffler and Lloyd 2010; Petinaki and Spiliopoulou 2012; Cuny *et al.* 2013). The geographic distribution of the studies in this review is likely to reflect political and professional momentum that supports surveillance and reporting of MDR in veterinary species in Europe, including the Netherlands (Mevius and Heederik 2014), or Sweden with the Swedish veterinary antimicrobial resistance monitoring program (SVARM) (Bengtsson *et al.* 2012). As the majority of the studies of MDR that have included horses were from Europe, the global relevance of this systematic review is somewhat limited. However, as the presence of MDR is not likely to be limited to European horses, it is important to have a baseline from which to compare future research from other regions.

Within this review, the format and design of the studies are varied, and range from case-control studies (Weese and Lefebvre 2007; Vincze *et al.* 2014) and longitudinal studies

(Williams *et al.* 2013; Van Balen *et al.* 2014), to prevalence studies (Busscher *et al.* 2006; Lee *et al.* 2014), and outbreak investigations (Bergstrom *et al.* 2012; Allen *et al.* 2013) or case reports (Abbott *et al.* 2005; Herdan *et al.* 2012).

The information provided in this review is potentially of use in further surveillance for MDR in horses, and for human health. Human health implications were the primary focus of multiple studies reported here (Weese 2004; Espie *et al.* 2005; Sung *et al.* 2008; Slama *et al.* 2010; Van den Eede *et al.* 2013b). This is an area studied, in part, through molecular epidemiologic studies (Ewers *et al.* 2014a; Ewers *et al.* 2014b; Schmiedel *et al.* 2014), and these rated highly on the scoring system (**Table 2.1)**. The studies with the highest scores (maximum of eight) contained detailed epidemiologic information and molecular typing (Maddox *et al.* 2012a; Maddox *et al.* 2012b), or detailed molecular typing with or without molecular epidemiology (Dierikx *et al.* 2012; Loncaric *et al.* 2014; Schmiedel *et al.* 2014). These results are likely confounded by the fact that recency of publication was not controlled for, as it was explicitly included as a scoring criterion.

Limitations to this review include restrictions that may have resulted from the search terms used in the study, which may have excluded potential information. However the express focus of this review was to look for the reporting of MDR in equine populations and it is likely that a broad range of articles in English are represented. Non-English studies were excluded and therefore there is a loss of reported information from journals not published in English; this is a likely source of bias in this review in favour of countries that primarily publish in English. Another limitation to the quality of this review is the high proportion of studies that were retrieved with the search terms and subsequently met the broad full-text inclusion criteria. The 75 studies reviewed were diverse and difficult to synthesise results from. This diversity of study design and focus may somewhat impede the ability to make definitive conclusions regarding specific bacterial isolates and the collation and comparison between important bacteria (i.e. MRSA or ESBL-producing Enterobacteriaceae). If this review were to be repeated, a broader search term might have been applied with a more focussed question driving the review.

With the search terms used for this review, the results have been focussed on staphylococci and Enterobacteriaceae, while other potentially harmful zoonotic bacteria such as MDR *Rhodococcus equi* or *Burkholderia mallei* were not retrieved and assessed.

This is likely of more significant clinical importance in the developing world where human disease caused by these organisms is more likely to occur, and a much lower proportion of results from these countries were included. Under-reporting of AMR in the developing world is a global concern in AMR stewardship (Vernet *et al.* 2014), and one identified by the WHO (Anonymous 2014a).

2.6 Conclusions

This review highlights the need for the continued and comprehensive reporting of MDR, especially where it relates to bacteria that are of potential public health concern. The utilisation of accepted protocols to investigate MDR pathogens in Europe should be emulated in other research communities. There is also the opportunity for the creation of guidelines in the veterinary profession not simply for use, but also for monitoring of resistance.

3. Antimicrobial sensitivity of bacteria isolated from neonatal foal samples submitted to a New Zealand veterinary pathology laboratory.

3.1 Abstract

This study was initiated to identify antimicrobial resistance (AMR) patterns of bacteria isolated from NZ foals. These findings may provide region specific information for the development of antimicrobial stewardship guidelines. A database search of submissions for bacterial culture from foals ≤3 weeks of age from April 2004 to December 2013 was performed. Culture results and sensitivities were compiled and demographic factors (age, sex, breed, region, sample source) were tabulated. Susceptibility results were as defined for the Kirby-Bauer disk diffusion susceptibility test. Multi-drug resistance (MDR) was defined as non-sensitivity to 3 or more of the core panel of antimicrobials evaluated (ceftiofur; enrofloxaxin; gentamicin; penicillin; tetracycline; trimethoprim-sulfamethoxazole); penicillin results were not included for Gram-negative bacterial isolates.

Submissions from 102 foals were examined, and 127 bacterial isolates were cultured from 64 (62.7%) submissions. Demographic data were similar between submissions that were culture positive compared to culture negative. Four bacterial groups (*Staphylococcus spp.*, *Streptococcus spp.*, *Enterococcus spp.* and *Escherichia coli*) accounted for 100 (78.7%) of all bacterial isolates cultured. At least one MDR isolate was cultured from 24 (37.5%) foals, and two or more MDR isolates were recovered from 8 (12.5%) foals.

A decreased *in vitro* antimicrobial susceptibility to commonly used antimicrobials in NZ foals was found. This is of concern from a treatment perspective; and also from a stewardship and monitoring perspective. More effective methods of capturing and recording information at a national level are recommended for the creation of national guidelines, and for future surveillance of AMR.

3.2 Introduction

Antimicrobial resistance is a major medical, political and public issue of concern (Prescott 2014). The stewardship of antimicrobial drugs in the veterinary profession, including the reporting and monitoring of AMR, and the implementation of coordinated interventions designed to improve and measure the appropriate use of antimicrobials, is an increasingly important part of the responsible use of antimicrobials (Bowen 2013; Prescott 2014). Guidelines have the potential to prevent inappropriate antimicrobial use, and thus reduce prescribing practices that may select for resistance (Dunowska *et al.* 2006). Guidelines for the rational use of antimicrobials enable practitioners to improve antimicrobial stewardship, and thus attempt to slow the development of AMR (Bengtsson *et al.* 2012). This is critically important to both equine and human health (Wilson 2001; Bowen 2013). However, these must be regionally relevant, and underpinned by evidence based veterinary medicine (Morley *et al.* 2005).

The risks of untreatable bacterial infections, including overwhelming sepsis, are especially great for neonatal equine populations (Palmer 2014; Theelen *et al.* 2014a). Bacterial colonisation occurring soon after birth has the potential to cause life-threatening infections, especially when immunologically compromised through the failure of transfer of passive immunity (i.e. insufficient quality or quantity of colostrum ingestion after birth) (McGuire *et al.* 1977; Vendrig and Fink-Gremmels 2012; Palmer 2014). Foals have been the subject of antimicrobial susceptibility studies and publications for this reason (Hirsh *et al.* 1993; Marsh and Palmer 2001; Russell *et al.* 2008; Theelen *et al.* 2014a). A recent international study looking at temporal trends in culture results from septic foals showed a significant decrease in susceptibility measurements noted for amikacin, ceftiofur, ceftoxime, gentamicin, imipenem and ticarcillin/clavulanic acid (Theelen *et al.*). However, the situation in NZ has yet to be described. This study aims to address this deficit by describing AMR of equine neonatal isolates from samples submitted to a NZ company, with veterinary pathology laboratories in the North Island between 2004 and 2013.

3.3 Materials and Methods

3.3.1 Data collection

Antimicrobial sensitivity records for bacterial isolates cultured from equine samples submitted to the New Zealand Veterinary Pathology (NZVP Ltd, Hamilton and Palmerston North laboratories, NZ) between April 2004 and December 2013 were assessed. All equine culture and sensitivity records submitted to the laboratory between the study dates were available for selection. Identifying client, horse and veterinarian information were removed from the records by NZVP in order to retain client confidentiality. Data collected included a unique accession number for each sample, the age of the horse on submission, gender, breed, organ or tissue source of submission, geographic origin within NZ and date of submission, along with the bacterial species of isolates cultured and the sensitivities of these isolates to routine laboratory antimicrobial panels.

3.3.2 Case selection

A database search of the records was conducted to extract information, using age as the selection criterion to identify the antimicrobial sensitivity profiles of isolates from foals (of recorded age $\leq$ 3 weeks). Samples were excluded if they were recorded as submitted post-mortem, including a group of submissions made as part of a perinatal mortality study (Wolfe 2009). Case information (excluding history) was limited to date of submission, age of foal (0-21 days-old), breed, animal species and sample source. Submissions were assumed to be from unique foals provided their sample accession numbers were different. Submissions listed as "foetus" or "neonate" were excluded from further review, as age was not defined. Submissions included were presumed to be from clinically affected cases. Horses with more than one sample submitted were assessed, and exclusion of isolates was made if two isolates from the same submission had an identical antibiogram. One sample per horse was assessed for foals that did not result in the growth of any isolates in any submission. The time-periods assigned to the foal-year (August 1st to July 31st the following year) were based upon the NZ Thoroughbred foaling season dates (Dicken *et al.* 2011; Waldron *et al.* 2011).

3.3.4 Culture and sensitivity, identification and classification

Aerobic culture results were selected; anaerobic and fungal isolates were not assessed. Anaerobic sensitivities were not part of the NZVP laboratory protocol. Kirby-Bauer disk-diffusion tests of cultured isolates based on a standardised protocol were documented (Bauer *et al.* 1966), and the definition of sensitivity was based on the Clinical Laboratory Standards Institute's (CLSI) recommendations for specific antimicrobial/bacterial isolate combinations (Cockerill *et al.* 2012). The laboratory selection of antimicrobials for testing was based either on NZVP's standard protocols, individual microbiologist selection, or clinician request. The number of antimicrobials tested against each isolate varied, and only those listed below were described in this study. Moderate or intermediate sensitivity, and resistant were both classed together as "not sensitive" for the purpose of this study.

3.3.5 Antimicrobial sensitivity and multi-drug resistance definition

Sensitivity to penicillin and gentamicin was defined as sensitivity of the cultured bacterial species to penicillin and/or gentamicin in combination for each bacterial isolate. Isolate MDR status was defined as non-sensitivity to three or more of a core panel of antimicrobials determined by the laboratory's protocol [ceftiofur, enrofloxacin, gentamicin, penicillin, tetracycline and trimethoprim-sulfamethoxazole (TMPS)]. Ampicillin and amoxicillin-clavulanic acid non-sensitivity were also described when available. Penicillin was removed from these criteria for Gram-negative bacteria, and isolates were required to be non-sensitive to three out of the remaining five antimicrobials. This is a modification of a previously described definition of MDR (Beard 2010).

3.3.6 Data analysis

Data were stored and manipulated in Microsoft Excel® (Microsoft Corporation, Redmond WA, USA, 2010). Submissions were stratified by foal-level signalment and demographic variables. These were age of animal, sex, breed, geographic region of submission, sample source, and culture type. The records for bacterial isolates were then examined with respect to sensitivity to antimicrobials including penicillin and gentamicin, MDR and demographic factors. Summative data were described by using counts, percentages and 95% confidence intervals (CI).

3.4 Results

In total, 160 foal accessions were included in the initial study dataset; 187 samples were associated with these accessions. Of these, 58 accessions were removed due to submission criteria not being met (i.e. age not specified as within range 0-21 days or the submission was known to not be from a clinical case. Of the 102 remaining submissions that met the inclusion criteria for the study, 9 foals had multiple samples submitted. After duplicated isolates with identical antibiograms were removed from the dataset, there were no foals with more than one sample for assessment. Over the ten-year study period 102 foals, each with one submission, were included in the study. Of these submissions, 64 (62.7%; 95% CI 53.4 – 72.1%) returned a positive aerobic bacterial culture result.

3.4.1 Overall submissions

Signalment data is summarised in **Table 3.1**. Horse signalment information (age, sex and breed) was not fully specified for a number of foal submissions. For 80 (78.4%) foals the age was not specified, but was recorded as less than three weeks old. Gender was not identified in 30/102 (29.4%) of submissions. Thoroughbred was the most common breed (68/102, 66.7%), whereas the rest were recorded as unknown/unspecified/mixed-breed, Standardbred, Miniature or Draught. The geographic distribution of the locations from which submissions were made centred on three major regions: Auckland, Waikato, and Manawatu-Wanganui. These three regions, the predominant Thoroughbred breeding regions of NZ (Waldron *et al.* 2011), accounted for 89.2% of all submissions (95% CI 83.2 - 95.2%; 91/102) and 87.5% of culture positive submissions (95% CI 79.4 – 95.6%; 56/64).

Table 3.1: Demographic information of eligible foal submissions in the study of antimicrobial sensitivity of bacteria isolated from neonatal foal samples submitted to a New Zealand veterinary pathology laboratory (2004-2013)

Demographics		Submissions: Culture Positive n	Submissions: All n	Culture Positive % (95% CI)	Relative Proportion % (95% CI)
Year of submission	2004	4	7	57.1 (20.5 - 93.8)	6.9 (2.0 - 11.8)
	2005	3	4	75.0 (32.6 - 117.4)	3.9 (0.1 - 7.7)
	2006	9	11	81.8 (59.0 - 104.6)	10.8 (4.8 - 16.8)
	2007	6	15	40.0 (15.2- 64.8)	14.7 (7.8 - 21.6)
	2008	8	12	66.7 (40.0 - 93.3)	11.8 (5.5 - 18.1)
	2009	9	14	64.3 (39.2- 89.4)	13.7 (7.0 - 20.4)
	2010	5	9	55.6 (23.1 - 88.0)	8.8 (3.3 - 14.3)
	2011	9	13	69.2 (44.1- 94.3)	12.7 (6.2 - 19.2)
	2012	9	11	81.8 (59.0 - 104.6)	10.8 (4.8 - 16.8)
	2013	2	6	33.3 (-4.4 – 71.0)	5.9 (1.3 - 10.5)
Age	<3weeks	47	80	58.8 (48.0 - 69.5)	78.4 (70.4 - 86.4)
	0-7 days	12	16	75.0 (53.8 - 96.2)	15.7 (8.6 - 22.8)
	8-21 days	5	6	83.3 (53.5 - 113.2)	5.9 (1.3 - 10.5)
Sex	Unknown	24	30	80.0 (65.7 - 94.3)	29.4 (20.6 - 38.2)
	Female	18	33	54.5 (37.6 - 71.5)	32.4 (23.3 - 41.5)
	Male	22	39	56.4 (40.8 – 72.0)	38.2 (28.8 - 47.6)
Region	Auckland	14	18	77.8 (58.6 – 97.0)	17.6 (10.2 – 25.0)
	Bay of Plenty	1	1	62.0 (48.5 - 75.5)	1.0 (-0.9- 2.9)
	Christchurch	1	1	-	1.0 (-0.9 - 2.9)
	Hawkes Bay	2	2	-	2.0 (-0.7 - 4.7)
	Manawatu-Wanganui	11	23	47.8 (27.4 - 68.2)	22.5 (14.4 - 30.6)
	Marlborough	1	1	50.0 (1.0 - 99.0)	1.0 (-0.9 - 2.9)
	Tasman	1	2	-	2.0 (-0.7 - 4.7)
	Waikato	31	50	50 (-19.3 – 119.3)	49.0 (39.3 - 58.7)
	Wellington	2	4	-	3.9 (0.1 - 7.7)
Breed	Other	16	25	64 (45.2 - 82.8)	24.5 (16.2 - 32.8)
	Thoroughbred	45	68	66.2 (54.9 - 77.4)	66.7 (57.6 - 75.8)
	Standardbred	3	5	60.0 (17.1 - 102.9)	4.9 (0.7 - 9.1)
	Miniature	0	2	-	2.0 (-0.7 - 4.7)
	Draught	0	2	-	2.0 (-0.7 - 4.7)
Specimen	Miscellaneous solid	21	25	84 (69.6 - 98.4)	24.5 (16.2 - 32.8)
	Miscellaneous fluid	14	25	56 (36.5 - 75.5)	24.5 (16.2 - 32.8)
	Joint /bone	15	36	41.6 (25.6 - 57.8)	35.3 (26.0 - 44.6)
	Ophthalmic	1	1	-	1.0 (-0.9 - 2.9)
	Respiratory	9	9	-	8.8 (3.3 - 14.3)
	Faecal/ Gastrointestinal	2	4	50.0 (1.0 - 99.0)	3.9 (0.1 - 7.7)
	Urogenital	2	2	-	2.0 (-0.7 - 4.7)

Demographics:
Year information is year of birth (i.e. 2004 represents official NZ foaling season August 1st 2004 - July 31st 2005) with the exception of the final year of the study (2013) which is August 1st 2013 – December 9th 2013); Breed "other" includes unknown and mixed or non-specified breed; miscellaneous solid or liquid is a sample of unidentified anatomic location;
% culture positive is No. culture positive/No. all foals submitting; relative proportion % is No. all foal submission/total foal submissions (i.e. each demographic factor/102 foals

3.4.2 Specimens

A number of the submissions were of unknown or unspecified anatomic origin (50/102; 49.0%; 95% CI 39.3 – 58.7%), half of which were documented as "swab" or "tissue" and half as "fluid" or "aspirate". Orthopaedic ("joint" or "bone") samples accounted for the largest proportion of known submissions (35.3%; 95% CI 26.0 44.6%) and the rest (15.7%; 95% CI 8.6 – 22.7%) were of ophthalmic, gastrointestinal, respiratory or urogenital origin (**Table 3.1**).

3.4.3 Culture results

Overall, 127 isolates were cultured from 64 foals and subjected to antimicrobial panel testing. Of these, 65.4% (95% CI 57.1 – 73.6%) were Gram-positive and 34.6% (95% CI 26.4 – 42.9%) were Gram-negative. Four genera accounted for 78.7% (95% CI 71.6 – 85.9%) of all isolates. These were *Streptococcus spp.* (25.2%; 95% CI 17.6 – 32.7%), *Staphylococcus spp.* (23.6%; 95% CI 16.2 – 31.0), *Enterococcus spp.* (9.4%; 95% CI 4.4 – 14.5%) %), *Escherichia coli* (20.5%; 95% CI 13.5 – 27.5%).

3.4.4 Sensitivity results

The number of antimicrobials each isolate was tested against ranged from 6 to 16, with a median of 7. A summary of selected *in vitro* sensitivities for the most commonly isolated bacterial species are given in **Table 3.2**. Overall, the sensitivity to ceftiofur was 67.7% (95% CI 59.5 – 76.0%); sensitivity to enrofloxacin was 92.9% (95% CI 88.4 – 97.4%); sensitivity to gentamicin was 73.0% (95% CI 65.3 – 80.8%); sensitivity to penicillin was 47.2% (95% CI 38.4 – 56.0%); sensitivity to TMPS was 65.1% (95% CI 56.8 – 73.4%); and sensitivity to tetracycline was 58.9% (95% CI 50.2 – 67.5%). Sensitivity to amoxicillin-clavulanic acid and ampicillin, where available, is also described in **Table 3.2**. No single antimicrobial was tested against all 127 isolates. In total 98.4% (125/127; 95% CI 96.3 – 100.6%) of isolates were subjected to susceptibility testing against penicillin and/or gentamicin; 81.6% (95% CI 74.8 – 88.4%) of these isolates were susceptible to this antimicrobial combination. Of 127 isolates, 126 met the criteria for identification of possible MDR. Overall, 3.2% (95% CI 0.1 – 6.2%) of isolates were resistant against all 5 or 6 of the major antimicrobials tested. Results of major MDR species are shown in **Table 3.3**. The MDR isolates came from 24 individual animals (37.5% of all culture positive foals; 95% CI 25.6 – 49.4%). Of these more than one MDR isolate was cultured in 8 foals (8/64; 12.5% of all foals; 95% CI 4.4 – 20.6%).

Table 3.2: Antimicrobial sensitivity data from positive cultures of 64 foals in the study of antimicrobial sensitivity of bacteria isolated from neonatal foal samples submitted to a New Zealand veterinary pathology laboratory (2004-2013)

	AmoxyClav		Ampicillin		Ceftiofur		Enrofloxacin		Gentamicin		Penicillin		Tetracycline		TMPS	
Bacteria	**S/ Total**	**S %**	**S/ Total**	**S %**	**S/ Total**	**S %**	**S/ Total**	**S %**	**S/ Total**	**S %**	**S/ Total**	**S %**	**S/ Total**	**S %**	**S/ Total**	**S %**
All Gram-positive	9/11	81.8	31/45	68.9	62/82	75.6	75/82	91.5	60/82	73.2	57/83	68.7	53/81	65.4	59/82	72.0
Staphylococcus spp	3/3	100	7/14	50.0	22/29	75.9	28/29	96.6	23/29	79.3	12/30	40.0	21/29	72.4	24/29	82.8
Streptococcus spp	N/T		18/19	94.7	30/32	93.8	28/32	87.5	22/32	68.8	30/32	93.8	20/31	64.5	22/32	68.8
Enterococcus spp	N/T		N/T		4/12	33.3	10/12	83.3	6/12	50.0	8/12	66.7	3/12	25.0	7/12	58.3
All Gram-negative	3/7	42.9	5/21	23.8	22/42	52.4	42/44	95.5	32/44	72.7	N/A		23/44	52.3	23/44	42.9
E. coli	2/6	33.3	5/14	35.7	13/24	54.2	26/26	100	20/26	76.9	N/A		9/25	46.2	12/26	33.3

Overall antimicrobial sensitivity. S % is percentage of isolates sensitive to the respective antimicrobials, and numbers in brackets are No. sensitive/Total. Only major species shown.

Abbreviations:

AmoxyClav – amoxicillin/clavulanate ; Amp – ampicillin; Ceft – ceftiofur; Enro – enrofloxacin; Gent – gentamicin; Pen – penicillin; Tet – tetracycline; TMPS – trimethoprim/sulfamethazole;

N/A- Not applicable; N/T- Not tested

Table 3.3: Multi-drug resistant (MDR) isolates in the study of antimicrobial sensitivity of bacteria isolated from neonatal foal samples submitted to a New Zealand veterinary pathology laboratory.

Isolates	Number Isolates Cultured N	% Isolates (95% CI)	MDR Isolates N	% MDR (95% CI)
Gram-positive total	83	65.4 (57.1- 73.6)	20	24.1 (14.9 - 33.3)
- ***Enterococcus spp****	12	9.4 (4.4 - 14.5)	6	50.0 (21.7 - 78.3)
- ***S.aureus****	23	18.1 (11.4 - 24.8)	6	26.1 (8.1 - 44.0)
- ***S.spp****	32	25.2 (17.6 - 32.7)	5	15.6 (3.0 - 28.2)
Gram-negative total	44	34.6 (26.4 - 42.9)	13	29.5 (16.1 - 43.0)
- ***E coli****	26	20.5 (13.5 - 27.5)	6	23.1 (6.9 - 39.3)
Combined total	127		33	26.0 (18.4 - 33.6)

MDR of predominant culture species only. % isolates is No. individual isolate/total No. isolates. %MDR is No. of MDR isolates for species/No. individual isolate

Enterococcus spp - Enterococcus species*; S.aureus - Staphylococcus aureus; S.spp – Streptococcus* species*; E coli - Escherichia coli*.

* Indicates major species isolated. No. of MDR isolates with corresponds with % of MDR/total isolates cultured for that species (or genera**)**

3.5 Discussion

Antimicrobial resistant bacterial isolates are frequently cultured from clinical samples in the North Island of New Zealand. This included resistance to many of the antimicrobials licenced for use in horses in this country (Anonymous 2013), as well as MDR in a substantial proportion (26%) of the isolates. The sensitivity of Gram-negative bacteria to each of the antimicrobials tested ranged from 24 to 95%, and for Gram-positive from 65 to 91%. Enrofloxacin was most effective against all bacterial isolates. The reduction in efficacy of commonly used antimicrobials may have a profound effect on the survival of compromised or sick individuals, particularly in foals (Palmer 2014). Consequently it is vital that these findings are used to support the creation of regionally relevant guidelines for the rational use of antimicrobials, to improve antimicrobial stewardship by equine veterinarians.

The number of isolates examined in this records based study was comparable to an Australian report, with 124 isolates (Russell *et al.* 2008). In this regard the current study is also comparable to an 8 year North American study in which positive blood cultures were obtained from 155 animals (Marsh and Palmer 2001). In the previous studies, samples were submitted for blood culture (Marsh and Palmer 2001) from foals presenting to referral veterinary hospitals (Russell *et al.* 2008; Theelen *et al.* 2014a). In the current study, samples submitted from a variety of primary and secondary referral veterinary practices. This expands the framework of information, as it describes the sensitivity of bacteria the foals are exposed to in what is more likely to be a community setting.

Infectious causes of mortality in NZ foals >48-hours old accounted for a substantial proportion of deaths (>80%) in animals examined during the 2007-2008 foaling season (Wolfe 2009). Broad-spectrum antimicrobial use is therefore commonly recommended for the treatment of at-risk foals or those suspected of sepsis, in order to attempt to reduce morbidity and mortality (Palmer 2014). Consequently, there are various recommendations for the most efficacious first-line treatment, most often beta-lactam/aminoglycoside combinations (Corley and Hollis 2009; Palmer 2014; Theelen *et al.* 2014a). In the current study 81.6% of tested isolates were sensitive to either penicillin or gentamicin. This result was higher than most other broad-spectrum antimicrobials when used singly (overall sensitivity to TMPS was 65.1%; and sensitivity to tetracycline

was 58.9%), and compared favourably to results of 72% (95% CI 64.3-80.6%) sensitivity to this combination in a similarly sized Australian study (Russell *et al.* 2008).

Enrofloxacin was the most efficacious antimicrobial against the bacterial isolates *in vitro* in the current study. Despite an overall sensitivity of 92.9% to enrofloxacin, it should be remembered that this is listed as a critically important antimicrobial by the World Health Organisation (Bowen 2013), and it is therefore not acceptable for this to be used as a first-line treatment (Anonymous 2012a). Quinilones also have the potential to cause arthropathies experimentally in foals (Vivrette *et al.* 2001) and therefore are not commonly recommended as a first-line treatment for neonatal sepsis (Wilson 2001). The detection of AMR in Gram-negative and positive isolates to enrofloxacin in these foals supports the need for the development of and adherence to specific antibiotic use guidelines, to preserve the efficacy of this drug for NZ equine patients.

Isolates with MDR were cultured from 37.5% of foal submissions, even after the removal of resistance to penicillin by Gram-negative bacteria from the data analyses. Multi-drug resistance has been previously identified using similar antimicrobial panels and criteria of resistance to three-or-more antimicrobials, with similar results in Australia and the United Kingdom (Russell *et al.* 2008; Johns and Adams 2013). Further knowledge and characterization of these MDR isolates would be useful in the future monitoring of resistance, especially with respect to risk factors (Johns and Adams 2013).

Previous studies in North America and Australia have identified changes in the types of bacteria isolated in foal populations with a decrease in total proportion of Gram-negative bacteria cultured, although overall more Gram-negative isolates have been cultured in these populations (Marsh and Palmer 2001; Russell *et al.* 2008; Theelen *et al.* 2014b). In the current study, a higher proportion of Gram-positive bacteria were cultured, and may be reflective of the lack of detail on the origin of some of the samples in the records, or the laboratory microbiologic protocols used. The results reflect the susceptibility of the bacteria these foals were exposed to, but it does not give an accurate representation of the clinical significance or patient outcomes. As relevant clinical information (including history) was not available from the data set, the author had no knowledge of previous treatments or current disease processes. There has been limited research into the susceptibility of pathogenic microbes found in clinically affected equines in NZ, despite work being done in other species (Petrovski *et al.* 2011; Pleydell *et al.* 2012; Karkaba *et*

al. 2013). Nevertheless, this study provides much needed insight into the current situation in NZ, and a starting point for the design of prospective bacterial sensitivity monitoring.

While the information from records used for this study reflected the demographics of the major equine subpopulations in NZ in terms of location and breed (Rosanowski *et al.* 2013) there was a lack of signalment information for some submissions, including the high proportion of unknown/unspecified submission parameters (notably sex, age, sample source). A greater limitation is the likely loss of submissions due to age not being specified at the time of sample submission. Consequently it is probable that the results from this study underrepresent the total number of submissions from foals during the study period. Further analyses, including analysis of the change in sensitivities and MDR over the study period may have been possible if larger numbers of foal submissions were available in the dataset.

These findings highlight the need for complete submission information at the laboratory level, in order for closer monitoring of resistant organisms. This requires the participation (and acceptance of responsibility) of veterinarians in practice, a workplace culture of accurate record-keeping, and client education and compliance monitoring (Hodgson *et al.* 2008; Wernli *et al.* 2011). Better compliance by submitting veterinarians with laboratory requirements not only improves the feedback and interpretations given to the clinician by the laboratory, but also provides invaluable information for MDR surveillance (Hodgson *et al.* 2008).

3.6 Conclusions

The results of this retrospective records based descriptive study indicate that there is AMR to commonly used antimicrobials in foals under veterinary care in NZ. Multi-drug resistance was found, indicating a need for regionally relevant antimicrobial use recommendations to be developed, taking into account the NZ situation. These guidelines will attempt to slow the development of AMR. This study highlights the importance of complete submission information to determine risk factors for MDR.

4. Antimicrobial sensitivities of aerobic isolates from respiratory samples of young New Zealand horses submitted to a veterinary pathology laboratory.

4.1Abstract

Decreased efficacy of commercially available veterinary antimicrobials and increased prevalence of multi-drug resistance (MDR) is of concern. A definitive veterinary diagnosis of bacterial disease (such as bronchopneumonia or pleuropneumonia) is recommended to first ensure that antimicrobial treatment is needed. The aim of this study is to describe and analyse bacterial culture and antimicrobial sensitivity data from respiratory samples submitted from young horses (4 weeks old to 3 years old) to a commercial NZ veterinary laboratory between April 2004 and July 2014. A retrospective database search for respiratory samples from young horses was conducted, and samples described with respect to demographic factors. The results of *in vitro* sensitivity testing by Kirby-Bauer disk diffusion were described and tabulated for the major bacterial species isolated. Multiple correspondence analysis was used to describe clustering of multi-drug resistance (MDR) and selected demographic variables. Veterinarians submitted respiratory samples from 289 eligible horses, bacteria was cultured from 237 submissions, with at least one aerobic bacterial isolate on which sensitivity testing was performed. Overall, 774 bacterial isolates were cultured, the majority of these were Gram-positive (67.6%; 95% CI 64.3% – 70.9%). *Streptococcus* species were the most common genus of bacteria isolated and accounted for 40.1% (95% CI 36.6 – 43.5%) of isolates cultured. Sensitivity of *Streptococcus* species to penicillin, gentamicin and ceftiofur was >85%; sensitivity of *Streptococcus* species to trimethoprim-sulfonamide (TMPS) was 52.6% (95% CI 47.0% – 58.2%). Overall Gram-negative sensitivity to ceftiofur, tetracycline, and TMPS was <75%. Multi-drug resistance was defined as resistance to three or more antimicrobials, and was found for 15.5% of isolates (95% CI 13.0 - 18.1%) and in 39.2% of horses (95% CI 33.0 - 45.5%). These results indicate that penicillin is an appropriate first-line antimicrobial for use in NZ horses where a bacterial respiratory infection is suspected while results of culture and sensitivity are pending. Continued monitoring of culture and sensitivity results at a local level should be used to inform future empirical antimicrobial selection.

4.2 Introduction

Worldwide there has been increased attention placed upon antimicrobial resistance (AMR) in the medical (Thomas *et al.* 2014) and veterinary (Prescott 2014) professions. Veterinary use of antimicrobials in horses has recently come under greater scrutiny, with the use of antimicrobials in respiratory disease identified as an area where inappropriate therapy occurs with a relatively high frequency (Weese and Sabino 2005; Hughes *et al.* 2013). In a survey using clinical case scenarios, 67.4% (763/1128) of United Kingdom veterinarians surveyed indicated that they would prescribe a trimethoprim-sulfonamide (TMPS) combination to a coughing pyrexic yearling, while 10.4% would prescribe penicillin; 2.9% oxytetracycline and 5.8% a 3rd or 4th generation cephalosporin (Hughes *et al.* 2013). These practices describe the treatment of horses with antimicrobials that are not likely to be effective (Hughes *et al.* 2013), but have the potential to increase the risk of AMR carriage (Maddox *et al.* 2011; Johns *et al.* 2012). The prescription practices of NZ equine veterinarians are not known at this time.

NZ is geographically isolated from much of the rest of the world, and it has stringent border biosecurity practices that have prevented a number of infectious equine diseases from entering the country (Rogers and Cogger 2010). However, this does not preclude the establishment of multi-resistant microbes within the country, including some of importance to human health (Herdan *et al.* 2012). Although there are few published studies or reports encompassing the NZ equine population, there have been recent concerns expressed about the emergence of multi-resistant bacteria cultured from some commercial stud farms (Herdan *et al.* 2012) and in the sick foal population (**Chapter 3**) in NZ.

Young horses are exposed and susceptible to respiratory disease (Waller 2014). Although less commonly associated with loss than musculoskeletal injury, respiratory disease accounts for substantial portion of retirement or spelling from racing in NZ (Perkins *et al.* 2005). Antimicrobial treatment of young racing horses in Canada has been reported, even without indications of bacterial infection being the cause of disease (Weese and Sabino 2005). This is a practice that may also be occurring in NZ, and may contribute to relatively high levels of oral potentiated sulfonamide use in NZ (Anonymous 2013).

Respiratory disease is a possible source of economic loss, especially for young performance horses (Dyson *et al.* 2008). This is not only confined to known contagious

pathogens such as *Rhodococcus equi*, *Streptococcus equi* ssp *equi*, *Streptococcus equi* ssp *zooepidemicus*, or equine herpes virus (EHV), but also includes losses associated with inflammatory respiratory disease (Wood *et al.* 2005a; Wood *et al.* 2005b; Couëtil *et al.* 2007). Antimicrobial treatment of young horses with airway inflammation in the absence of bacterial disease has been shown in Canadian racehorses, with 69% of horses referred with non-septic airway disease prescribed antibiotics before being seen at a referral hospital (Weese and Sabino 2005). It is important that bacterial respiratory infections are correctly identified and diagnosed (Couëtil *et al.* 2007), and laboratory results should be used in conjunction with the clinical picture to justify the clinical use of antimicrobials (Morley *et al.* 2005).

It is also important to have an understanding of the susceptibility of bacterial pathogens at a regional level, to underpin the development of regionally relevant guidelines for the prudent use of antimicrobials (Wilson 2001; Morley *et al.* 2005; Bowen 2013). This study aims to examine the patterns of sensitivity to commonly used antimicrobials and multi-drug resistance (MDR) in young NZ horses to provide a rationale for selection of appropriate antimicrobial therapy for suspected or confirmed bacterial respiratory disease.

4.3 Materials and methods

4.3.1 Data collection

Records of antimicrobial sensitivities (*in vitro*) of bacterial isolates cultured from equine samples submitted to New Zealand Veterinary Pathology (NZVP Ltd, Auckland, Hamilton and Palmerston North laboratories, NZ) between April 2004 and July 2014 were assessed. All equine culture and sensitivity records submitted to the laboratory between the study dates were available for selection, although no identifying client or horse information was available. Unique accession numbers were used to identify samples, and each set of samples was assumed to be from different animals. Clinical histories were not available in the database.

4.3.2 Case selection

The bacterial sensitivity of isolates from equines between four-weeks of age to three-years-old listed in the database were selected. The age of animals was divided according to the categories used by the laboratory on submission forms. The age group one-month

to 23-months included submissions listed as “weaner (weaning)” and “yearling”. Only samples described as respiratory (i.e. “broncho -alveolar lavage” “lung” “lung swab” “lymph node swab” “nasal discharge” “nasal swab” “pharyngeal swab” “pleural fluid” “respiratory swab” “sinus” “sinus swab” “throat swab” “thoracic fluid” “tracheal swab” “tracheal wash”) were included for description and analysis. Horses with more than one sample submitted were assessed, and exclusion of an isolate was made if two isolates from the same submission had an identical antibiogram. For the purposes of comparing demographic information, one sample per horse (if only negative culture results were obtained) was included in the dataset for analysis of demographic information.

4.3.3 Culture and sensitivity, identification and classification

The methods used to culture and determine antimicrobial sensitivity have been described previously (see **Chapter 3.2**). Briefly, the laboratory selection of antimicrobials for testing was based either on standard NZVP protocols, individual microbiologist selection, or submitting clinician request. Aerobic culture results were selected for analysis; anaerobic and fungal isolates were not assessed. Disk-diffusion tests of cultured isolates were based on a standardised protocol (Bauer *et al.* 1966), and the definition of sensitivity was based on the CLSI (Clinical Laboratory Standards Institute) recommendations for specific antimicrobial/bacterial isolate combinations (Cockerill *et al.* 2012).

The antimicrobials examined included ceftiofur, enrofloxacin, gentamicin, penicillin, tetracycline, and TMPS. Moderate or intermediate sensitivity, and resistant were both classed together as “not sensitive”. Multidrug resistance was defined as an isolate being resistant to three or more of the following antimicrobials (Beard 2010), each representing a unique drug class: ceftiofur, enrofloxacin, gentamicin, penicillin, tetracycline, TMPS. One *E. coli* isolate was removed from the dataset due to the results of testing with three antimicrobials not being available.

4.3.4 Data analysis

Data were stored and manipulated in Microsoft Excel® and Microsoft Access® (Microsoft Corporation, Redmond WA, USA, 2010). Demographic and signalment variables included region of origin, age and breed. The anatomic origin (if known) and type of submitting sample was described. The data for bacterial isolates described in the records were then examined with respect to sensitivity to antimicrobials and demographic factors, in particular age and region. Data were described by using counts, percentages and 95% confidence intervals (CI). Pearson's chi-squared or Fisher's exact tests were performed on isolates with respect to MDR status and select submission factors to determine p-values.

Multiple correspondence analysis (MCA) (Greenacre and Blasius 2006) was performed to visualise demographic factors with respect to multi-drug resistance on a two-dimensional plot. For each bacterial isolate, the demographic factors of "region" "age" and "date" were included, as was MDR status. The dates were recoded into two categories: April 2004-2008 (inclusive), and 2009-July 2014 (inclusive). The analysis was adjusted to account for inflation of the Burt Matrix using the joint method (Greenacre and Blasius 2006). All statistical analysis and MCA were conducted in using STATA version 13.1® software (StataCorp, College Station TX, USA, 2013).

4.4 Results

Over the 10 year study period, records were available for 289 respiratory samples submitted for culture; from 237 (82.0%) of these submissions aerobic bacteria were cultured and antimicrobial sensitivities were recorded.

Table 4.1. Demographic submission information from 289 horses from which respiratory samples for culture and sensitivity were submitted to a New Zealand laboratory (2004-2014).

Demographic Groups		Culture Positive N	Total Submissions n	Proportion Positive % (95% CI)
Region	Auckland	61	70	87.1 (79.2-95.0)
	Waikato	135	166	81.3 (75.4-87.2)
	North Island (other)	29	37	78.4 (65.1-91.6)
	South Island (other)	12	16	75.0 (53.8-96.2)
Year	2004	6	6	100
	2005	8	8	100
	2006	8	8	100
	2007	13	13	100
	2008	18	19	94.7 (84.6-104.8)
	2009	33	46	71.7 (58.7-84.7)
	2010	25	45	55.6 (41.1-70.1)
	2011	34	38	89.5 (79.8-99.2)
	2012	26	26	100
	2013	50	56	89.3 (81.2-97.4)
	2014	16	24	66.7 (47.8-85.6)
Age	1 to 23 months	129	162	79.6 (73.4-85.8)
	2 years	56	67	83.6 (74.7-92.5)
	3 years	52	60	86.7 (78.1-95.3)
Breed	Standardbred	19	22	86.4 (72.0-100.7)
	Thoroughbred	172	216	79.6 (74.3-85.0)
	Other breed	13	16	81.3 (62.1-100.4)
	Unknown	33	35	94.3 (86.6-102.0)
Sex	Female	97	115	84.3 (77.7-91.0)
	Male	113	131	86.6 (80.4-92.2)
	Unknown	27	43	62.8 (48.3-77.2)
All Submissions	Total	237	289	82.0 (77.6-86.4)

Region "South Island (other)" includes all regions in the South Island; Region "North Island (other)" includes Bay of Plenty, Manawatu-Wanganui, Northland, Wellington; 2014 is January-July 2014.
Proportion positive indicates Culture positive/Total submissions, as a percent.

4.4.1 Demographics

Demographic information is summarised in **Table 4.1**. Submissions from the Waikato region accounted for the majority of respiratory samples (166/289; 57.4%; 95% CI 51.7 – 63.1%). There were year-to-year variations in sample submission, ranging from 6/289 (2.1%; 95% CI 0.4 – 3.7%) in 2004 to 56/289 (19.4%; 95% CI 14.8 – 23.9%) in 2013. In the age group 4-weeks to 23-months, there were 162/289 (56.1%; 95% CI 50.3 – 61.8%) submissions. Two-year-olds accounted for 67/289 (23.2%; 95% CI 18.3 – 28.0%) of all submissions, and three-year-olds accounted for 60/289 (20.8%; 95% CI 16.1 – 25.4%) of submissions. Breeds (from which samples were submitted for culture) included Arabian, Miniature horse, pony, Shire or draught, Standardbred, station or cross-bred, Thoroughbred, Trakehner, Warmblood, and unknown breed. Thoroughbreds accounted for the majority of submissions (216/289; 74.7%; 95% CI 69.7 – 79.8%). Sex was not documented in 43/289 (14.9%; 95% CI 10.8 – 19.0%) of submissions, and there were 131/289 (45.3%; 95% CI 39.6 – 51.1%) submissions from males and 115/289 (39.8%; 95% CI 34.1 – 45.4%) from females.

4.4.2 Samples

Samples for which sensitivities were not recorded included those which cultured an anaerobic bacterium or a fungus (without also culturing aerobic bacteria), those which cultured mixed bacterial growth that were not subsequently speciated and sensitivity tested, and those for which selective culture for *Rhodococcus equi* and *Streptococcus equi* ssp *equi* were negative. In total 52/289 (18.0%; 95% CI 13.6 – 22.4%) of respiratory samples submitted from horses were not culture positive (i.e. had no sensitivities to antimicrobials recorded); 6/52 (15.4%; 95% CI 5.6 – 25.2%) had two submitted samples that were both culture-negative. Of the positive samples, 119/237 (50.2%; 95% CI 43.8 – 56.6%) were from nasal swabs and tracheal samples ("swab" or "wash") accounted for 98/237 (41.4%; 95% CI 35.1 – 47.6%) of samples. A single bacterial species was cultured from 26/237 (11.0%; 95% CI 7.0 – 14.9%) submissions; two to four bacterial species were cultured from 175/237 (73.8%; 95% CI 68.2 – 79.4%); five to seven bacterial species were cultured from 36/237 (15.2%; 95% CI 10.6 – 19.8%) of submissions; and 1/237 (0.4%; 95% CI -0.4 – 1.2%) had eleven bacterial species isolated that were tested for antimicrobial sensitivities.

4.4.3 Culture results

A total of 774 unique bacterial isolates were cultured from 237 horses with positive growth from submitted samples. Of these isolates, 523/774 (67.6%; 95% CI 64.3 – 70.9%) were Gram-positive; *Staphylococcus* species accounted for 119/523 (22.8%; 95% CI 19.2 – 26.3%) of these isolates, of which 65/119 (54.6%; 95% CI 45.7 – 63.6%) were *Staphylococcus aureus*. *Streptococcus* species constituted 310/523 (59.3%; 95% CI 55.1 – 63.5%) of all Gram-positive isolates. Of these 125/310(40.3%; 95% CI 34.9 – 45.8%) were identified as *Streptococcus equi* ssp *zooepidemicus*. *Enterococcus* species accounted for 18/523 (1.6%; 95% CI 1.9 – 5.0%) of cultured isolates. Gram-negative bacterial isolates accounted for 251/774 (32.4%; 95% CI 29.1 – 35.7%) of isolates. Enterobacteriaciae constituted 164/251 (65.3%; 95% CI 59.5 – 71.2%) of all Gram-negative isolates. Of these 61/164 (37.2%; 95% CI 29.8 – 44.6%) were identified as *Escherichia coli*. *Pseudomonas* species accounted for 32/251 (4.1%; 95% CI 8.6 – 16.9%) of Gram-negative isolates, and *Actinobacillus* species and *Pasturella* species accounted for 9/251 (2.3%; 95% CI 1.3 – 5.9%), and 2/251 (1.1%; 95% CI -0.3 – 1.9%) respectively.

4.4.4 Sensitivity results

Overall sensitivity results are described in **Table 4.2**. Antimicrobial susceptibility of Gram-positive isolates were <75% for tetracycline and TMPS, and >90% for gentamicin only. Streptococcal sensitivity to penicillin was >97%. The lowest overall sensitivity found for a Gram-negative bacterium was to ceftiofur (55.6%).

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Table 4.2. Antimicrobial sensitivity of isolates from 237 equine respiratory submissions (2004-2014)

	Ceftiofur		Enrofloxacin		Gentamicin		Penicillin		Tetracycline		TMPS	
Bacteria	Sensitive/ Total	Sensitive % (95% CI)	Sensitive/ Total	Sensitive % (95% CI)	Sensitive/ Total	Sensitive % (95% CI)	Sensitive/ Total	Sensitive % (95% CI)	Sensitive/ Total	Sensitive % (95% CI)	Sensitive/ Total	Sensitive % (95% CI)
All Gram-positive	431/513	84.0 (80.8-87.2)	450/523	86.0 (83.0-89.0)	482/523	92.2 (89.9-94.5)	411/523	78.6 (75.1-82.1)	359/523	68.6 (64.6-72.6)	297/523	56.8 (52.6-61.0)
Staph spp	102/118	86.4 (80.2-92.6)	116/119	97.5 (94.7-100.3)	111/119	93.3 (88.8-97.8)	68/119	57.1 (48.2-66.0)	97/119	81.5 (74.5-88.5)	98/119	82.4 (75.6-89.2)
S.spp	294/303	97.0 (95.1-98.9)	248/310	80.0 (75.5-84.5)	284/310	91.6 (88.5-94.7)	301/310	97.1 (95.2-99.0)	183/310	59.0 (53.5-64.5)	163/310	52.6 (47.0-58.2)
Rest Gram-positive	35/92	38.0 (28.1-47.9)	86/94	91.5 (85.9-97.1)	87/94	92.6 (87.3-97.9)	42/94	44.7 (34.6-54.8)	79/94	84.0 (76.6-91.4)	36/94	38.3 (28.5-48.1)
All Gram-negative	138/248	55.6 (49.4-61.8)	237/250	94.8 (92.0-97.6)	216/250	86.4 (82.2-90.6)	-	-	173/250	69.2 (63.5-74.9)	147/250	58.8 (52.7-64.9)
E. coli	41/61	67.2 (55.4-79.0)	60/60	100	52/60	86.7 (78.1-95.3)	-	-	42/60	70.0 (58.4-81.6)	32/60	53.3 (40.7-65.9)
Rest Gram-negative	97/187	51.9 (44.7-59.1)	177/190	93.2 (89.6-96.8)	164/190	86.3 (81.4-91.2)	-	-	131/190	68.9 (62.3-75.5)	115/190	60.5 (53.5-67.5)

Abbreviations:
Staph spp = *Staphylococcus* species; *S.spp* = *Streptococcus* species *E. coli* = *Escherichia coli* ; TMPS = trimethoprim-sulfonamide; - = testing not indicated

4.4.5 Multi-drug resistance

Multi-drug resistance was recorded for the 773 eligible isolates. Of these, 120/773 (15.5%; 95% CI 13.0 - 18.1%) were resistant to 3 or more antimicrobial families. Multi-drug resistant isolates were cultured from 93/237 (39.2%; 95% CI 33.0 - 45.5%) of horses (range one to four MDR isolates per horse). Of all Gram-positive isolates, 55/523 were MDR (10.5%; 95% CI 7.9 – 13.1%). Within specific genera of Gram-positive isolates, *Enterococcus* species included 3/18 MDR isolates (16.7%; 0 – 33.9%), *Staphylococcus* species 12/119 MDR isolates (10.1%; 95% CI 4.7 – 15.5%)**,** and *Streptococcus* species 12/310 MDR isolates (3.9%; 95% CI 1.7 – 6.0%). Overall 65/250 (26.0%; 95% CI 20.6 – 31.4%) Gram-negative bacteria cultured were MDR. In the family *Enterobacteriaciae* there were 39/163 MDR isolates cultured (23.9%; 95% CI 17.4 – 30.5%) and 18/32 *Pseudomonas* species cultured were MDR (56.3%; 95% CI 39.1 – 73.4%).

4.4.6 Statistical analysis

The age of horse (p=0.046, Pearson's χ^2 test) and date of submission (divided into two time periods of 2004-2008 and 2009-2014, p=0.003, Pearson's χ^2 test) had a significant associations with MDR status. Region (p=0.602, Fisher's exact test), sex (p=0.403, Pearson's χ^2 test) and breed (p=0.211, Fisher's exact test) were not significantly associated with the occurrence of MDR in this dataset.

4.4.7 Multiple correspondence analysis

Figure 4.1 shows the results of multiple correspondence analysis, which was used to graphically depict associations between selected demographic factors and MDR. The plot shows that non-MDR isolates ("No") lie close to the centre, and this represents the most common (or average) result, indicating most isolates were not MDR. Also shown in the plot is a clustering of 2-year-olds, submission years 2009-2014, and the Waikato region with MDR isolates ("Yes"). In total, 95% of the variance is explained in two dimensions, with most of the variance shown in dimension 1. Variables contributing most to the variation in the analysis were date (dimension 1) and region (dimension 2).

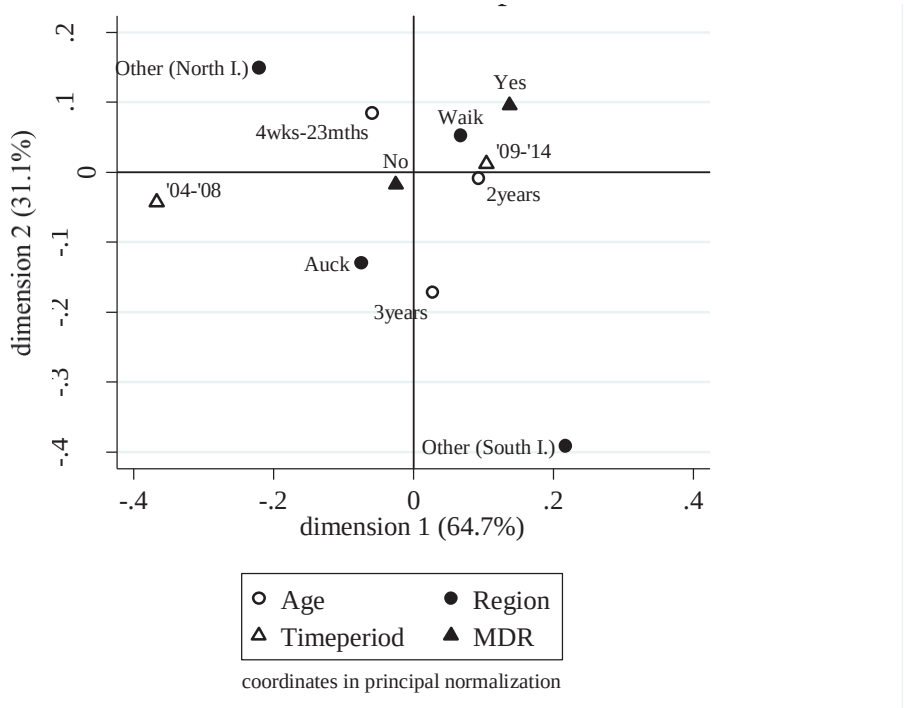

Figure 4.1: Multiple component analysis (MCA) in two dimensions, using a joint method, of 773 bacterial isolates cultured from NZ horses 2004-2014.

Age = age of horse (variables are 4 weeks – 23 months; 2 years; 3 years) associated with each bacterial isolate

MDR = Multi-drug resistant to ≥3 antimicrobial groups (variables are yes; no)

Region = region of origin of the bacterial isolates (variables are Auck= Auckland; Other (North I.)= North Island (Other); Other (South I.)= South Island (Other); Waik= Waikato)

Timeperiod = year-group of submission (variables are '04-'08= 2004-2008; '09-'14=2009-2014

4.5 Discussion

This study provides evidence that medications such as TMPS may not be effective against bacterial respiratory pathogens in young horses in NZ. However, penicillin, gentamicin and ceftifour were effective *in vitro* against multiple species of Gram-positive bacteria in most cases, with gentamicin and enrofloxacin commonly found to be effective *in vitro* against Gram-negative bacteria.

Bacteria associated with a known or potentially causal role in equine respiratory disease seen in this study included *Streptococcus* species, *Actinobacillus* and *Pasturella* species, and cultures of other bacterial that require interpretation within the clinical context of the submission (Hughes *et al.* 2013). Of these bacterial species, *Streptococcus* species accounted for 310/774 of all isolates cultured, while *Actinobacillus* and *Pasturella* were 9/774 and 2/774 respectively. This suggests that streptococcal infections are more common than other causes of bacterial respiratory infection (or colonisation) in NZ, but also may reflect the diagnostic practices of veterinarians in this country although comparable studies have not been published.

Ceftiofur has been recommended for the treatment of respiratory streptococcal infections in horses (Haggett and Wilson 2008). However, the results of the current study do not support any therapeutic advantage over the use of penicillin. *Streptococcus spp* are generally susceptible to penicillin (Erol *et al.* 2012; Giguère *et al.* 2013), and there was little appreciable difference between penicillin and ceftiofur sensitivity in this study (ceftiofur 95.1 – 98.9% versus penicillin 95.2 – 99.0%). Additionally, it has been recommended that ceftiofur, a 3rd generation cephalosporin, and enrofloxacin not be used as a first-line antimicrobial as it they have been identified as critically important antimicrobials by the World Health Organisation (Bowen 2013).

Overall the sensitivity of bacteria to TMPS was low, with Gram-positive sensitivity at 56.8% and the Gram-negative sensitivity 58.8%. Staphylococcal bacteria were susceptible to TMPS (82.4%); however, their association with clinical respiratory infections is not common (Cardwell *et al.* 2013). Staphylococcus species are more likely to be associated with contamination from the upper respiratory tract (Hughes 2013; Van den Eede *et al.* 2013a). In NZ, TMPS is one of the few antimicrobials that is available in an oral formulation and accounts for a high proportion antibiotic sales (Anonymous 2013) and suspected on-farm misuse (Rosanowski *et al.* 2014).

In a recent retrospective study of diagnostic samples of beta-haemolytic *Streptococcus* submitted to a United States (USA) laboratory over a 10-year period, a statistically significant increase in resistance of *Streptococcus equisimilis* to both gentamicin and tetracyline was noted (Erol *et al.* 2012). In the current study, the overall sensitivity of streptococcal species to gentamicin was 88.5 – 94.7% (95% CI), and overlaps with (although is slightly higher than) the sensitivity reported in the USA study of between 83.3% and 91.2% (Erol *et al.* 2012). It is possible that the result reflects the classification of bacteria, as there is intrinsic variation in the sensitivities to antimicrobials that different species of *Streptococcus* exhibit (Giguère *et al.* 2013). Gentamicin is not an appropriate first choice for the treatment of respiratory infections in horses, despite bacteria cultured in this study having a sensitivity of 92.2% (Gram-positive) to 86.4% (Gram-negative) to the antimicrobial. However, gentamicin does not achieve therapeutic concentrations in respiratory secretions (McKenzie III and Murray 2000; Winther 2012). However in the face of inflammation and increased blood flow, systemic gentamicin is likely to penetrate affected lung tissue and have chemotherapeutic effect (Clarke *et al.* 1996; Panidis *et al.* 2005), so may be justified on the basis of culture and sensitivity.

Bacteria that were resistant to three-or-more antimicrobials appeared to be relatively common in the study population, with at least one MDR isolate cultured from 39.2% of horses. The age of the horse and the year of submission were both significantly associated with MDR status and this was reflected in the MCA plot. Multiple correspondence analysis is a unique way to describe ordinal and categorical data, as it allows the visualisation of associations between multiple variables (Greenacre and Blasius 2006). This is a method not commonly reported in veterinary research, although it has been effectively utilised to describe focal bone mineral density patterns (Bogers *et al.* 2014). Based on the number of different variables potentially associated with MDR, it was used here to assess multiple factors for associations, without assessment of statistical significance. In this dataset, there was clustering of isolates from the Waikato, with 2-year-old horses and the submission years 2009-2014. This is an indication that submission of MDR isolates was common in this age group of animals, and these may be potential risk factors for MDR carriage and infection. Further investigation of this is warranted from both a treatment, and public health perspective (Beard 2010).

Increased AMR in host bacteria following the use of antimicrobials has been described in equine populations (Dunowska *et al.* 2006; Maddox *et al.* 2011), and is therefore likely to be a contributing factor to the high proportion of horses culturing an MDR isolate. The overuse and misuse of antimicrobials in equine medicine has been described in Canada and the United Kingdom, especially in the treatment of respiratory conditions (Weese and Sabino 2005; Hughes *et al.* 2013). While there was no knowledge of pre-treatment or overuse of antimicrobials in the animals from which samples for culture and sensitivity were taken for this study, previous reports have identified that antibiotic use (prior to examination by a veterinarian) may be occurring on some NZ stud farms (Rosanowski *et al.* 2014).

Some of the limitations associated with using data from laboratories have been discussed previously (see **Chapter 3.4**), including the inability to relate antimicrobial sensitivities to an accurate and well described case history (Morley *et al.* 2005). Additionally, a likely bias exists from the origin of samples, with a majority of samples submitting from the Waikato (57.4%). This bias somewhat reflects the location of the greatest concentration of horses in the commercial population in NZ (Rosanowski *et al.* 2012; Rosanowski *et al.* 2013). However it also reflects the location of the laboratories used in this study. The laboratories from which the data were obtained are situated in the North Island, and while a small proportion of samples originated from the South Island (**Table 4.1**), any true regional differences in antimicrobial sensitivity are not able to be accurately described. Breed variations are likely to be emphasised by this same regional bias, as well as the economic utility of racehorse breeds in the age range chosen for this study. Respiratory samples were likely lost from this study due to incomplete information regarding the source of samples, information that was not completed by the submitting veterinarian or clinic. As there was no evidence of association between "unknown" submission information and the culture of MDR isolates, it is possible that the missing sensitivity data would have been more likely to not be MDR. Provisions of clinical history, especially previous treatment, as well as signalment information are important not only so the laboratory can provide comprehensive feedback, but also to allow for the continued monitoring of antimicrobial sensitivity patterns (Hodgson *et al.* 2008).

The samples in this study came predominantly from nasal (50.2%) and tracheal (41.4%) samples, and while respiratory disease is typically stratified into "upper" and "lower"

airway disease, the exact mode of collection of the samples used in this study was not specified. The implication is that even if a sample has been labelled as tracheal, bronchial or lung, there is a significant likelihood that upper airway floral contamination is also present (Cardwell *et al.* 2013). Bacterial isolates from all sample sources were described together, as the potential clinical implications to keeping them separate are lost in the limitations of the study methodology. Even a stringent laboratory protocol is also subject to variations year-to-year and between laboratories; there are inherent limitations to any retrospective study of antimicrobial sensitivity or resistance (Feary *et al.* 2005). Nevertheless this is a valuable and necessary contribution to understanding of general clinical antimicrobial sensitivity and resistance in this country.

4.6 Conclusions

The results of this study confirm that penicillin is an appropriate first-line antimicrobial to use in most NZ's horses where a Gram-positive bacterial respiratory infection is suspected while results of culture and sensitivity are pending. On-going monitoring of culture and sensitivity results at a local level should be done to ensure guidelines reflect regional antimicrobial sensitivities, and therefore inform appropriate antimicrobial selection in the future. Decreased efficacy of commercially available veterinary antimicrobials and MDR is of concern, and appropriate diagnosis and treatment is recommended to first ensure that antimicrobial treatment is warranted. The continued monitoring and surveillance of antimicrobial sensitivity and resistance in NZ is warranted.

5. General Discussion

5.1 Introduction

Horses occupy a unique niche in our lives. They frequently have close contact with people as companions, have a potentially high monetary value as a commercial commodity, or in the cases of some animals, they have the potential to enter the food chain in meat products. These three diverse aspects of the equine industry mean that increased AMR in horses is likely to have economic, emotional and human public health implications.

Within the confines of this thesis, the aims have been both broad and focussed. In **Chapter 2**, a global approach to MDR was made in order to give boundaries and framework to the narrowed objectives of **Chapter 3** and **Chapter 4**. NZ is small and geographically isolated from most of the developed world, with stringent biosecurity legislation with the aim to protect agriculture and ecology (Crump *et al.* 2001). However, even though MDR pathogens are recognised as biosecurity risks (Crump *et al.* 2001), they are impractical to screen for at international borders. Antimicrobial resistant organisms including those that are MDR cause infectious diseases occur in both human (Thomas *et al.* 2014) and animal (Herdan *et al.* 2012; Karkaba *et al.* 2013) populations in NZ. These infections are likely to occur predominantly from locally selected resistance. The stated objectives of this thesis were targeted at describing a proportion of equine microbiology submissions to a national commercial laboratory (i.e. NZVP). The current lack of substantial information from NZ was the driving factor for this thesis, and there is substantial opportunity for further scientific work to be done in this area in the future.

The antimicrobial sensitivities of organisms cultured from horses described in this thesis have overall good sensitivity (>80%) to gentamicin and enrofloxacin, while sensitivities to ceftiofur are overall lower than most other reported sensitivities (**Table 1.1**, **Table 3.2**, **Table 4.2**). Less than 70% sensitivity of *Escherichia coli* to ceftiofur in the data used for this thesis was reported, compared to >90% sensitivity of reported in other populations (Wilson 2001; Clark *et al.* 2008; Giguère *et al.* 2013; Goncagul and Intas 2013). This may suggest ESBL-production by bacteria in some NZ horses, as resistance to 3rd or 4th generation cephalosporins are used to screen for potential ESBL-production (Schmiedel *et al.* 2014), and this is of public health concern.

In light of a case of multiple MDR bacterial infection in a foal outlined in **Chapter 1.2** (and also noted in 8/64 culture-positive foals in **Chapter 3**) the potential for MDR bacterial infection in exists in this country for young horses, as it does in other countries (Weese *et al.* 2006). Multi-drug resistance was an integral part of the motivation for the common theme connecting the chapters of this thesis in **Chapters 2**, **3** and **4**; however it is not the only area of relevance in the thesis. The systematic review in **Chapter 2** attempted to describe the recent status of MDR in horses worldwide, and there were five published studies from Australasian horses over the 2004-2014 publication period included in the review. This is likely to substantially underrepresent the NZ situation. Unreported AMR and MDR are present in NZ horses (**Chapter 3** and **Chapter 4**), although the degree to which this is clinically or zoonotically significant is still not known.

5.2 Limitations of thesis

The most substantial limitations of this thesis are intrinsically related to the nature of the data (and therefore study design) of the descriptive chapters. Missing and unknown data were largely attributed to a lack of submission information given to the laboratory (such as horse age, sex, breed, and sample source). Clinical histories were also not available in the given dataset, and from the author's personal experience these are not always supplied to the laboratory by veterinarians. This meant that no information regarding previous treatment with antibiotics was known.

Antimicrobial treatment and hospitalisation have both been associated with increased risk of AMR (Dunowska *et al.* 2006; Bryan *et al.* 2010) and MDR (Weese and Lefebvre 2007; Vincze *et al.* 2014). However, it was not within the aims of this thesis to assess clinical outcomes and associate bacterial culture and colonisation with previous treatment or disease. Anatomic location, namely the respiratory tract in **Chapter 4**, was used to define bacterial origin. This was, however, used as a proxy for disease rather than clinical diagnosis of respiratory disease. While this limits the power of the clinical applicability of culture results with respect to disease, it still provides valuable information regarding antimicrobial sensitivities of bacteria cultured from equine respiratory tracts.

If the database was examined from the perspective of bacterial isolates, rather than the horse-level factors of age (**Chapter 3** and **4**) and submission type (**Chapter 4**), all bulk sensitivity data would have been included. As the utility of this information would have been lower (epidemiologically), demographic factors were used to select submissions for analysis.

Due to a difference in focus between **Chapter 3** and **Chapter 4,** MDR was defined in terms of non-sensitivity (**Chapter 3**), and resistance (**Chapter 4).** Penicillin was also excluded from the criteria for Gram-negatives for (**Chapter 3**); this was a decision based on clinical practicality and applicability of the results. However, the MDR definition was simplified for the extended descriptive analysis of MDR in **Chapter 4**; this definition was specified as resistance to 3-or more of six classes of antimicrobial. Examples of differing definitions for MDR include: two-or-more antimicrobial classes (Zhao *et al.* 2007), three-or-more antimicrobial classes (Karczmarczyk *et al.* 2011), four-or-more antimicrobial classes (Maddox *et al.* 2012a), and MDR to five-or-more antimicrobial classes (Singh 2009a) Non-sensitivity of isolates as part of a definition of MDR also has precedent, as in Busscher *et al.* (2006) where it is defined as intermediate or resistant sensitivity to four-or more antimicrobials.

5.4 Future directions

The challenges to be faced in the dystopian future and "post-antibiotic age" are real and without solution at this time (Prescott 2014). Infectious bacterial disease, apparently conquered in the mid-twentieth century, is likely to once again become a significant cause of morbidity worldwide (Levy and Marshall 2004). The paradigm shift that is now taking place follows advances in molecular understanding of microbial pathogens (Prescott 2014), and is analogous to the difference between carpet-bombing and drone strike tactics in aerial warfare. In NZ equine veterinary terms, it is likely to first involve research into on-farm epidemiology, including molecular description and analysis. Successive research in NZ may then be focussed on increasing understanding of both clinical (as in **Chapter 3** and **4**) and population-level microbial sensitivities. These may take the form of observational studies and molecular typing of MDR pathogens that have the potential to impact on human populations.

Another important vector for future research will be a qualitative (and potentially quantitative) survey of antimicrobial use by equine veterinarians. Results of similar surveys have been described from companion animal veterinarians in this country (Pleydell *et al.* 2012), or among equine veterinarians in the United Kingdom (Hughes *et al.* 2013). This will give some quantification of use for monitoring purposes, and also has the potential to be part of increasing education and awareness of antimicrobial stewardship in the NZ veterinary community.

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