FIGHTING ANTIMICROBIAL RESISTANCE IN FOAL SEPSIS

DOES THE GUT CONSPIRE AGAINST US?

Mathijs Jacques Pierre Theelen

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COLOFON
Fighting antimicrobial resistance in foal sepsis: does the gut conspire against us?
Mathijs J.P. Theelen
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Fighting antimicrobial resistance in foal sepsis

Does the gut conspire against us?

De strijd tegen antibioticaresistentie bij veulens met sepsis
Worden we tegengewerkt door de darm?

(met een samenvatting in het Nederlands)

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**DIVERSITY
IS WHAT MAKES LIFE
INTERESTING**

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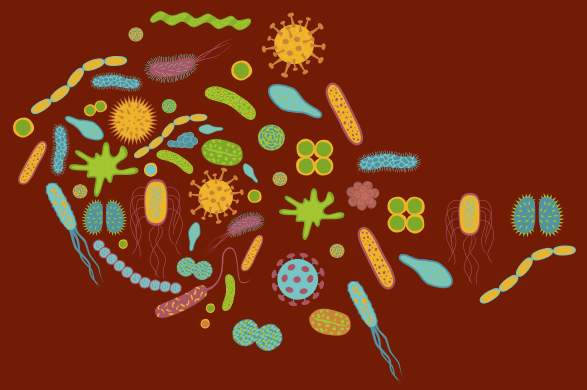
CHAPTER 1

General introduction and scope of this thesis



INTRODUCTION

Infectious diseases are common in neonatal foals affecting 8.3% of foals before the age of 30 days [1]. Foals are more susceptible to infectious diseases as they have immature innate and adaptive immune responses compared to adult horses [2]. Several risk factors for infectious diseases have been identified in foals, such as birth complications and inadequate colostrum intake [3-5]. Sepsis is one of the most serious infectious conditions in neonatal foals and is associated with high mortality [6, 7]. Timely antimicrobial treatment can potentially avoid rapid clinical deterioration. However, concerns are rising about development of antimicrobial resistance which complicates treatment of foals with sepsis [8]. In order to preserve antimicrobials for future use to treat serious bacterial infections in humans as well as animals, including foals with sepsis, judicious use of antimicrobials is key. Antimicrobial stewardship, defined as the judicious use of antimicrobials weighed against the requirement to treat a presenting clinical condition in an individual patient, has been advocated in equine medicine [9].

SEPSIS IN NEONATAL FOALS

Substantial advances have been made in medical management of critically ill foals in recent years, but despite these advances, sepsis is still one of the leading causes of death in neonatal foals [6, 7]. Survival rates of foals diagnosed with sepsis vary widely and range from 10% to 71% in studies performed at different institutes and in different time periods [10-14]. Sepsis can present as primary disease, but is also observed frequently as comorbidity to other neonatal problems such as prematurity or neonatal maladjustment syndrome and negatively affects prognosis [15]. Sepsis results from the dysregulation of the systemic host response to cascading inflammatory and anti-inflammatory mediators induced by infecting organisms and is often defined as systemic inflammatory response syndrome (SIRS) caused by infection [16]. Several risk factors for development of sepsis have been identified. Insufficient intake of good quality colostrum resulting in failure of passive transfer (FPT: inadequate immunoglobulins in the blood of the neonatal foal) is one of the most important risk factors and is associated with increased mortality [4, 5, 11]. Bacteria can enter the body via a variety of entry portals, such as the umbilicus, the respiratory tract or disrupted skin or mucous membranes. However, in the first 24 hours of life, the 'open gut' that allows for absorption of immunoglobins also poses a risk for translocation of bacteria from the gastro-intestinal tract into the bloodstream [17]. Gastrointestinal defence in new-born foals is limited in comparison to adult horses, due to an immature epithelial barrier function and deficits in both innate and adaptive immune responses. Consequently, foals are at increased risk of disturbance to mucosal homeostasis during initial intestinal colonisation that

may lead to excessive inflammation and bacterial translocation into the bloodstream, resulting in sepsis. It has been suggested this is the most common route for development of sepsis in foals. While many foals admitted to equine hospitals show clinical signs consistent with 'suspected sepsis', definite diagnosis of sepsis is complicated. Blood cultures are considered the gold standard. However, up to 40% of blood cultures collected from foals with sepsis were found to be false negative in a study comparing blood culture results to samples collected at necropsy [18]. False negatives can result from samples collected after antimicrobial treatment has started or if low blood volumes are used [19]. Blood cultures can also be false positive, in case of sample contamination or potential transient bacteremia without clinical implications [20]. Therefore, it is important that blood culture results are interpreted in the light of presence of clinical signs suggestive of sepsis. A weighted sepsis scoring system is therefore often used as a diagnostic tool for sepsis in neonatal foals, but this has a relatively low sensitivity and specificity [21, 22]. Progression of sepsis is often rapid and sepsis can lead to multiple organ dysfunction syndrome (MODS), multiple organ failure syndrome (MOFS), septic shock and ultimately the death of the foal within hours [15]. Culture and antimicrobial susceptibility testing results are usually not available until at least 48 hours after submission of the sample. The rapid clinical deterioration in foals with sepsis is a challenge to the treating veterinarian and warrants immediate initiation of (antimicrobial) treatment, while awaiting culture and susceptibility testing results.

TREATMENT OF SEPSIS IN FOALS: THE IMPORTANCE OF ANTIMICROBIALS

The most common form of sepsis in foals is bacterial sepsis [23]. Therefore, in attempt to control the infection, antimicrobials are the cornerstone of treatment in foals with sepsis. Other important aspects of treatment of sepsis in foals are anti-inflammatory treatment, cardiovascular support, respiratory support and nutritional support [15]. Because of the immature immune system and the large variation in bacteria that can cause sepsis in foals, initial antimicrobial treatment should be bactericidal and broad-spectrum. Drug selection is often based on historic data of causative organisms and their susceptibility patterns. Most studies until now have identified *Escherichia coli* as the most common causative organism isolated from foals with sepsis. Prevalence of other bacteria such as *Enterococcus* spp., *Streptococcus* spp., *Actinobacillus* spp., *Enterobacter* spp., *Staphylococcus* spp. and *Klebsiella* spp. vary widely between studies conducted in different geographic locations and in different time periods. [8, 13, 23-27]. Temporal trends in prevalence of bacteria causing sepsis in foals have been identified [23]. A decrease in enteric gram-negative organisms, *Salmonella* spp. and *Actinobacillus* spp. isolated from blood cultures was observed. This could potentially

necessitate updates in treatment protocols for foal sepsis. However, temporal data on prevalence of bacteria isolated from foals with sepsis is scarce. Furthermore, many of the studies mentioned above included a limited number of isolates. Based on current literature, a combination of ampicillin and an aminoglycoside is recommended for initial antimicrobial treatment in foals suspected of sepsis, while awaiting culture and susceptibility testing results [8]. This recommendation is based on susceptibility data on isolate level. However, polymicrobic infections, in which more than one bacterial species is cultured from one sample, occur frequently in foals with sepsis, ranging from 8% to 45% [26, 27]. Cumulative susceptibility data at foal level could, therefore, provide the clinician with more clinically useful information on which to base selection of antimicrobials for initial treatment. In human patients with sepsis, correct initial antimicrobial treatment (all causative organisms are susceptible to the combination of antimicrobials administered) had a positive effect on survival [28]. The same is likely true for foals with sepsis, although it is currently unknown to what extent antimicrobial treatment choices affect outcome. Many foals improve after initial antimicrobial treatment has been started. However, some foals fail to show clinical improvement. In those cases, clinicians often adjust antimicrobial therapy based on culture and susceptibility testing results from samples collected at hospital admission. It is, however, not known if the bacterial species infecting the foal at the time and the antimicrobial susceptibility profile of these bacteria is still the same as those identified in samples collected at hospital admission. Therefore, it might be better to collect a new sample for culture and susceptibility testing to ensure correct treatment. Currently, no data have been published on culture and susceptibility testing results from samples collected during hospitalization and after the start of antimicrobial treatment in foals with sepsis while these results might differ significantly from those of samples collected at hospital admission. Clinicians could use this information to guide them in selecting antimicrobial drugs for treatment in cases of foal sepsis that do not respond to initial treatment.

EMERGENCE OF ANTIMICROBIAL RESISTANCE

Antimicrobial resistance (AMR) is a growing problem in both human as well as veterinary medicine and poses a threat to effective treatment of bacterial infections [29]. Several studies have reported on emergence of antimicrobial resistant bacteria in equine medicine [30, 31]. Only one study reported on temporal trends in antimicrobial resistance in bacteria isolated from foals [23]. A decrease in percentage of isolates susceptible to enrofloxacin was observed. The authors conclude that antimicrobial resistance to commonly used antimicrobial drugs to treat foals with sepsis, such as penicillin, ampicillin and aminoglycosides, did not develop during the study period

(1982-2007). However, the study provides limited detail as data on susceptibility of bacteria is only presented at the level of Gram-positive, enteric Gram-negative and non-enteric Gram-negative bacteria and data on species level is not provided. Also, in that study, isolates were classified as either susceptible or resistant and no data on minimum inhibitory concentrations (MICs) were presented. Presentation of MIC data has the benefit of allowing for early detection of trends in gradual development of antimicrobial resistance as median MICs may increase without leading to changes in the proportion of isolates classified as susceptible [32]. Therefore, there is a need for studies that present MIC data rather than reporting only percentages susceptible and resistant isolates. To assess the emergence of antimicrobial resistance in equine neonatal medicine there is a need for more studies, including sufficient numbers of isolates, over a prolonged period of time to allow for detection of temporal trends, by using consistent laboratory protocols and interpretation criteria to provide accurate and detailed information, including MIC data. The information collected in these studies can be used to monitor development of antimicrobial resistance in bacteria causing sepsis in foals. Systematic (national and international) surveillance of culture and susceptibility results would allow for even better detection of temporal trends in antimicrobial resistance at an early stage. However, currently these data are not (yet) collected in a standardised and harmonised way and the data are not consistently made available to veterinarians. Differences in methods (disc diffusion vs. microdilution) and application of different interpretation criteria (MIC breakpoints) further hamper accurate interpretation of the data on a large scale. In the equine industry, there is a need for a more structured system to monitor the development of antimicrobial resistance. In such a system, it would be important to separate reporting on results from samples collected in the field or at hospital admission (community-acquired pathogens) and those collected during hospitalization (hospital-acquired pathogens) to provide clinicians with information applicable to their own working situation on which to base their selection of antimicrobials for treatment in horses.

CONSEQUENCES OF ANTIMICROBIAL RESISTANCE IN EQUINE (NEONATAL) MEDICINE

Until now, no studies have been published on the effects of antimicrobial resistance on outcome, complications or treatment cost in foal sepsis. Infections caused by antimicrobial resistant bacteria in foals with sepsis are difficult to treat and might have life-threatening consequences. Increasing prevalence of antimicrobial resistant isolates could drive veterinarians to use antimicrobials classified as ‘alternative’ or even ‘restricted’ instead of ‘first-line’ antimicrobials. A questionnaire to characterize antimicrobial prescribing patterns of equine veterinarians in the UK showed

that veterinarians working in a referral practice were more likely to prescribe 3rd and 4th generation cephalosporins and fluoroquinolones in hypothetical clinical case scenarios than first-opinion equine practitioners [33]. The authors hypothesize that clinicians working in referral centers might be more likely to prescribe restricted antimicrobials because they have been more exposed to multidrug resistant infections. Increased use of drugs that are classified as critically important antimicrobials for human medicine by the World Health Organization (WHO) [34] that should be reserved for use in human medicine, potentially contributes to further development and spread of antimicrobial resistance. Another potential consequence of the emergence of antimicrobial resistant bacteria in foals treated in neonatal intensive care units (NICU) in equine hospitals is that those bacteria might also spread to the environment and form reservoirs of resistant bacteria within the hospital. These bacteria subsequently may cause healthcare-associated infections (HAIs) in other patients with limited treatment options. In human medicine, critically ill patients admitted to intensive care units are at risk of developing HAIs, frequently related to particular surgical and medical procedures, which often involve specific species or strains of bacteria that are resistant to many antimicrobial drugs and are also present in the hospital environment [35]. Until now, no data on prevalence of HAIs in equine neonatal medicine and the causative organisms and their susceptibility patterns have been published.

THE INTESTINAL MICROBIOME AND ANTIMICROBIAL RESISTANCE

The equine gastrointestinal tract harbors a complex polymicrobial community, of which bacteria form the largest part [36]. This community plays an essential role in digestion. The different microorganisms associated with a distinct space are called the microbiota (the bacterial component of which is often studied by use of 16S rRNA gene sequencing), whereas the corresponding entity of genetic material is referred to as the microbiome (studied by the use of shotgun metagenomic sequencing) [37]. A well-functioning gastrointestinal tract and a healthy intestinal microbiota community are essential for equine health and disturbances are associated with disease, such as diarrhea [38]. Until recently, the equine hindgut microbiota was relatively poorly characterized. By using next generation sequencing techniques, Firmicutes and Bacteroidetes have been identified as the most abundant bacterial phyla present in the equine intestinal tract of healthy horses [38, 39]. Some studies have evaluated the effect of diet on faecal microbiota composition [40-43]. Others have compared faecal microbiota composition of diseased horses to that of a healthy control group [38]. However, as a result of the use of different DNA isolation and sequencing techniques, combined with many other potential factors of influence that differ between studies, information on

what is considered a 'healthy' or 'normal' equine intestinal microbiota composition and which factors shape it, is currently limited. In humans, several distinct types of intestinal microbiota composition (enterotypes) can be distinguished in healthy individuals [44, 45]. Furthermore, it is known that environmental factors (e.g. receiving breastfeeding as an infant, educational level, geographic location) and host factors (e.g. gender, age) affect intestinal microbiota composition [44, 46, 47]. Use of antimicrobials in horses carries the risk of development of antimicrobial-associated diarrhoea and this might result from dysbacteriosis or overgrowth of pathogenic strains of bacteria [48]. Currently, no studies using next generation sequencing (NGS) techniques have been performed in horses to assess the effect of antimicrobials on the intestinal microbiota. Bacteria in the equine gastrointestinal tract carry genetic information encoding for metabolic pathways that are essential for digestion, but they also carry other genes, such as antimicrobial resistance genes (ARGs). ARGs are naturally present in environmental bacteria and were identified in ancient environmental samples far predating the discovery of antimicrobials [49]. All the ARGs in a certain environment, of both pathogenic and non-pathogenic bacteria, are called the resistome [50]. Use of antimicrobials places selection pressure on bacteria, including those in the intestines, which can lead to increases in relative abundance of ARGs and a higher gut resistance potential [51]. The presence of antimicrobial resistant bacteria in the intestinal microbiota doesn't necessarily have a negative impact on the host's health. However, if they cause an infection, it might be difficult to treat. Human clinical patients with intestinal overgrowth of vancomycin-resistant *Enterococcus* spp. after antimicrobial treatment often subsequently develop life-threatening bloodstream infections, demonstrating the potential of antimicrobial resistant bacteria originating from the gut to cause infections to the host, including sepsis [52]. Bacteria in the equine hindgut of healthy horses carry ARGs in the absence of antimicrobial treatment [53]. Antimicrobial treatment likely causes these to increase in relative abundance, as has been demonstrated in humans [54]. The bacteria and the ARGs they carry can form a reservoir and potentially cause infection in the host. Furthermore, these resistant bacteria can also spread to the environment by faecal excretion and subsequently cause infections in other animals or humans. This is especially important in a hospital setting in which contamination of the environment with antimicrobial resistant bacteria could be a source of healthcare-associated infections in other (already immunocompromised) patients [55]. Currently, no NGS studies have been published regarding the potential effects of antimicrobial treatment on the equine faecal resistome. This might be relevant not only for horses, but also from a One Health perspective as there is a close interaction between horses, their owners and the environment.

ONE HEALTH

Antimicrobial resistance is a problem that affects both human as well as veterinary medicine [29]. Antimicrobial resistant bacteria can spread from animals to humans and the environment and vice versa are not restricted to ecological compartments [56]. Therefore, antimicrobial resistance is a true One Health problem and a One Health approach should be adopted when studying AMR or when designing policies for antimicrobial usage. Legislation for prescribing antimicrobials in veterinary medicine is becoming increasingly restrictive in an attempt to preserve antimicrobials for use in human medicine to treat serious bacterial infections. Veterinarians are encouraged to minimise their use of antimicrobials. Antimicrobial stewardship is the judicious use of antimicrobials weighed against the requirement to treat a presenting clinical condition in an individual patient. Antimicrobial stewardship programs designed for veterinary medicine can help to reduce the use of antimicrobials and thereby the development and spread of antimicrobial resistance.

AIMS OF THIS THESIS

The first main objective of this thesis is to study antimicrobial susceptibility and development of resistance in bacteria isolated from foals with sepsis in order to provide guidance to clinicians in selecting antimicrobial drugs for initial treatment. In order to achieve this goal, we have formulated several subsidiary aims:

- To describe temporal trends in prevalence and antimicrobial susceptibility of bacteria isolated from foals with sepsis over a time period of more than three decades
- To evaluate the potential effect of type of infections (single organism vs. polymicrobic infections) on likelihood of survival
- To evaluate the effect of initial antimicrobial treatment on likelihood of survival
- To evaluate differences in culture and susceptibility testing results between samples collected at hospital admission and those collected after ≥48 hours of hospitalization
- To determine the most likely origin of positive samples collected after ≥48 hours of hospitalization to gain insight into potential healthcare-associated infections in foals treated in a neonatal intensive care unit

The second main objective of this thesis is to evaluate the potential role of the intestinal microbiome and resistome as a reservoir of antimicrobial resistance. In order to achieve this goal, we have also formulated several subsidiary aims:

- To describe the composition of the equine faecal microbiota in healthy horses and ponies under normal housing and management conditions
- To evaluate the relative influence of several host- and environmental factors on the faecal microbiota composition in horses
- To evaluate the cumulative short- and long-term effect of transportation, hospitalization, oral treatment with trimethoprim sulfadiazine (TMS) and discharge from the hospital on the faecal microbiome and resistome

All studies included in this thesis aim to contribute to the scientific knowledge that can be used to design or further improve antimicrobial stewardship programs for equine (neonatal) medicine.

OUTLINE OF THIS THESIS

In Chapter 2 the results of a study aimed at detecting temporal trends in prevalence of bacteria isolated from foals with sepsis are discussed. Bacteriological culture results of 588 foals diagnosed with sepsis between 1979 and 2010 are presented and temporal trends in prevalence of bacterial isolates are described.

In Chapter 3 temporal trends in antimicrobial susceptibility of bacteria isolated from foals with sepsis are presented. The same samples as used for the study presented in Chapter 2 are included. This chapter provides insight into the emergence of antimicrobial resistance in equine neonatal medicine over a time period of more than three decades.

In Chapter 4 the relative importance of antimicrobial drugs in treatment of foals with sepsis is discussed. We evaluate differences in outcome (survival) between foals that were treated with antimicrobials to which all of the cultured bacteria at hospital admission were susceptible (correct initial antimicrobial treatment) versus foals that were treated with antimicrobials to which at least one of the cultured bacteria at hospital admission was resistant (incorrect initial antimicrobial treatment). In this chapter, the effect of polymicrobic vs. single organism infections on outcome is also presented.

In Chapter 5 the effect of hospitalization on bacterial culture and susceptibility testing results is assessed by comparing culture results from samples collected at hospital admission with those collected after more than 48 hours of hospitalization. Also, we are presenting the most likely origin of the positive bacterial cultures collected after more than 48 hours of hospitalization by differentiating between potential treatment failures, suspected acquired antimicrobial resistance by causative organisms, anti-

microbial resistant infections detected at hospital admission and healthcare-associated infections.

In Chapter 6 the composition of the equine faecal microbiome in healthy horses and ponies under standard housing and management conditions is presented as well as the host- and environmental factors that affect this composition.

In Chapter 7 the short- and long-term effects of trimethoprim-sulfadiazine (TMS) on the equine faecal microbiota and the faecal resistome are presented. In this longitudinal study six healthy Welsh ponies are sampled at the farm, after transportation to the hospital, during hospitalization, during treatment with TMS and for six months after discharge from the hospital. In this study the effects of TMS on the faecal microbiota composition and the resistome are presented. This study provides insight into the duration and extent of excretion of ARGs via the faeces of horses after oral TMS treatment.

In Chapter 8, based on the current scientific evidence combined with the results of the studies included in this thesis, practical guidelines for implementation of antimicrobial stewardship in equine practice are provided with a special focus on equine neonatal medicine as appropriate.

In Chapter 9, the general discussion, the findings in this thesis are summarized and discussed in a broader perspective. Also, potential areas of interest for future research are discussed.

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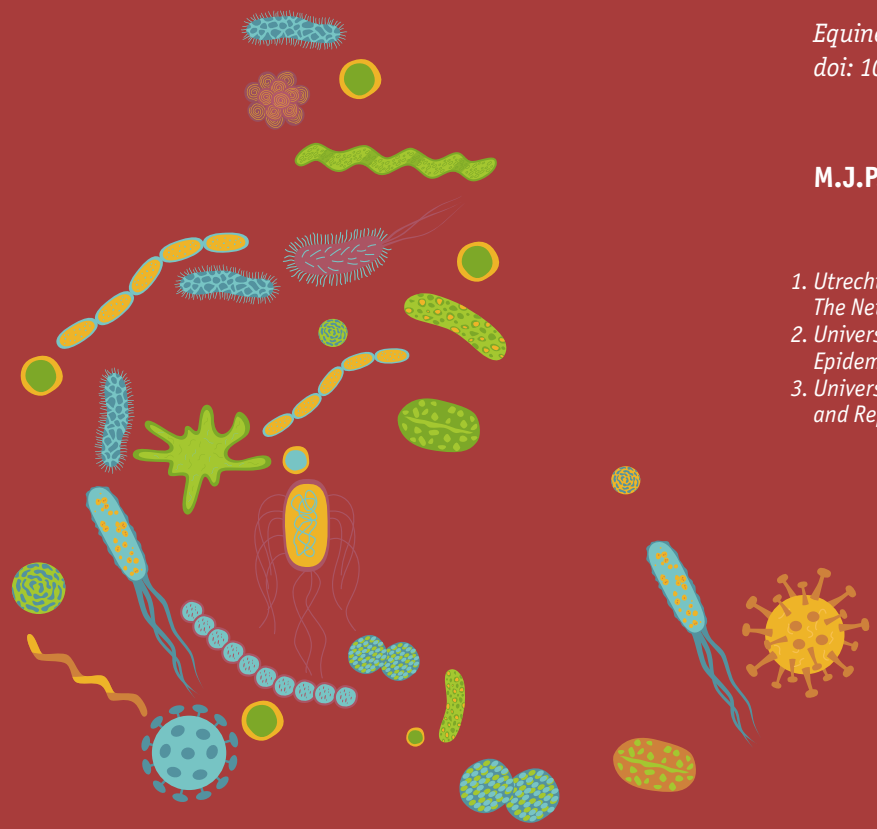
CHAPTER 2

Temporal trends in prevalence of bacteria isolated from foals with sepsis: 1979 – 2010



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ABSTRACT

Reasons for performing the study: Sepsis is an important cause of death in foals. Knowledge of which pathogens are likely to be involved is important for selection of antimicrobial drugs for initial treatment.

Objectives: To identify temporal trends in prevalence of bacteria isolated from foals with sepsis between 1979 and 2010.

Study design: Retrospective review of medical records.

Methods: All foals ≤30 days of age presented to the Veterinary Medical Teaching Hospital (VMTH) at the University of California, Davis between 1979 and 2010, with a diagnosis of sepsis confirmed by culture of bacteria from blood or internal organs (ante mortem or at necropsy), were included in the study. Conventional microbiological methods were used to identify isolated organisms. The Cochran-Armitage trend test was used for statistical analysis.

Results: The percentage of Gram-positive isolates increased significantly over the years. The percentage Enterobacteriaceae, and *Klebsiella* spp. in particular, decreased over time. *Enterococcus* spp. isolates were cultured more often in recent years.

Conclusions: Whereas Gram-negative bacteria, particularly Enterobacteriaceae, remain the most common isolates from neonatal foals with sepsis, the prevalence of Gram-positive bacteria is increasing. This trend underlines the importance of including antimicrobial drugs active against both Gram-positive and Gram-negative bacteria in treatment protocols while awaiting the results of bacteriological culture and susceptibility tests. The increased prevalence of *Enterococcus* spp. is of concern because antimicrobial susceptibility patterns for enterococci are unpredictable and enterococci can also act as donors of antimicrobial resistance genes to other bacteria.

***Keywords**: Horse; Gram-negative bacteria; Gram-positive bacteria; Enterococcus spp.; Sepsis; Neonatology*

INTRODUCTION

Bacterial infection is a leading cause of death in foals during the first few weeks of life in North America [1]. Signs of sepsis in the foal can initially be subtle and share features in common with other infectious and noninfectious conditions. Onset and nature of clinical signs, which frequently progress rapidly, are influenced by the pathogen involved, immune status of the foal and other factors [2, 3]. Infection can occur in utero or soon after birth via portals of entry that include the umbilicus, respiratory tract and wounds, although the gastrointestinal (GI) tract is the predominant portal of entry for infection [4-7]. Failure of passive transfer (FPT) of colostral antibody has long been recognised as a risk factor for the development of sepsis [8, 9]. Ingestion of adequate amounts of colostrum during the first few hours of life also plays an important nonimmunological role in preventing acquisition of infection by 'closing the neonatal gut' to translocation of macromolecules, including bacteria [10]. Previous reports documenting that Gram-negative enteric bacteria are the predominant isolates from neonatal foals with sepsis, provide further evidence of the importance of the GI tract as a portal of entry for bacteria [2, 3, 5, 11-20].

Because signs of sepsis typically progress rapidly and frequently lead to death, aggressive early treatment with appropriate antimicrobials and diligent supportive care are necessary to successfully manage infected foals, while awaiting the results of culture and susceptibility tests performed on samples collected from the foal. Selection of antimicrobials for initial treatment should be based, at least in part, on historical information regarding the bacterial species most likely to be isolated from infected foals from a particular farm, facility or geographic location and the susceptibility of these isolates to particular antimicrobials, together with clinical indicators that may be associated with infection with specific groups or species of bacteria [2, 3, 5, 6, 11-20]. Because the antimicrobial activity of different classes of antimicrobial drugs may be predominantly or exclusively restricted to bacteria with specific Gramstaining characteristics, distinguishing foals infected with Gram-positive bacteria from those infected with Gram-negative bacteria would potentially be helpful in guiding selection of antimicrobials for initial treatment [2].

This study represents an extension of work that has been ongoing at the University of California (UC), Davis, USA, for many years, the overall objective of which is to generate quantitative antimicrobial susceptibility data to guide rational selection of antimicrobial drugs for inclusion in treatment protocols for foals with sepsis. The specific aim of the current study is to determine the prevalence of bacteria isolated from foals with sepsis and to evaluate potential temporal trends.

MATERIALS AND METHODS

Case selection

The case records of foals ≤30 days of age, presented to the William R. Pritchard Veterinary Medical Teaching Hospital (VMTH), University of California, Davis, USA, between January 1979 and December 2010, were reviewed. Data recorded in the medical record at admission and during hospitalisation of the foal were retrieved from the Veterinary Medical and Administrative Computing System (VMACS), as used by VMTH personnel at UC Davis, or from stored paper records if the data had not been entered into VMACS.

Records for those foals with a diagnosis of sepsis confirmed by culture of bacteria from blood or multiple internal organs were selected for further evaluation. Foals from which samples were collected by tracheal wash or by swabbing the umbilicus after surgical preparation, were also selected for further evaluation. Cases were included only if they showed clinical or laboratory signs consistent with systemic sepsis (fever [>38.9°C], neutropenia or neutrophilia [<4.0 x 10^9/l or >12.0 x 10^9/l], increased band neutrophil count [>0.05 x 10^9/l], toxic changes in neutrophils, fibrinogenaemia [>4.0 g/l], hypoglycaemia [<4.4 mmol/l], metabolic acidosis, scleral injection, petechiation, anterior uveitis, diarrhoea, respiratory distress or joint swelling). A total of 588 foals met the criteria for inclusion in the study.

Bacterial culture, identification and classification

Samples retrieved from foals with sepsis originated from several locations and 1-3 blood cultures were obtained per foal. Blood (5-10 ml) was aseptically collected from the jugular or cephalic vein or through jugular catheters at the time of placement using aseptic technique (often for first cultures, during recent years) for bacteriologic culture, after removal of hair with a safety razor and preparation of the site with povidone-iodine scrub solution and alcohol. Culture of blood was performed using broth inoculation, with or without antibiotic resin (Trypticase Soy Broth)[a] or by the lysis-centrifugation method (Isolator)[b].

Foals that died or were subjected to euthanasia were examined post mortem, during which samples from internal organs (e.g. liver, kidney, spleen, brain, body cavity or joint) were retrieved aseptically for bacteriological culture. All samples collected ante- or post mortem were submitted to the VMTH Microbiology Diagnostic Laboratory (MDL) at UC Davis for bacterial culture, identification and susceptibility testing. Conventional microbiological methods were used to identify isolated organisms.

When multiple isolates belonging to different bacterial species were cultured from one sample or from samples taken at different points in time or from different locations in a particular foal, all isolates were included in the present study. When multiple isolates of the same bacterial species were retrieved, they were considered to be the same isolate if their colony morphology, biochemical characteristics and antimicrobial susceptibility patterns were identical. Otherwise, isolates from different samples were considered to be different strains of the same bacterial species and both were included in the present study. Using the above criteria, a total of 1091 bacterial isolates from 588 foals were included.

Criteria for determination of time periods for evaluation of temporal trends

Three time periods were established in order to identify potentially significant temporal trends in prevalence of bacterial isolates. Time periods were selected to take into account changes in approaches to antimicrobial use in neonatal foals at UC Davis during the 31 years of the study, as well as the desirability of including similar numbers of bacterial isolates in each time period. The time periods selected were 1979-1990, 1991-1997, and 1998-2010. Prior to 1990, gentamicin was the aminoglycoside antimicrobial of choice for inclusion in treatment regimens for sepsis in foals. A change in the approach to initial antimicrobial therapy was made in 1990 based on publication of a study documenting that a substantially higher proportion of Enterobacteriaceae isolated from foals with sepsis were susceptible to amikacin than to gentamicin [17]. In 1997, an article was published advocating the prophylactic use of antimicrobials in foals that were born unobserved or had recognised risk factors [4]. Subsequent to this publication, veterinarians at the VMTH and in the referral area increased their prophylactic use of antimicrobials in neonatal foals.

Changes in the specific antimicrobial drugs and dosing regimens used to treat foal with sepsis at the VMTH over the years are outlined below:

1970s:

Penicillin G (20,000 – 40,000 iu/kg bwt i.v. q. 6 h) and kanamycin (5 mg/kg bwt i.m. q. 8h) or trimethoprim/sulfamethoxazole (30 mg/kg bwt per os q. 12 h).

Late 1970s and 1980s:

Gentamicin (2.2 mg/kg bwt i.v. q. 8 h or 3.3 mg/kg bwt i.v. q. 12 h) and penicillin G (20,000 – 40,000 iu/kg bwt i.v. q. 6 h) or ampicillin (20 mg/kg bwt i.v. q. 6-8 h).

1990s - present:
Amikacin (7 mg/kg bwt i.v. q. 8 h or 10 mg/kg bwt i.v. q. 12 h until 1995, and 21-25 mg/kg bwt i.v. q 24 h from 1995 until the present) and ampicillin (20 mg/kg bwt i.v. q. 6-8 h), or ceftiofur sodium (5-10 mg/kg bwt i.v. or i.m. q. 12 h).

Data analysis

The Cochran-Armitage trend test was performed to determine temporal differences in prevalence of bacterial isolates between the 3 timeframes: 1979-1990; 1991-1997; 1998-2010. Analysis of this data was performed using commercial software (StatXact Version 9.0)[c]. Results were considered significant if the P value was ≤ 0.05.

RESULTS

Prevalence of bacterial isolates

A total of 1091 bacteria were isolated from 588 foals (Table 1). Three hundred and nine foals (52,6%) had a mixed infection. The mean number of isolates per foal was 1.85 and the median was 1 isolate. Seven hundred and sixty-six (70.2%) of the isolates were Gram-negative bacteria, whereas 325 (29.8%) were Gram-positive bacteria.

Table 1. Prevalence of bacteria causing sepsis in foals admitted to the VMTH, UC Davis, USA, 1979 – 2010

Bacterial species	No. isolates	Percentage of total isolates (%)
Gram-negative bacteria	766	70.2
Enterobacteriaceae	523	47.9
Escherichia coli	314	28.8
Klebsiella spp.	80	7.3
Enterobacter spp.	42	3.8
Salmonella spp.	32	2.9
Proteus spp.	24	2.2
Actinobacillus spp.	152	13.9
Pseudomonas spp.	27	2.5
Other Gram-negative isolates	64	5.9
Gram-positive bacteria	325	29.8
Streptococcus spp.	161	14.6
β-haemolytic streptococci.	103	9.4
Enterococcus spp.	79	7.2
Staphylococcus spp.	58	5.3
Coagulase-positive staphylococci	31	2.8
Coagulase-negative staphylococci	24	2.2
Other Gram-positive isolates	27	2.5

Escherichia coli was the bacterial species most frequently isolated, accounting for 28.8% of all isolates. Other Gram-negative bacteria cultured included *Actinobacillus* spp. (13.9%), *Klebsiella* spp. (7.3%), *Enterobacter* spp. (3.8%), *Salmonella* spp. (2.9%), *Pseudomonas* spp. (2.5%) *and Proteus* spp. (2.2%). Gram-positive organisms included *Streptococcus* spp. (14.8%; 9.4% β-haemolytic streptococci and 5.3% other *Streptococcus* spp.*)*, *Enterococcus* spp. (7.2%) and *Staphylococcus* spp. (5.3%). Other bacteria were cultured less frequently (<1.1%).

Temporal trends in prevalence of bacterial isolates

The numbers of isolates in each time period were as follows: 1979-1990: 328 isolates, 1991-1997: 415 isolates, 1998-2010: 348 isolates. Whereas *E. coli*, *Actinobacillus* spp. and *Streptococcus* spp. remained the most prevalent isolates in all time periods, significant temporal changes were observed with regard to the prevalence of several bacterial species or groups of bacteria (Table 2).

The percentage of Gram-positive isolates increased significantly over the years (1979-1990: 25.9%, 1991-1997: 27.7%, 1998-2010: 35.9%, P value 0.0042).

The percentage of Enterobacteriaceae decreased between the time periods 1991-1997 and 1998 and 2010 (1979-1990: 49.1%, 1991-1997: 52.5%, 1998-2010: 41.4%, P value 0.041). The relative frequency of isolation of *Klebsiella* spp. also decreased over time (1979-1990: 10.4%, 1991-1997: 6.3%, 1998-2010: 5.7%, P value 0.027). *Enterococcus* spp. was cultured more often in the most recent time period: 1998-2010 (1979-1990: 5.5%, 1991-1997: 4.6%, 1998-2010: 12.1%, P value 0.0008). No significant trends in prevalence over time were found for other bacterial species.

Table 2. Temporal trends in prevalence of bacteria causing sepsis in foals admitted to the VMTH, UC Davis, USA, 1979 – 2010

Bacterial species	1979-1990 (n = 328)		1991-1997 (n = 415)		1998-2010 (n = 348)	
	No. isolates	Percentage of total isolates (%)	No. isolates	Percentage of total isolates (%)	No. isolates	Percentage of total isolates (%)
Gram-negative bacteria*	243	74.1	300	72.3	223	64.1
*Enterobacteriaceae**	161	49.1	218	52.5	144	41.4
Escherichia coli	90	27.4	145	34.9	78	22.4
Klebsiella spp.*	34	10.4	26	6.3	20	5.7
Enterobacter spp.	13	4.0	18	4.3	11	3.2
Salmonella spp.	12	3.7	8	1.9	12	3.4
Proteus spp.	7	2.1	8	1.9	9	2.6
Actinobacillus spp.	53	16.2	56	13.5	43	12.4
Pseudomonas spp.	13	4.0	4	1.0	10	2.9
Other Gram-negative isolates	16	4.9	22	5.3	26	7.5
Gram-positive bacteria*	85	25.9	115	27.7	125	35.9
Streptococcus spp.	47	14.3	63	15.2	51	14.7
β-haemolytic streptococci	29	8.8	37	8.9	37	10.6
Enterococcus spp.*	18	5.5	19	4.6	42	12.1
Staphylococcus spp.	14	4.3	22	5.3	22	6.3
Coagulase positive staphylococci	8	2.4	11	2.7	12	3.4
Coagulase negative staphylococci	6	1.8	10	2.4	8	2.3
Other Gram-positive isolates	6	1.8	11	2.7	10	2.9

* *Statistically significant trend P<0.05.*

DISCUSSION

Prevalence of bacterial isolates

Several retrospective studies have evaluated the most common organisms isolated from both blood culture and necropsy specimens from foals with sepsis over the years [2, 3, 5, 11-20]. The subsets of foals included in some of these studies were also included in the current study [2, 17, 20]. The current study covers a longer time span and includes more bacterial isolates than previously published studies.

Although the relative frequency of isolation of different bacterial species varies between different studies, *E. coli* is the bacterial species isolated most commonly from foals with sepsis in all studies published to date. In the current study, the next most commonly isolated species, after *E. coli*, were *Streptococcus* spp., *Actinobacillus* spp., *Klebsiella* spp., *Enterococcus* spp., *Staphylococcus* spp. *and Enterobacter* spp. Potential reasons for differences in results between different studies include different time periods over which the studies were completed, geographical variation in bacterial populations, climate, differences in management practices on breeding farms that may select for or against infection with different bacterial species, differences in use patterns of antimicrobials for both prophylactic and therapeutic purposes, the specific age range of foals included in the studies, the specific samples for which culture information was included and differences in laboratory techniques used between different laboratories or within the same laboratory over different years.

Several aspects of the design of this study could have influenced the results obtained. Prior administration of antimicrobial drugs and hospitalisation before sampling are 2 factors that potentially influence prevalence of bacteria isolated from horses. Not all isolates included in the current study originated from samples collected at the time of admission; several originated from foals that had already been hospitalised for a variable period and others were isolated from samples collected at necropsy. The foals from which these isolates were cultured had typically, although not consistently, received antimicrobial treatment before the samples were obtained. Data on antimicrobial treatment before admission were not consistently available for all cases and could not therefore be taken into account in this study. Additionally, it is not known whether the site of sample collection has any influence on prevalence of bacterial isolates. Such an analysis was judged to be infeasible in this study because of the low number of isolates from most sites.

Temporal trends in prevalence of bacterial isolates

The relative frequency with which Gram-positive organisms were cultured from foals with sepsis increased significantly over time in this study. This finding is consistent with results of one previous study performed between 1986 and 2000 in Georgia, USA [19]. The results of studies performed during the 1980s and the first half of the 1990s in the USA (Florida and Texas) and Australia, in which percentages of Gram-positive isolates of <20% were reported, and more recent studies from the USA (Florida and Pennsylvania) and Australia in which percentages of Gram-positive isolates of 25% or more were reported, also support a trend of increased prevalence of Gram-positive isolates in foals with sepsis [5, 11-16, 18]. The same trend has occurred in human medicine, where from 1979 through 1987, Gram-negative bacteria were the predominant organisms causing sepsis in man, whereas Gram-positive bacteria were reported to predominate in each subsequent year [21].

When comparing results of different prevalence studies it is important to recognise that the collection site of the specimen potentially influences the prevalence of Gram-positive bacteria. A study, published in 1989, showed that proportional distribution of species of bacteria isolated from blood samples was different from the distribution of bacteria isolated from internal organs at post mortem [20]. The same study also showed that blood cultures were more accurate in identifying the presence of Gram-positive isolates than in detecting Gram-negative bacteria. Forty-three per cent of the isolated Gram-negative bacteria (including 60% of the *E. coli* bacteria) in that study went undetected by blood culture alone but were cultured at necropsy, as compared with only 10% of the Gram-positive bacteria that went undetected by blood culture alone.

Several studies that reported prevalence of bacterial isolates originating from foals with sepsis included only blood culture positive cases, which may have led to under-representation of Gram-negative bacteria in comparison with Gram-positive bacteria [14, 16, 18].

The temporal decrease in Gram-negative isolates in general and Enterobacteriaceae in particular, as a percentage of total isolates could potentially be the result of more extensive use in recent years of antimicrobial drugs such as aminoglycosides or third generation cephalosporins that have a predominantly Gram-negative spectrum of activity. This conclusion is supported by the finding in the current study that the most profound change occurred after prophylactic use of antimicrobials with a Gram-negative spectrum of activity became commonplace in foals in the USA in the late 1990s. A decrease in prevalence of Gram-negative enteric organisms in foals with sepsis was

also reported in a study performed between 1982 and 2007 in Florida, USA [18]. It has been hypothesised that the prophylactic use of antimicrobials in newborn foals helps prevent establishment of systemic infection with Gram-negative enteric bacteria that translocate across the intestinal barrier while it is still permeable for large molecules, such as immunoglobulin, but also bacteria [4].

Another possible explanation for the increase in prevalence of Gram-positive organisms is that the emergence and evolution of neonatal intensive care units and advanced critical care techniques in the last 2 decades has allowed critically ill foals that would have previously died or been subjected to euthanasia, to be treated. These foals have relatively long periods of hospitalisation and are therefore potentially more likely to acquire nosocomial infection, which in man often involves Gram-positive bacteria [22, 23]. Because most field-acquired infections are thought to occur through translocation of bacteria from the GI tract during the first few hours of life, they more often involve Enterobacteriaceae [4, 6, 10]. Unfortunately, historical data that would help distinguish between field-acquired and nosocomial infection was not available in the present study and therefore this hypothesis could not be tested. Further research will be necessary to support or refute this hypothesis.

Another potential explanation for the temporal increase in the proportion of Gram-positive isolates is the development of resistance to antimicrobial drugs among Gram-positive isolates [24]. If this is the case, the recent emergence of Enterobacteriaceae that elaborate β-lactamases that inactivate extended spectrum β -lactam antimicrobials and render them resistant to third generation cephalosporins, raises the potential for Gram-negative organisms to again increase in prevalence as a cause of sepsis in the future [24, 25].

In the current study, *Klebsiella* spp. were isolated less frequently in recent years. *Klebsiella* spp. isolates were typically highly susceptible to amikacin and ceftiofur, drugs commonly used in equine practice. Other studies have reported a decrease in the proportion of *Salmonella* spp., *Actinobacillus* spp. and *Streptococcus* spp. isolates in recent years [18, 19]. These trends were not observed in the current study.

The most striking finding in the current study was the substantial increase in prevalence of *Enterococcus* spp. isolates between 1998 and 2010, a trend also reported in a study performed in Georgia USA between 1996 and 2000 [19]. The same trend has been seen in human medicine, where *E. faecium* has increased in importance as a cause of bloodstream infections [26]. *E. faecium* is known to have a higher rate of antimicrobial resistance than other enterococci. In the current study, *E. faecium* was the most

commonly isolated *Enterococcus* species (*E. faecium* n = 37, *E. faecalis* n = 21, other enterococci n = 21); therefore, it is possible that the increased prevalence of enterococci observed in this study could be the result of increased antimicrobial selection pressure for resistance. *Enterococcus* spp. are intrinsically resistant to several antimicrobial drugs and also readily accumulate mutations and exogenous genes that confer additional resistance through plasmids and transposons [27, 28]. Consequently, the susceptibility pattern of *Enterococcus* spp. isolates is unpredictable, which creates a therapeutic challenge for clinicians.

Extensive use of antimicrobials places strong selection pressure on bacterial isolates, favouring selection of resistant species such as *Enterococcus* spp. In a study performed in mice it was demonstrated that treatment with antibiotics perturbs the normal commensal microbiota and sets the stage for intestinal domination by bacteria associated with hospital acquired infections, including *Enterococcus* spp., preceding bloodstream infection [29]. *Enterococcus* spp. are not only a threat to equine health; enterococci from animal origin can also act as donors of antimicrobial resistance genes for other pathogenic enterococci in man [28]. Restrictive and well-considered use of antimicrobial drugs is therefore crucial.

Because regional differences in prevalence of bacteria occur, the trends observed in the current study might be unique to the hospital where this study was performed. However, several trends are similar to those found in studies performed in other parts of the world, suggesting that the results of the current study may be applicable to other geographic locations [18, 19].

Collection of blood and other samples for bacterial culture remains an important component of the diagnostic work-up of foals suspected of having sepsis and also provides data to allow temporal trends in prevalence of individual bacterial species, as well as their antimicrobial susceptibility profiles, to be monitored. Observed trends form the basis for adjustment over time of first choice antimicrobial drugs to initiate treatment of foals with sepsis.

The increasing prevalence of Gram-positive bacteria as a cause of sepsis in neonatal foals, underlines the importance of including antimicrobial drugs active against both Gram-positive and Gram-negative bacteria in treatment protocols while awaiting the results of bacteriological culture and susceptibility tests. The increased prevalence of *Enterococcus* spp. should be of concern to clinicians because enterococci can have very unpredictable susceptibility patterns and can also act as donors of antimicrobial

resistance genes to other bacteria. When managing neonatal foals, excellent hygiene and other measures are necessary to reduce the potential for nosocomial infection.

Authors' declaration of interests

No competing interests have been declared.

Ethical animal research

Ethical review not required by this journal; it is a retrospective study based on clinical records.

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Authorship

All authors have made significant contributions to the completion of this study and have played an import role in drafting and/or revising the final article. All authors have approved the final version submitted for publication.

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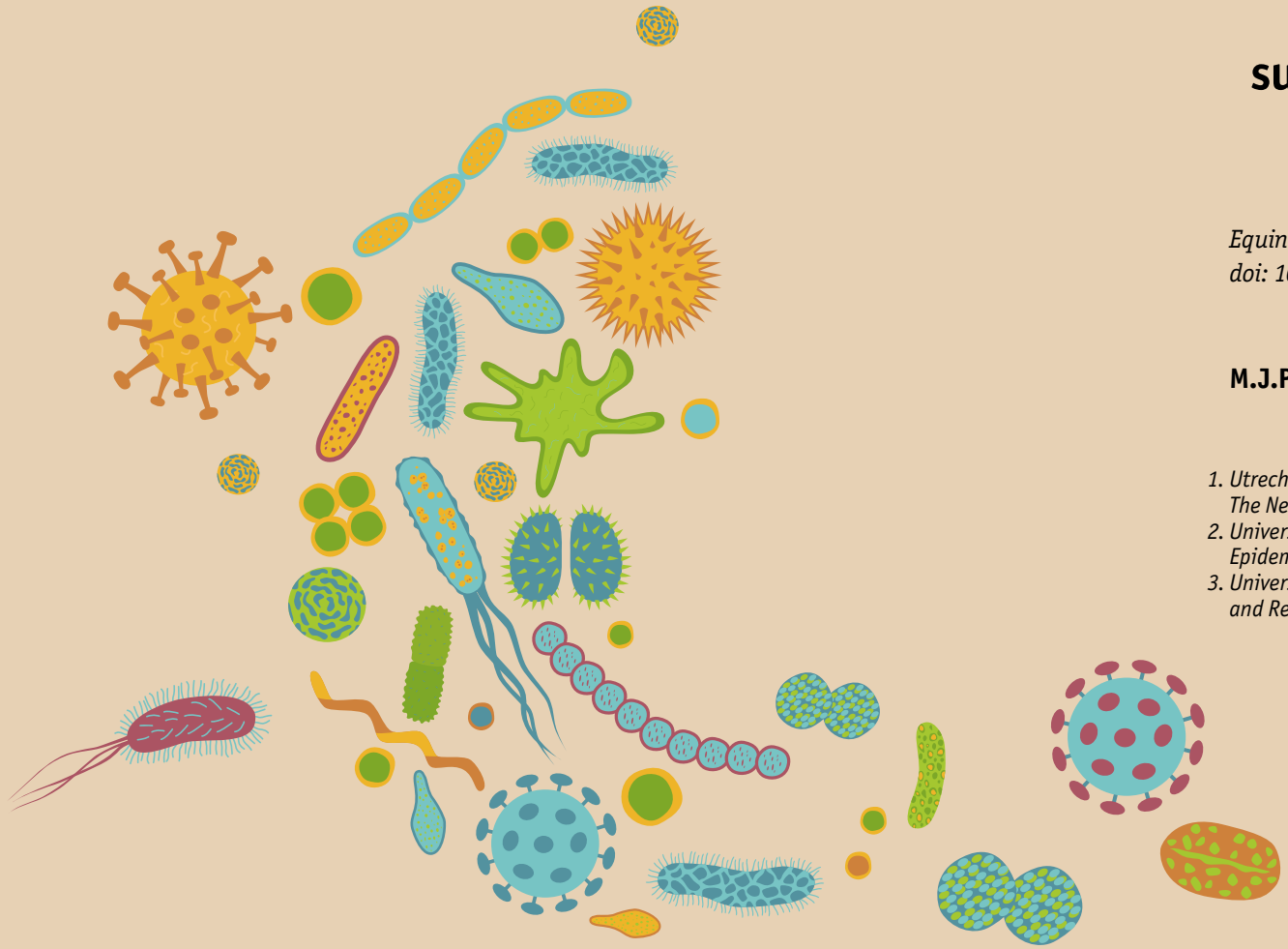

CHAPTER 3

Temporal trends in *in vitro* antimicrobial susceptibility patterns of bacteria isolated from foals with sepsis: 1979 – 2010

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ABSTRACT

Reasons for performing the study: Monitoring the development of antimicrobial resistance is important for the rational selection of appropriate antimicrobial drugs to initiate treatment of foals with sepsis.

Objectives: To identify temporal trends in antimicrobial susceptibility patterns of bacteria isolated from foals with sepsis.

Study design: Retrospective review of medical records.

Methods: Foals aged <30 days with a diagnosis of sepsis, confirmed by culture of bacteria, were included. Susceptibility data, expressed as minimum inhibitory concentrations (MICs) (MIC_{50}, $MIC_{90,}$ MIC range) and percentage of isolates that were susceptible to a particular antimicrobial drug, were compared for bacteria isolated from foals during 3 different time periods: 1979-1990, 1991-1997 and 1998-2010. The Cochran-Armitage trend test and the Jonckheere-Terpstra test were used for statistical analysis.

Results: A total of 1091 bacterial isolates were cultured from 588 foals. Enterobacteriaceae, *Actinobacillus* spp. and β-haemolytic *Streptococcus* spp. showed a decrease in percentage of isolates susceptible to gentamicin over time. Enterobacteriaceae, *Actinobacillus* spp. and β-haemolytic *Streptococcus* spp. showed an increase in MIC values for amikacin. Enterobacteriaceae showed a decrease in percentage of isolates susceptible to ceftiofur. *Enterococcus* spp. and *Pseudomonas* spp. showed increased MIC values to ceftiofur. Enterobacteriaceae showed increased MIC values to ceftizoxime. *Enterococcus* spp. became more resistant to imipenem and showed increased MIC values to ticarcillin/clavulanic acid. In contrast, several trends in increased susceptibility were also seen.

Conclusions: Based on these in vitro results, the combination of amikacin and ampicillin remains an appropriate choice for initiating treatment of sepsis in foals while awaiting culture and susceptibility test results, although increasing development of resistance to amikacin was demonstrated. The decrease in in vitro activity of ceftiofur against Enterobacteriaceae is of concern. Similarly, the development of resistance of *Enterococcus* spp. to imipenem is an important finding that warrants monitoring in the future. Judicious use of antimicrobials is therefore crucial.

***Keywords**: Horse; Neonate; Temporal trends; Septicaemia; Enterococcus spp.*

INTRODUCTION

Genes coding for antimicrobial resistance are present in bacteria cultured from horses [1, 2]. The emergence of bacteria that are resistant to antimicrobials could have significant health implications for horses, including foals, and must be monitored by susceptibility testing of bacteria isolated from appropriate samples collected from clinical patients, as well as from animals free from disease [1, 2]. Selection of antimicrobials for initial treatment is typically based on knowledge of susceptibility patterns of bacteria previously isolated from horses with the same or similar disease syndromes. Several factors, including hospitalisation and prior use of antimicrobials on the farm of origin, influence these susceptibility patterns [3-5]. Over time, trends of increasing or decreasing susceptibility of bacterial isolates to particular antimicrobial drugs can be observed. It is, therefore, necessary to regularly re-evaluate antimicrobial susceptibility profiles in order to provide the clinician with up-to-date information on which to base rational selection of antimicrobials for use in initial treatment protocols. This is particularly important when treating rapidly progressive life-threatening infections such as sepsis in foals.

Antimicrobial susceptibility patterns of bacterial isolates from foals with sepsis have been presented in several reports dating back to 1982 [6-12]. However, only 2 reports describe temporal trends in susceptibility patterns of bacterial isolates, and in only one of these studies are susceptibility data specified to the level of individual bacterial species [7, 12]. None reported susceptibility data in the form of minimum inhibitory concentrations (MICs).

This study represents an extension of work that has been ongoing at the University of California (UC) Davis for many years, the overall objective of which is to generate quantitative antimicrobial susceptibility data to guide rational selection of antimicrobial drugs for inclusion in treatment protocols for foals with sepsis. The goal of the current study was to document temporal trends in antimicrobial susceptibility patterns of bacteria isolated from foals diagnosed with sepsis.

MATERIALS AND METHODS

Case selection

The case records of foals ≤30 days of age presented to the William R. Pritchard Veterinary Medical Teaching Hospital (VMTH), UC Davis, USA, between January 1979 and December 2010, were reviewed. Data recorded in the medical record at admission and during hospitalisation of the foal were retrieved from the Veterinary Medical and

Administrative Computing System (VMACS), as used by VMTH personnel at UC Davis, or from stored paper records if the data had not been entered into VMACS.

Records for those foals with a diagnosis of sepsis confirmed by culture of bacteria from blood or multiple internal organs were selected for further evaluation. Foals from which samples were collected by tracheal wash or by swabbing the umbilicus after surgical preparation, were also selected for further evaluation. Cases were included only if they showed clinical or laboratory signs of systemic sepsis (fever [>38,9 °C], neutropenia or neutrophilia [<4.0 x 10^9/l or >12.0 x 10^9/l], increased band neutrophil count [>0.05 x 10^9/l], toxic changes in neutrophils, hyperfibrinogenaemia [>4.0 g/l], hypoglycaemia [<4.4 mmol/l], metabolic acidosis, scleral injection, petechiation, anterior uveitis, diarrhoea, respiratory distress or joint swelling). A total of 588 foals met the criteria for inclusion in the study.

Bacterial culture, identification and classification

Samples retrieved from foals with sepsis originated from several locations. For blood culture, up to 3 blood samples (5-10 ml each) were collected aseptically from the jugular or the cephalic vein, either by venepuncture or through an i.v. catheter. Culture of blood was performed using broth inoculation, with or without antibiotic resin (Tryp-

ticase Soy Broth)[a], or by the lysis-centrifugation method (Isolator)[b].

Foals that died or were subjected to euthanasia were given post mortem examination, during which samples from internal organs (e.g. liver, kidney, spleen, brain, body cavity or joint) were retrieved aseptically for bacteriological culture. All samples collected ante- or post mortem were submitted to the VMTH Microbiology Diagnostic Laboratory (MDL) at UC Davis for bacterial culture and identification using conventional microbiological methods. All isolates, including those collected at necropsy, were saved as frozen stabilates at -80°C in skimmed milk or on Microbank beads[c] and were available for later susceptibility testing.

Antimicrobial susceptibility testing

Susceptibility testing of isolates was performed using the microdilution Sensititre[d] procedure, following Clinical Laboratory Standards Institute (CLSI) protocols [13, 14]. One bacterial colony was inoculated into brain-heart infusion broth and incubated for 4 h at 35°C. A small amount of this inoculated broth was then added to 0.85% NaCl solution to achieve a 0.5 McFarland Standard concentration, as measured using a nephlometer. A 10 µl sample of this suspension was then added to Mueller Hinton broth, and Sensititre plates (prior to 1988, plates were prepared manually) were inoculated with 100 µl of the Mueller Hinton broth in each well. The MIC was recorded as the lowest concentration of antimicrobial drug that inhibited visible growth of bacteria.

The following bacterial strains were run weekly as controls in accordance with the standard quality control procedures in place at the MDL: *Staphylococcus aureus ATCC 29213, Enterococcus faecalis ATCC 29212, Escherichia coli ATCC 25922, E. coli ATCC 35218* and *Pseudomonas aeruginosa ATCC 27853*.

Sensititre plates were custom made for the UC Davis MDL. Bacterial isolates were tested for susceptibility to 15 antimicrobial drugs. An isolate was considered to be susceptible to a particular antimicrobial drug if its MIC value was less than or equal to the clinical MIC susceptibility breakpoint for that antimicrobial, as determined by the CLSI, occasionally modified by equine-specific interpretations based on research performed in horses [13, 14].

For bacteria isolated from samples collected ante mortem, antimicrobial susceptibility testing was performed at the time of the patient's visit. Because not all the antimicrobial drugs included in this study were available during the earlier time periods, it was necessary to retrieve some isolates from the lyophilised repository at a later date for susceptibility testing against these antimicrobials. For example, ceftiofur was not added to the panel of antimicrobials tested until 1988, and imipenem was not added until 1992. Bacteria isolated from samples collected at necropsy were all susceptibility tested retrospectively after re-culturing from the lyophilised repository and confirming the identity of the isolate.

When multiple isolates of the same bacterial species were retrieved from different samples or locations in a particular patient, they were considered to be the same isolate if their colony morphology, biochemical characteristics and antimicrobial susceptibility patterns were identical. Otherwise, isolates from different sites were considered to be different strains of the same bacterial species and both were included in the present study. Using the above criteria, a total of 1091 bacterial isolates were cultured from 588 foals.

Antimicrobial drugs

The following 15 antimicrobial drugs were analysed for activity against specific species of bacteria: amikacin, ampicillin, ceftiofur, ceftizoxime, cefalothin, chloramphenicol, enrofloxacin, erythromycin, gentamicin, imipenem, penicillin, rifampicin, tetracycline, ticarcillin/clavulanic acid and trimethoprim/sulfamethoxazole (TMS). The drugs included in this study that are designated as critically important antimi-

crobials by the World Health Organization were: ceftiofur, ceftizoxime, enrofloxacin, erythromycin and imipenem [15, 16].

Time periods for evaluation of temporal trends

The following 3 time periods were established in order to identify potentially significant temporal trends in the antimicrobial susceptibility profiles of bacterial isolates: 1979-1990, 1991-1997, and 1998-2010. These time periods were selected to take into account changes in approaches to antimicrobial use in neonatal foals at UC Davis during the 31 years of the study.

Data analysis

The Jonckheere-Terpstra test was used to detect significant temporal changes over time in MIC_{50} and MIC_{90}, and the Cochran-Armitage trend test was used to detect significant temporal trends in the percentage of isolates susceptible (%S) to a particular antimicrobial over the 3 time periods, Analysis of these data was performed using commercial software (StatXact Version 9.0)[e]. Results were considered significant if $P \leq 0.05$.

RESULTS

Cumulative susceptibility patterns of bacterial isolates over the entire timeframe of the study (1979-2010)

Escherichia coli and *Klebsiella* spp. isolates were highly susceptible (>90%) in vitro to amikacin, ceftiofur, ceftizoxime, enrofloxacin and imipenem. *Enterobacter* spp. isolates were highly susceptible in vitro to amikacin, enrofloxacin and imipenem and *Salmonella* spp. isolates were highly susceptible in vitro to amikacin, ceftizoxime, cefalothin, enrofloxacin and imipenem. More than 87.5% of *Actinobacillus* spp. isolates were susceptible to all antimicrobials tested, except for erythromycin and penicillin. *Pseudomonas* spp. isolates were found to be highly resistant to most of the antimicrobials tested, although amikacin (88.9%), ticarcillin/clavulanic acid (88.0%) and imipenem (82.6%) were active against >80% of the isolates.

With regard to Gram-positive isolates, more than 90% of β-haemolytic *Streptococcus* spp. isolates were susceptible in vitro to cefalothin, ceftizoxime, imipenem, ceftiofur, rifampicin, penicillin, chloramphenicol, ampicillin, erythromycin and TMS. More than 90% of *Staphylococcus* spp. isolates were susceptible to imipenem, amikacin, cefalothin and enrofloxacin. In addition, more than 90% of coagulase-negative *Staphylococcus* spp. isolates were susceptible to chloramphenicol and rifampicin. *Enterococcus* spp. isolates were highly resistant to most antimicrobials tested in the current study; none

of the antimicrobials showed a susceptibility percentage of >90%. Ampicillin (72.4% susceptible) and chloramphenicol (75.7%) showed the highest level of in vitro activity against *Enterococcus* spp.

Cumulative susceptibility data on Gram-negative, Gram-positive and all isolates combined are presented in Table 1. Susceptibility data specified to the level of individual bacterial species are presented in Supporting Information Items S1-S12.

Temporal trends in susceptibility patterns of bacterial isolates

The numbers of isolates included in each period of time were as follows: 1979-1990: 328 isolates, 1991-1997: 415 isolates, 1998-2010: 348 isolates. Two different temporal trends were analysed. The first was a significant difference in the percentage of isolates that were categorised as being susceptible or resistant to a particular antimicrobial drug based on the clinical MIC breakpoint for susceptibility. The second temporal trend was a significant change in MIC values over time, reflected in an increase or decrease in values for MIC_{50}, MIC_{90} and MIC range limits. An increase in MIC values potentially indicates a trend towards the incremental development of resistance, but is not always reflected as a significant change in the percentage of isolates that are categorised as susceptible or resistant based on the clinical MIC breakpoint. A temporal trend of increased MIC values could potentially be regarded as an 'early warning' sign for the development of resistance [17]. The opposite can also be seen: a decrease in MIC values over time may indicate a trend towards the development of increased susceptibility.

Statistically significant temporal trends in increased MIC values and decreased percentage of isolates classified as being susceptible to particular antimicrobial drugs can be found in Table 2. Enterobacteriaceae (and the subgroup *Enterobacter* spp.), *Actinobacillus* spp. and β-haemolytic *Streptococcus* spp. showed an increase in MIC values for amikacin. In the group β-haemolytic *Streptococcus* spp. this trend was reflected in a decrease in the percentage of susceptible isolates. For the other aminoglycoside, gentamicin, increased MIC values were found in the group of the Enterobacteriaceae (and the subgroup *E. coli*) and in *Actinobacillus* spp. A decreased percentage of isolates in the group Enterobacteriaceae (and the subgroup *Salmonella* spp.), *Actinobacillus* spp. and β-haemolytic *Streptococcus* spp. were susceptible to gentamicin over time.

Table 1. Temporal and cumulative susceptibility of Gram-negative, Gram-positive and all isolates 1979-2010

	Gram-negative isolates				Gram-positive isolates				All isolates			
Antimicrobial Drug	**1979-1990 %S (n)**	**1991-1997 %S (n)**	**1998-2010 %S (n)**	**Total %S (n)**	**1979-1990 %S (n)**	**1991-1997 %S (n)**	**1998-2010 %S (n)**	**Total %S (n)**	**1979-1990 %S (n)**	**1991-1997 %S (n)**	**1998-2010 %S (n)**	**Total %S (n)**
Amikacin	98.5% (202)	95.9% (271)	96.3% (190)	96.8% (663)	54.8%* (42)	45.8%* (59)	39.7%* (58)	45.9%* (159)	91.0%* (244)	87.0%* (330)	83.1%* (248)	87.0%* (822)
Ampicillin	55.1% (225)	59.4% (271)	53.4% (193)	56.3% (689)	81.1% (53)	76.1% (71)	77.8% (90)	78.0% (214)	60.1% (278)	62.9% (342)	61.1% (283)	61.5% (903)
Ceftiofur	91.7% (156)	94.1% (272)	84.7% (190)	90.6% (618)	86.5%* (37)	92.7%* (55)	93.0%* (57)	91.3%* (149)	90.7%* (193)	93.9%* (327)	86.6%* (247)	90.7%* (767)
Ceftizoxime	91.0% (188)	95.6% (272)	91.7% (181)	93.1% (641)	90.0%* (40)	94.8%* (58)	92.9%* (56)	92.9%* (154)	90.8%* (228)	95.5%* (330)	92.0%* (237)	93.1%* (795)
Cefalothin	64.1% (209)	48.2% (272)	66.0% (156)	57.8% (637)	100%* (37)	96.5%* (57)	100%* (47)	98.6%* (141)	69.5%* (246)	56.5%* (329)	73.9%* (203)	65.2%* (778)
Chloramphenicol	74.0% (227)	69.9% (276)	75.3% (182)	72.7% (685)	80.3% (61)	91.0% (78)	89.2% (93)	87.5% (232)	75.3% (288)	74.6% (354)	80.0% (275)	76.4% (917)
Enrofloxacin	92.6% (150)	97.1% (272)	94.0% (182)	95.0% (604)	58.7% (46)	59.7% (77)	54.9% (91)	57.5% (214)	84.7% (196)	88.8% (349)	81.0% (273)	85.2% (818)
Erythromycin	NA	NA	NA	NA	61.8% (55)	70.3% (74)	63.4% (93)	65.3% (222)	NA	NA	NA	NA
Gentamicin	82.4% (221)	77.7% (264)	71.4% (189)	77.4% (674)	57.5%* (40)	43.9%* (57)	32.2%* (59)	42.9%* (156)	78.5%* (261)	71.7%* (321)	62.1%* (248)	71.0%* (830)
Imipenem	99.3% (149)	99.6% (247)	98.1% (160)	99.1% (556)	95.8% (48)	91.2% (68)	83.8% (80)	89.3% (196)	98.5% (197)	97.8% (315)	93.3% (240)	96.5% (752)
Penicillin	NA	NA	NA	NA	72.5%* (40)	72.4%* (58)	82.8%* (58)	76.3%* (156)	NA	NA	NA	NA
Rifampicin	NA	NA	NA	NA	76.5% (51)	75.3% (77)	69.8% (96)	73.2% (224)	NA	NA	NA	NA
Tetracycline	57.1% (217)	62.8% (266)	72.1% (183)	63.5% (666)	63.2% (57)	63.2% (76)	66.3% (95)	64.5% (228)	58.4% (274)	62.9% (342)	70.1% (278)	63.8% (894)
Ticarcillin/ clavulanic acid	82.1% (151)	79.1% (277)	82.7% (196)	80.9% (624)	NA	NA	NA	NA	NA	NA	NA	NA
Trimethoprim/ sulfamethoxazole	69.1% (220)	70.6% (272)	68.1% (191)	69.4% (683)	90.5%* (42)	87.9%* (58)	84.5%* (58)	87.3%* (158)	72.5%* (262)	73.6%* (330)	71.9%* (249)	72.8%* (841)

*n = number of isolates tested; %S = percentage of susceptible isolates; NA = not available; * = Enterococcus spp. isolates not included*

Table 2. Statistically significant temporal trends (P<0.05) in increased minimum inhibitory concentrations (MIC) values and decreased susceptibility of bacterial isolates to antimicrobial drugs

Antimicrobial drug	Increase in MIC values	Decrease in percentage susceptible isolates
Amikacin	Enterobacteriaceae *Enterobacter* spp. *Actinobacillus* spp. β-hemolytic *Streptococcus* spp.	β-hemolytic *Streptococcus* spp.
Ceftiofur	Enterobacteriaceae *Pseudomonas* spp. *Enterococcus* spp.	Enterobacteriaceae *E. coli*
Ceftizoxime	Enterobacteriaceae *E. coli* *Salmonella* spp.	
Gentamicin	Enterobacteriaceae *E. coli* *Actinobacillus* spp.	Enterobacteriaceae *Salmonella* spp. *Actinobacillus* spp. β-hemolytic *Streptococcus* spp.
Imipenem		*Enterococcus* spp.
Ticarcillin/ Clavulanic acid	*Enterococcus* spp.	

Concerning the cephalosporins, an increase in MIC values for ceftiofur was observed for Enterobacteriaceae, *Pseudomonas* spp. and *Enterococcus* spp. In the group of Enterobacteriaceae (and the subgroup *E. coli*), a decreased percentage of isolates were susceptible to ceftiofur over time. Increased MIC values for ceftizoxime were found for Enterobacteriaceae (and subgroups *E. coli* and *Salmonella* spp.). These increased MIC values were not, however, reflected in a decreased percentage of susceptible isolates in these groups. *Enterococcus* spp. showed a significant decrease in percentage of isolates susceptible to imipenem over time and also showed increased MIC values for ticarcillin/clavulanic acid.

Statistically significant temporal trends in decreased MIC values and increased antimicrobial susceptibility can be found in Table 3. Enterobacteriaceae (and the subgroups *E. coli* and *Salmonella* spp.) showed an increase in percentage of isolates susceptible to tetracycline over time. *Actinobacillus* spp. showed an increase in percentage of isolates susceptible to ampicillin, penicillin and rifampicin and also showed a decrease in MIC values for erythromycin. *Staphylococcus* spp. (and the subgroup coagulase-positive *Staphylococcus* spp.) showed an increase in percentage of isolates susceptible to chloramphenicol. Coagulase-negative *Staphylococcus* spp. showed an increase in percentage of isolates susceptible to ceftiofur and penicillin. *Pseudomonas* spp. showed an increase in percentage of isolates susceptible to gentamicin and *Klebsiella* spp. showed a decrease in MIC values for ampicillin.

Table 3. Statistically significant temporal trends (P<0.05) in decreased minimum inhibitory concentrations (MIC) values and increased susceptibility of bacterial isolates to antimicrobial drugs

Antimicrobial drug	Decrease in MIC values	Increase in percentage susceptible isolates
Ampicillin	*Klebsiella* spp.	*Actinobacillus* spp.
Ceftiofur		Coagulase-negative *Staphylococcus* spp.
Chloramphenicol		*Staphylococcus* spp. Coagulase-positive *Staphylococcus* spp.
Erythromycin	*Actinobacillus* spp.	
Gentamicin		*Pseudomonas* spp.
Penicillin	*Actinobacillus* spp.	*Actinobacillus* spp. Coagulase-negative *Staphylococcus* spp.
Rifampicin	*Actinobacillus* spp.	*Actinobacillus* spp.
Tetracycline	Enterobacteriaceae *E. coli* *Klebsiella* spp.	Enterobacteriaceae *E. coli* *Klebsiella* spp.

Temporal susceptibility data on Gram-negative, Gram-positive and all isolates combined are presented in Table 1. The complete data on susceptibility of isolates in each time group, specified to the level of bacterial species and including the temporal differences are presented in Supporting Information Item S1-S12.

DISCUSSION

Some of the drugs evaluated in this study are classified as critically important antimicrobials by the World Health Organization, which means they are regarded as critically important to human health [15]. As clearly outlined in the antimicrobial stewardship policy of the Equine Veterinary Journal, these drugs should be reserved for cases where no other alternatives are effective and only after appropriate susceptibility testing or when evidence for their use in certain diseases is compelling [16].

Cumulative susceptibility of bacterial isolates 1979-2010

The finding that more than 90% of Enterobacteriaceae in this study were susceptible in vitro to amikacin confirms its utility as a first choice antimicrobial for treating Gram-negative sepsis in foals. Enterobacteriaceae also showed a high level of susceptibility to enrofloxacin, imipenem and, to a lesser extent, ceftizoxime and ceftiofur, indicating that these drugs could be useful alternatives to amikacin under special circumstances in which the use of an aminoglycoside is contraindicated or the infecting organism is resistant to amikacin. Imipenem should be reserved to treat highly resistant infections in horses and should not be used as a first choice antimicrobial drug [18]. Enrofloxacin may induce arthropathy when used in neonatal foals;

therefore, its use should be avoided unless other options are not feasible [19, 20]. Third generation cephalosporin antimicrobials such as ceftiofur or ceftizoxime are therefore preferred for use in cases where administration of an aminoglycoside is contraindicated, such as in foals with azotaemia, dehydration or renal failure, or in those that are being treated concurrently with other nephrotoxic drugs.

Gram-positive organisms showed considerable differences in susceptibility patterns. As ampicillin is the drug most commonly used to treat Gram-positive sepsis in foals, it should be recognised that only β-haemolytic streptococci showed a high percentage of isolates to be susceptible to this drug (94.6%: 1979-2010). Ampicillin is also one of the drugs with the highest level of in vitro susceptibility (72.4%: 1979-2010) against *Enterococcus* spp. Data on the susceptibility of *Enterococcus* spp. to penicillin are not presented because the breakpoint currently used for *Enterococcus* spp. was outside the range of concentrations tested during several of the years included in this study. Although *Enterococcus* spp. isolates have been reported to be highly susceptible in vitro to aminoglycosides, cephalosporins and TMS, it has also been reported that in vitro results for these antimicrobials often do not translate into effectiveness in vivo [13, 14]. Therefore, only MIC values, and not %S *Enterococcus* spp. isolates, are reported for these drugs in the Supporting Information Items. *Enterococcus* spp. are known to be intrinsically resistant to several antimicrobial drugs, and also readily accumulate mutations and exogenous genes that confer additional resistance through plasmids and transposons [21, 22]. The susceptibility pattern of *Enterococcus* spp. isolates is highly unpredictable and can pose a real therapeutic challenge for clinicians [21, 22]. Extensive use of antimicrobials imposes a strong selection pressure on *Enterococcus* spp. and other bacterial species and favours the selection of resistant strains [23].

When interpreting the results presented for β-haemolytic streptococci, one should keep in mind that despite in vitro activity of TMS against β-haemolytic streptococci, this drug has previously been shown to be ineffective in eradicating *Streptococcus equi* ssp. *zooepidemicus* in vivo in horses [24, 25].

Although several published studies have reported susceptibility data for bacteria isolated from foals with sepsis [6-12], few studies have presented susceptibility data for individual bacterial species [6, 9-12], and none have reported MIC values for these isolates. Additionally, the number of isolates tested in most of these studies was relatively low (Brewer and Koterba [10] n = 108; Marsh and Palmer [6] n = 203; Russell et al [9] n = 124; Hollis et al [11] n = 75), making meaningful comparisons with the results of our study difficult [6, 9-11]. Another factor that complicates comparison between studies is the method used to determine susceptibility – either disc diffusion techniques or a breakpoint inhibitory concentration system – and the breakpoints used. It is important to recognise that breakpoints are subject to revision by CLSI over time and therefore could be the primary cause of differences in percentages of bacteria reported as being susceptible when studies performed in different years and at different facilities are compared. Because the actual interpretive breakpoints used in the studies referenced above were not stated, it is not possible to determine whether this important factor influenced the results obtained [6, 9-11]. This underlines the importance of presenting MIC values in reports on antimicrobial susceptibility because it allows the data to be reinterpreted when recommended interpretive breakpoints for susceptibility change over time. The number of Gram-negative enteric bacteria included in the study by Sanchez et al. [12] was higher (n = 274), permitting a more meaningful comparison. Enterobacteriaceae isolated in our study appeared to be less susceptible to chloramphenicol (68.6% vs. 84.6%), gentamicin (75.3% vs. 92.1%), tetracycline (56% vs. 76.4%) and TMS (63.8% vs. 80.4%) than in the study reported by Sanchez et al. [12]. Percentages of Gram-negative enteric isolates that were susceptible to amikacin, ampicillin, ceftiofur, enrofloxacin and imipenem were similar in both studies. The differences between the results of the cited study and our study could reflect geographical or management factors or differences in usage of antimicrobial drugs that could affect the selection pressure for resistance. The year in which a study is completed is also likely to influence susceptibility results because temporal changes in susceptibility can be expected, as documented in the current study. Because our study and the one reported by Sanchez et al. [12] were conducted over a similar timeframe, this factor is unlikely to account for observed differences in results between the 2 studies.

Temporal trends in susceptibility patterns

Three time periods were established in order to identify potentially significant temporal trends in antimicrobial susceptibility profiles of bacterial isolates. Time periods were selected to take into account changes in approaches to antimicrobial use in neonatal foals at UC Davis during the 31 years of the study. The time periods selected were 1979-1990, 1991-1997 and 1998-2010. Prior to 1990, gentamicin was the aminoglycoside antimicrobial of choice for inclusion in treatment regimens for sepsis in foals. A change in the approach to initial antimicrobial therapy was made in 1990 based on the publication of a study by Wilson et al. [8] documenting that a substantially higher proportion of Enterobacteriaceae isolated from foals with sepsis were susceptible to amikacin than to gentamicin. In 1997, Madigan published an article in which he advocated the use of antibiotics prophylactically in foals that were born unobserved or had recognised risk factors [26]. This publication caused veterinarians in the field and at the VMTH to increase the prophylactic use of antibiotics in neonatal foals.

Changes in the commonly prescribed or first line antimicrobial drugs and dosing regimens used to treat foal sepsis at the VMTH over the years are outlined below:

1970s:
Penicillin G (20,000 – 40,000 iu/kg bwt i.v. q. 6 h) and kanamycin (5 mg/kg bwt i.m. q. 8 h) or trimethoprim/sulfamethoxazole (30 mg/kg bwt per os q. 12 h)

Late 1970s and 1980s:
Gentamicin (2.2 mg/kg bwt i.v. q. 8 h or 3.3 mg/kg bwt i.v. q. 12 h) and penicillin G (20,000 – 40,000 iu/kg bwt i.v. q. 6 h) or ampicillin (20 mg/kg bwt i.v. q. 6-8 h).

1990s - present:
Amikacin (7 mg/kg bwt i.v. q. 8 h or 10mg/kg bwt i.v. q. 12 h until 1995, and 21-25 mg/kg i.v. q 24 h from 1995 until the present) and ampicillin (20 mg/kg bwt iv q. 6-8 h), or ceftiofur sodium (5-10 mg/kg bwt i.v. or i.m. q. 12 h).

The breakpoint inhibitory concentration system was used consistently throughout all years of our study, as were the breakpoints used to classify isolates as susceptible or resistant. Therefore, observed temporal changes in susceptibility data could not be ascribed to changes in methodology or to revisions of CLSI-recommended breakpoints over time. An additional feature of the current study was that susceptibility results were reported as quantitative MIC ranges, MIC_{50} and MIC_{90}, as well as in the form of categorical (susceptible vs. resistant) data based on established breakpoints. There are several advantages to this approach [17, 27]. First, the development of bacterial resistance to a particular antimicrobial may be incremental and result in changes in MIC range, MIC_{50} and MIC_{90} for that bacterial species, but not a change in the percentage of isolates classified as susceptible or resistant based on a particular breakpoint. These parameters may therefore be more sensitive early indicators of the development of resistance. Additionally, CLSI-recommended changes in MIC breakpoints over time do not influence raw MIC values, whereas they may influence calculated percentages of susceptible and resistant organisms. From a clinical standpoint, another important advantage of quantitative MIC data over qualitative susceptibility data is that the dose and dosing frequency for a particular antimicrobial can be adjusted, either up or down, to better customise the treatment protocol to the specific bacterial isolate from a particular case.

Several aspects of the design of this study could have influenced the results obtained. Susceptibility testing was not performed at the same time for all isolates included in this study. Some isolates were tested at the time of collection; others were analysed

after storage as frozen stabilates at -80°C. Whereas storage is unlikely to have influenced antimicrobial susceptibility test results, the true impact is not known. Prior administration of antimicrobial drugs and hospitalisation before sampling can influence susceptibility profiles of bacteria isolated from horses [5]. Not all isolates included in the current study originated from samples that were collected at the time of admission: several were from foals that had already been hospitalised for a variable period and others were isolated from samples collected at necropsy. The foals from which these isolates were cultured had typically, although not consistently, received antimicrobial treatment before the samples were obtained. Data on antimicrobial treatment before admission were not consistently available for all cases and could not therefore be taken into account. Whereas the inclusion of bacteria isolated from samples collected at necropsy from foals that died or were subjected to euthanasia may have influenced the overall susceptibility test results for the entire timeframe of the study, it is unlikely that inclusion of these isolates influenced the observed temporal trends in antimicrobial susceptibility. The proportion of isolates originating from necropsy samples was similar for all 3 time periods (1979-1990: 29.6%; 1991-1997: 31.3%; 1998-2010: 20.4%). It is not known whether the site of sample collection has any influence on susceptibility patterns of bacterial isolates. Such an analysis was judged to be infeasible in this study because of the low number of isolates from most sites.

The development of resistance to aminoglycosides was clearly evident over the years of this study. Decreased susceptibility of Enterobacteriaceae (and the subgroup *Salmonella* spp.), *Actinobacillus* spp. and β-haemolytic streptococci to gentamicin probably reflects the extensive use of gentamicin in foals and mature horses in California during the 1970s and 1980s, and has led clinicians in most parts of the USA to replace gentamicin with amikacin as the first choice antimicrobial for treating foal sepsis while awaiting culture and susceptibility test results. It should be recognised, however, that clinicians in some other parts of the world continue to regard gentamicin as the drug of choice for treating foals with Gram-negative sepsis. Such an approach is rational as long as the susceptibility of Gram-negative bacterial isolates to gentamicin in the particular location is carefully monitored.

The increase in MIC values of Enterobacteriaceae (and the subgroup *Enterobacter* spp.), *Actinobacillus* spp. and β-haemolytic *Streptococcus* spp. for amikacin, the drug currently used most commonly to treat Gram-negative infections in foals in the USA, as detected in this study, is of concern. Although for Enterobacteriaceae (and the subgroup *Enterobacter* spp.) and *Actinobacillus* spp. the change in MIC values has not yet led to a significant reduction in the percentage of bacteria that are classified as susceptible to amikacin, this finding may be an 'early warning' sign for the develop-

ment of resistance to amikacin [17]. The use of amikacin has expanded since the early 1990s, partially in response to a publication by Wilson et al. [8] reporting that a higher portion of Enterobacteriaceae were susceptible to amikacin than to gentamicin. The increased use of amikacin has presumably induced a selection pressure for resistance. Cross-resistance among aminoglycosides has been reported, although this is rarely seen for amikacin [28]. The number of amikacin-inactivating enzymes elaborated by bacteria is much lower than the number of inactivating enzymes for gentamicin, which may explain why the decrease in susceptibility of Gram-negative organisms to gentamicin occurs more rapidly than does the development of resistance to amikacin [28]. Ongoing monitoring of resistance will be important to determine whether amikacin will remain a reliable first choice antimicrobial for treating foal sepsis in the future.

Owing to the nephrotoxic potential of aminoglycosides, third generation cephalosporins are commonly used as an alternative antimicrobial treatment in foals affected by azotaemia, dehydration or renal failure. Ceftiofur is the most commonly used drug in this class. Enterobacteriaceae as a group (and the subgroup *E. coli*), showed a decrease in percentage susceptible isolates to ceftiofur over time. Other bacteria, such as *Pseudomonas* spp. and *Enterococcus* spp., showed a significant increase in MIC values for ceftiofur, indicating that incremental development of resistance is occurring. This decrease in in vitro activity of ceftiofur is of concern and suggests that ceftiofur will potentially be less effective for treating foals with sepsis caused by Gram-negative enteric organisms than was the case in years past.

Of all the major species of Gram-positive bacteria, *Enterococcus* spp. showed the most significant temporal trends in both MIC values and percentage of susceptible isolates, demonstrating the development of resistance. Particularly noteworthy is the finding that *Enterococcus* spp. showed a decrease in percentage of isolates susceptible to imipenem over time, despite imipenem not being used commonly on horse farms or in our hospital (<5 foals/year in our hospital). Imipenem is used more often in human medicine but is reserved for treatment of special cases in which no other drugs appear to be effective [18]. One hypothesis to explain the above finding would be nosocomial transmission of imipenem-resistant *Enterococcus* spp. from man to horses in the horse farm or equine hospital environment. Proof of this hypothesis would require further research. Besides increased resistance to imipenem, *Enterococcus* spp. also showed a significant increase in MIC values in more recent years for ceftiofur and ticarcillin/clavulanic acid, potentially indicating a trend towards the development of resistance. Resistant *Enterococcus* spp. isolates not only pose a risk to equine health but are also of serious concern in human health care [22]. Sepsis caused by *Enterococcus* spp. in man is often nosocomial in origin and has become more prevalent in recent years [29, 30].

Despite the frequent use of TMS in equine practice, no significant changes in susceptibility were found in the present study.

In contrast to the observed increases in resistance to antimicrobial drugs, reduced resistance (increased susceptibility) was also noted for some antimicrobial drugs, particularly those that are no longer used commonly in foals in California. Enterobacteriaceae (and subgroups *E. coli* and *Klebsiella* spp.) showed an increased percentage of isolates susceptible to tetracycline, to the extent that almost 70% of Enterobacteriaceae were susceptible to tetracycline in 1998-2010. Whereas 70% susceptibility is not sufficient to make tetracycline a suitable alternative first-choice antimicrobial for treating sepsis in foals, this finding is consistent with the results of one previous study [12], and suggests that reduced use of this drug may have led to less selection pressure for the emergence of resistance and, in turn, favoured the re-emergence of susceptible strains. *Staphylococcus* spp. (and subgroup coagulase-positive *Staphylococcus* spp.) showed an increase in percentage of isolates susceptible to chloramphenicol. The use of chloramphenicol is prohibited in large parts of the world owing to legislation and, in our hospital, its use to treat infections in foals has been infrequent during the past 20 years. The sparse use of this drug may have reduced or reversed the selection pressure for the emergence of resistance, resulting in an increase in percentage of staphylococci susceptible to chloramphenicol.

Coagulase-negative *Staphylococcus* spp. showed an increase in percentage susceptible isolates to ceftiofur and penicillin over time, even though both drugs are used commonly in our hospital. The reason for this seemingly paradoxical finding is not clear.

Implications for antimicrobial use in foals

Guidelines and consensus statements have been published in recent years to educate veterinarians about the importance of judicious and rational use of antimicrobials, to assist them in the process of rational antimicrobial selection, and to reduce the likelihood of development of antimicrobial resistance [16, 18]. Although culture and susceptibility testing of bacteria isolated from the particular case prior to initiation of treatment provides the best evidence on which to base antimicrobial selection, these publications acknowledge that antimicrobial drugs will always need to be used empirically in some patients [16, 18]. Empirical choice of antimicrobial drugs should be based on previous experience, knowledge of the agents most likely to be recovered from a particular species with disease in a particular organ system, and knowledge of local resistance patterns [16, 18]. Empirical use of antimicrobials is clearly justified in initial treatment protocols for rapidly progressive, life-threatening conditions such

as systemic sepsis, while awaiting the results of culture and susceptibility tests on samples collected at admission. Historical information regarding the predominant bacterial isolates from septic foals and their susceptibility to antimicrobials in a particular geographic location, as presented in this study, is therefore important to guide the empirical selection of antimicrobials to initiate treatment of foal sepsis.

Based on the in vitro results of this study, the current first choice combination of amikacin with ampicillin remains an appropriate initial treatment for foal sepsis. Amikacin continues to have a high level of in vitro activity against Gram-negative isolates and *Staphylococcus* spp. Amikacin is strongly preferred over gentamicin, owing to the high level of in vitro resistance of several species of bacteria to gentamicin. Ampicillin remains highly active in vitro against β-haemolytic *Streptococcus* spp. and is one of the drugs with the highest in vitro activity against *Enterococcus* spp. (1998-2010: 70.7% susceptible).

The decrease in in vitro activity of ceftiofur against Enterobacteriaceae over time is of concern and suggests that ceftiofur will potentially be less effective for treating foals with sepsis caused by Gram-negative enteric organisms than was the case in years past. Trimethoprim/sulfamethoxazole is not sufficiently active in vitro against many of the tested isolates to recommend its use to initiate treatment of sepsis in foals.

A high percentage of bacteria were found to be susceptible to imipenem in the current study; therefore, this drug might seem to be an attractive alternative for treatment of bacterial infections in foals. Use of imipenem should, however, be strictly limited to those cases in which infecting bacteria have been shown to be resistant to all feasible alternatives, or other antimicrobial drugs have proven to be ineffective.

It should be recognised that the findings of the current study may be unique to the hospital population at UC Davis. As noted above, climatic conditions and management factors, including the selection pressure imposed by antimicrobial use, will influence resistance patterns of bacteria in the local environment. Together with the knowledge that the susceptibility patterns of Enterobacteriaceae and *Enterococcus* spp., 2 of the most important groups of pathogens causing neonatal sepsis, are inherently unpredictable, the importance of determining the susceptibility of each individual isolate retrieved from a foal with sepsis cannot be overstated. Additionally, the observed changes in susceptibility patterns of groups of bacteria to antimicrobial drugs over time underscores the need for continuous monitoring, as well as judicious antimicrobial use.

Authors' declaration of interests

No competing interests have been declared.

Ethical animal research

Ethical review not required by this journal: it is a retrospective study based on clinical records.

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Authorship

All authors made significant contributions to the completion of this study and have played an important role in drafting and/or revising the final article. All authors have approved the final version submitted for publication.

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SUPPORTING INFORMATION ITEMS

Supporting Information Item S1. Temporal and cumulative susceptibility data of Enterobacteriaceae isolated from foals with sepsis, UC Davis, USA, 1979-2010

Antimicrobial drug	Break-point	1979-1990					1991-1997					1998-2010					Total (1979-2010)				
		N	Range	MIC_{50}	MIC_{90}	%S	N	Range	MIC_{50}	MIC_{90}	%S	N	Range	MIC_{50}	MIC_{90}	%S	N	Range	MIC_{50}	MIC_{90}	%S
Amikacin [b]	16	139	≤0.5 – 32	1*	4*	99.3	211	≤0.5 - >64	2*	8*	95.3	137	≤0.5 – 32	2*	8*	97.1	487	≤0.5 - >64	2	8	96.9
Ampicillin [a]	8	161	1 - >16	16	>16	48.5	213	0.5 - >16	8	>16	50.2	143	0.5 - >16	16	>16	43.4	517	0.5 - >16	16	>16	47.8
Ceftiofur [a]	2	111	≤0.25 - >8	0.5*	1*	95.5*	212	≤0.25 - >8	0.5*	1*	94.3*	139	≤0.25 - >8	≤0.25*	4*	86.3*	462	≤0.25 - >8	0.5	2	92.2
Ceftizoxime [a]	8	133	≤0.5 - >32	≤0.5*	≤0.5*	96.2	212	≤0.5 - >32	≤0.5*	<0.5*	95.8	134	≤0.5 - >32	≤0.5*	4*	94.0	479	≤0.5 - >32	≤0.5	1	95.4
Cefalothin [a,b]	8	150	≤2 - >16	8	>16	58.0	212	≤2 - >16	16	>16	35.4	115	≤2 - >16	8	>16	60.0	477	≤2 - >16	16	>16	48.4
Chloramphenicol	8	161	≤4 - >16	8	>16	70.8	216	≤4 - >16	8	>16	64.4	135	≤4 - >16	8	>16	72.6	512	≤4 - >16	8	>16	68.6
Enrofloxacin	0.5	105	≤0.25	≤0.25	≤0.25	100	212	≤0.25 - >2	≤0.25	<0.25	97.6	137	≤0.25 - >2	≤0.25	≤0.25	97.1	454	≤0.25 - >2	≤0.25	≤0.25	98.0
Gentamicin [b]	2	157	≤0.25 - >16	0.5*	16*	82.2*	204	≤0.25 - >16	0.5*	>16*	73.5*	136	≤0.25 - >16	0.5*	>16*	69.9*	497	≤0.25 - >16	0.5	>16	75.3
Imipenem	4	107	≤1 – 4	≤1	≤1	100	196	≤1 – 16	≤1	≤1	99.5	115	≤1 – 2	≤1	≤1	100	418	≤1 – 16	≤1	≤1	99.8
Tetracycline	4	155	≤1 - >8	8*	>8*	47.7*	207	≤1 - >8	4*	>8*	54.6*	136	≤1 - >8	2*	>8*	67.7*	498	≤1 - >8	4	>8	56.0
Ticarcillin/ Clavulanic Acid [a,d]	16	107	≤16 - >64	≤16	>64	76.6	217	≤16 - >64	≤16	>64	73.7	143	≤16 - >64	≤16	64	77.6	467	≤16 - >64	≤16	>64	75.6
Trimethoprim/ Sulfamethoxazole [c]	2	155	≤0.25 - >4	≤0.25	>4	63.2	212	≤0.25 - >4	≤0.25	>4	64.6	138	≤0.25 - >4	≤0.25	>4	63.0	505	≤0.25 - >4	≤0.25	>4	63.8

Supporting Information Item S2. Temporal and cumulative susceptibility data of E. coli isolated from foals with sepsis, UC Davis, USA, 1979-2010

Antimicrobial drug	Break-point	1979-1990					1991-1997					1998-2010					Total (1979-2010)				
		N	Range	MIC_{50}	MIC_{90}	%S	N	Range	MIC_{50}	MIC_{90}	%S	N	Range	MIC_{50}	MIC_{90}	%S	N	Range	MIC_{50}	MIC_{90}	%S
Amikacin	16	88	≤2 – 32	≤2	4	98.9	144	≤2 - >64	≤2	8	96.5	75	≤2 – 32	≤2	4	98.7	307	≤2 - >64	≤2	8	97.7
Ampicillin [a]	8	90	1 - >16	4	>16	67.8	142	0.5 - >16	4	>16	66.2	78	1 - >16	4	>16	61.5	310	0.5 - >16	4	>16	65.5
Ceftiofur [a]	2	62	≤0.25 – 2	≤0.25	0.5	100*	141	≤0.25 - >8	0.5	1	95.7*	77	≤0.25 – 8	≤0.25	4	89.6*	280	≤0.25 - >8	0.5	1	95.0
Ceftizoxime [a]	8	75	≤0.5 – 1	≤0.5*	≤0.5*	100	141	≤0.5 - >128	≤0.5*	≤0.5*	93.6	72	≤0.5 – 16	≤0.5*	4*	98.6	288	≤0.5 - >128	≤0.5	≤0.5	97.9
Cefalothin [a]	8	82	≤2 - >16	8	>16	59.8	141	≤2 - >16	16	>16	35.5	62	≤2 - >16	8	>16	62.9	285	≤2 - >16	16	>16	48.4
Chloramphenicol	8	90	≤4 - >16	8	>16	78.9	144	≤4 - >16	8	>16	70.8	74	≤4 - >16	8	>16	82.4	308	≤4 - >16	8	>16	76.0
Enrofloxacin	0.5	57	≤0.25	≤0.25	≤0.25	100	141	≤0.25 - >2	≤0.25	≤0.25	97.2	75	≤0.25 - >2	≤0.25	≤0.25	98.7	273	≤0.25 - >2	≤0.25	≤0.25	98.2
Gentamicin	2	90	≤0.25 - >16	0.5*	4*	88.9	135	≤0.25 - >16	0.5*	>16*	79.3	75	≤0.25 - >16	0.5*	>16*	77.3	300	≤0.25 - >16	0.5	>16	81.7
Imipenem	4	56	≤1	≤1	≤1	100	133	≤1 – 16	≤1	≤1	99.3	64	≤1	≤1	≤1	100	253	≤1 – 16	≤1	≤1	99.6
Tetracycline	4	87	≤1 - >8	4*	>8*	51.7*	136	≤1 - >8	2*	>8*	61.0*	73	≤1 - >8	2*	>8*	69.9*	296	≤1 - >8	4	>8	60.5
Ticarcillin/ Clavulanic Acid [a,d]	16	56	≤16 - >128	≤16	64	85.7	144	≤16 - >128	≤16	64	79.2	75	≤16 – 128	≤16	32	89.3	275	≤16 - >128	≤16	64	83.3
Trimethoprim/ Sulfamethoxazole [c]	2	86	≤0.25 - >4	≤0.25	>4	67.4	141	≤0.25 - >4	≤0.25	>4	67.4	75	≤0.25 - >4	≤0.25	>4	66.7	302	≤0.25 - >4	≤0.25	>4	67.2

Supporting Information Item S3. Temporal and cumulative susceptibility data of Klebsiella spp. isolated from foals with sepsis, UC Davis, USA, 1979-2010

Antimicrobial drug	Break-point	1979-1990					1991-1997					1998-2010					Total (1979-2010)				
		N	Range	MIC_{50}	MIC_{90}	%S	N	Range	MIC_{50}	MIC_{90}	%S	N	Range	MIC_{50}	MIC_{90}	%S	N	Range	MIC_{50}	MIC_{90}	%S
Amikacin	16	31	≤0.5 – 2	1	2	100	26	≤0.5 – 64	1	16	96.2	20	≤0.5 – 8	1	2	100	77	≤0.5 - 64	1	8	98.7
Ampicillin [a]	8	34	8 - >16	>16*	>16*	8.8	25	16 - >16	>16*	>16*	0	20	4 - >16	16*	>16*	10.0	79	4 - >16	>16	>16	6.3
Ceftiofur [a]	2	27	≤0.25 – 2	0.5	1	100	25	≤0.25 - >8	0.5	2	96.0	20	≤0.25 – 0.5	≤0.25	0.5	100	72	≤0.25 - >8	0.5	1	98.6
Ceftizoxime [a]	8	30	≤0.5	≤0.5	≤0.5	100	26	≤0.5 - >64	≤0.5	≤0.5	96.2	20	≤0.5 – 1	≤0.5	≤0.5	100	76	≤0.5 - >64	≤0.5	≤0.5	98.7
Cefalothin [a]	8	34	≤2 - >16	8	>16	67.7	25	≤2 - >16	4	>16	56.0	19	≤2 - >16	4	>16	79.0	78	≤2 - >16	4	>16	66.7
Chloramphenicol	8	34	≤4 - >16	8	>16	52.9	25	≤4 - >16	8	>16	52.0	20	≤4 - >16	≤4	>16	80.0	79	≤4 - >16	8	>16	59.5
Enrofloxacin	0.5	27	≤0.25	≤0.25	≤0.25	100	25	≤0.25 – 1	≤0.25	≤0.25	96.0	20	≤0.25	≤0.25	≤0.25	100	72	≤0.25 – 1	≤0.25	≤0.25	98.6
Gentamicin	2	33	≤0.25 - >16	≤0.25	8	72.7	25	≤0.25 - >16	1	>16	60.0	20	≤0.25 - >16	≤0.25	>16	75.0	78	≤0.25 - >16	0.5	>16	69.2
Imipenem	4	28	≤1	≤1	≤1	100	25	≤1 – 2	≤1	≤1	100	19	≤1 – 2	≤1	≤1	100	72	≤1 – 2	≤1	≤1	100
Tetracycline	4	33	≤1 - >8	4*	>8*	51.5*	25	≤1 - >8	2*	>8*	52.0*	20	≤1 - >8	≤1*	4*	90.0*	78	≤1 - >8	2	>8	61.5
Ticarcillin/ Clavulanic Acid [a,d]	16	28	≤16 - >128	≤16	64	67.9	26	≤16 - >128	≤16	>128	57.7	20	≤16 - >128	≤16	≤16	95.0	74	≤16 - >128	≤16	>128	71.6
Trimethoprim/ Sulfamethoxazole [c]	2	34	≤0.25 - >4	0.5	>4	55.9	25	≤0.25 - >4	0.5	>4	60.0	20	≤0.25 - >4	≤0.25	>4	75.0	79	≤0.25 - >4	≤0.25	>4	62.0

Supporting Information Item S4. Temporal and cumulative susceptibility data of Enterobacter spp. isolated from foals with sepsis, UC Davis, USA, 1979-2010

Antimicrobial drug	Break-point	1979-1990					1991-1997					1998-2010					Total (1979-2010)				
		N	Range	MIC_{50}	MIC_{90}	%S	N	Range	MIC_{50}	MIC_{90}	%S	N	Range	MIC_{50}	MIC_{90}	%S	N	Range	MIC_{50}	MIC_{90}	%S
Amikacin	16	12	≤0.5 – 2	1*	2*	100	16	≤0.5 – 16	1*	16*	100	10	≤0.5 – 32	8*	16*	90.0	38	≤0.5 – 32	1	16	97.4
Ampicillin	8	13	1 - >16	>16	>16	23.1	17	2 - >16	>16	>16	11.8	11	0.5 - >16	>16	>16	9.1	41	0.5 - >16	>16	>16	14.6
Ceftiofur	2	11	≤0.25 - >8	2	>8	54.5	17	≤0.25 - >8	1	>8	82.4	9	≤0.25 - >8	0.5	>8	55.6	37	≤0.25 - >8	1	>8	67.6
Ceftizoxime	8	12	≤1 - >32	≤1	>32	58.3	18	≤1 - >32	≤1	>32	88.9	10	≤1 - >32	≤1	16	80.0	40	≤1 - >32	≤1	>32	77.5
Cefalothin	8	12	≤2 - >16	>16	>16	16.7	17	4 - >16	>16	>16	11.8	8	≤2 - >16	>16	>16	25.0	37	≤2 - >16	>16	>16	16.2
Chloramphenicol	8	13	≤4 - >16	16	>16	46.2	18	≤4 - >16	16	>16	33.3	11	≤4 - >16	>16	>16	36.4	42	≤4 - >16	>16	>16	38.1
Enrofloxacin	0.5	9	≤0.25	≤0.25	≤0.25	100	18	≤0.25	≤0.25	≤0.25	100	8	≤0.25	≤0.25	≤0.25	100	35	≤0.25	≤0.25	≤0.25	100
Gentamicin	2	13	≤0.25 - >16	≤0.25	>16	53.8	17	≤0.25 - >16	8	>16	41.2	9	≤0.25 - >16	>16	>16	33.3	39	≤0.25 - >16	16	>16	43.6
Imipenem	4	11	≤1	≤1	≤1	100	17	≤1 – 2	≤1	≤1	100	9	≤1	≤1	≤1	100	37	≤1 – 2	≤1	≤1	100
Tetracycline	4	13	≤2 - >8	8	>8	38.5	17	≤2 - >8	>8	>8	35.3	11	≤2 - >8	≤2	>8	63.6	41	≤2 - >8	8	>8	43.9
Ticarcillin/ Clavulanic Acid [d]	16	11	≤16 - >64	>64	>64	45.5	18	≤16 - >64	≤16	>64	50.0	10	≤16 - >64	32	>64	40.0	39	≤16 - >64	32	>64	46.2
Trimethoprim/ Sulfamethoxazole [c]	2	13	≤0.5 - >4	1	>4	53.8	17	≤0.5 - >4	>4	>4	35.3	11	≤0.5 - >4	>4	>4	27.3	41	≤0.5 - >4	>4	>4	39.0

Supporting Information Item S5. Temporal and cumulative susceptibility data of Salmonella spp. isolated from foals with sepsis, UC Davis, USA, 1979-2010

Antimicrobial drug	Break-point	1979-1990					1991-1997					1998-2010					Total (1979-2010)				
		N	Range	MIC_{50}	MIC_{90}	%S	N	Range	MIC_{50}	MIC_{90}	%S	N	Range	MIC_{50}	MIC_{90}	%S	N	Range	MIC_{50}	MIC_{90}	%S
Amikacin [b]	16	12	≤2 – 8	≤2	4	100	8	≤2 – 8	≤2	8	100	12	≤2 – 8	≤2	8	100	32	≤2 – 8	≤2	8	100
Ampicillin	8	12	1 - >16	1	>16	75.0	8	1 - >16	1	>16	75.0	11	1 - >16	>16	>16	45.5	31	1 - >16	2	>16	64.5
Ceftiofur	2	5	0.25 – 1	1	1	100	8	0.25 - >8	1	>8	87.5	11	0.5 – 4	0.5	4	72.7	24	0.25 - >8	1	4	83.3
Ceftizoxime	8	7	≤0.5	≤0.5*	≤0.5*	100	8	≤0.5 – 2	≤0.5*	2*	100	12	≤0.5 – 128	≤0.5*	8*	91.7	27	≤0.5 – 128	≤0.5	4	96.3
Cefalothin [b]	8	12	≤2 – 32	4	8	91.7	8	≤2 – 16	4	16	87.5	7	≤2 – 4	2	4	100	27	≤2 – 32	4	8	92.6
Chloramphenicol	8	12	≤4 - >16	8	>16	75.0	8	≤4 - >16	8	>16	75.0	9	≤4 - >16	≤4	>16	55.6	29	≤4 - >16	8	>16	69.0
Enrofloxacin	0.5	6	≤0.25	≤0.25	≤0.25	100	8	≤0.25	≤0.25	≤0.25	100	11	≤0.25 – 2	≤0.25	2	81.8	25	≤0.25 – 2	≤0.25	≤0.25	92.0
Gentamicin [b]	2	10	≤0.25 - >16	≤0.25	1	90.0*	8	0.5 – 2	0.5	2	100*	11	≤0.25 - >16	>16	>16	45.5*	29	≤0.25 - >16	0.5	>16	75.9
Imipenem	4	6	≤1	≤1	≤1	100	6	≤1	≤1	≤1	100	6	≤1	≤1	≤1	100	18	≤1	≤1	≤1	100
Tetracycline	4	12	≤1 - >8	8	>8	41.7	8	≤1 - >8	2	>8	75.0	11	≤1 - >8	4	>8	63.6	31	≤1 - >8	2	>8	58.1
Ticarcillin/ Clavulanic Acid [d]	16	6	≤16 - >128	≤16	>128	83.3	8	≤16 - >128	≤16	>128	75.0	12	≤16 – 128	≤16	64	50.0	26	≤16 - >128	≤16	>128	65.4
Trimethoprim/ Sulfamethoxazol [c]	2	12	≤0.25 - >4	≤0.25	>4	75.0	8	≤0.25	≤0.25	≤0.25	100	11	≤0.25 - >4	>4	>4	45.5	31	≤0.25 - >4	≤0.25	>4	71.0

Supporting Information Item S6. Temporal and cumulative susceptibility data of Actinobacillus spp. isolated from foals with sepsis, UC Davis, USA, 1979-2010

Antimicrobial drug	Break-point	1979-1990					1991-1997					1998-2010					Total (1979-2010)				
		N	Range	MIC_{50}	MIC_{90}	%S	N	Range	MIC_{50}	MIC_{90}	%S	N	Range	MIC_{50}	MIC_{90}	%S	N	Range	MIC_{50}	MIC_{90}	%S
Amikacin	16	50	≤0.5 - >32	4*	8*	98.0	56	≤0.5 – 32	4*	8*	98.2	43	≤0.5 - >32	8*	16*	97.7	149	≤0.5 - >32	4	8	98.0
Ampicillin	8	51	≤0.25 - >32	≤0.25	16	88.2*	56	≤0.25 - >32	≤0.25	1	94.6*	41	≤0.25 – 1	≤0.25	0.5	100*	148	≤0.25 - >32	≤0.25	1	93.9
Ceftiofur	2	36	≤0.25	≤0.25	≤0.25	100	56	≤0.25 – 1	≤0.25	≤0.25	100	41	≤0.25 – 2	≤0.25	≤0.25	100	133	≤0.25 – 2	≤0.25	≤0.25	100
Ceftizoxime	2	42	≤0.5	≤0.5	≤0.5	100	56	≤0.5	≤0.5	≤0.5	100	39	≤0.5 – 1	≤0.5	≤0.5	100	137	≤0.5 – 1	≤0.5	≤0.5	100
Cefalothin	8	48	≤2 – 16	≤2	≤2	97.9	56	≤2 – 32	≤2	≤2	98.2	35	≤2 – 16	≤2	≤2	97.1	139	≤2 – 32	≤2	≤2	97.8
Chloramphenicol	8	53	≤4 – 16	≤4	≤4	98.1	56	≤4 - >32	≤4	≤4	96.4	38	≤4	≤4	≤4	100	147	≤4 - >32	≤4	≤4	98.0
Enrofloxacin	0.5	33	≤0.25 - >2	≤0.25	≤0.25	97.0	56	≤0.25 – 2	≤0.25	≤0.25	98.2	36	≤0.25 – 1	≤0.25	≤0.25	97.2	125	≤0.25 - >2	≤0.25	≤0.25	97.6
Erythromycin	0.5	50	≤0.25 - >2	1*	>2*	16.0	55	≤0.25 - >2	2*	>2*	10.9	39	≤0.25 - >2	1*	2*	25.6	144	≤0.25 - >2	1	2	16.7
Gentamicin	2	51	≤0.25 – 4	1*	1*	98.0*	56	≤0.25 – 4	1*	2*	94.6*	43	≤0.25 – 16	2*	4*	76.7*	150	≤0.25 – 16	1	2	90.7
Imipenem	4	31	≤1	≤1	≤1	100	47	≤1 – 2	≤1	≤1	100	37	≤1	≤1	≤1	100	115	≤1 – 2	≤1	≤1	100
Penicillin	0.12	50	≤0.12 - >8	0.25*	1*	6.0*	56	≤0.12 - >8	0.25*	2*	26.8*	41	≤0.12 – 2	≤0.12*	0.5*	53.7*	147	≤0.12 - >8	0.25	1	27.2
Rifampicin	1	36	≤0.25 - >4	1*	2*	77.8*	55	≤0.25 - >4	0.5*	2*	85.5*	37	≤0.25 – 1	0.5*	1*	100*	128	≤0.25 - >4	0.5	2	87.5
Tetracycline	4	49	≤1 - >8	≤1	≤1	98.0	55	≤1 - >8	≤1	≤1	96.4	39	≤1 – 2	≤1	≤1	100	143	≤1 - >8	≤1	≤1	97.9
Ticarcillin/ Clavulanic Acid [d]	16	33	≤16 – 128	≤16	≤16	97.0	56	≤16 – 64	≤16	≤16	98.2	43	≤16	≤16	≤16	100	132	≤16 – 128	≤16	≤16	98.5
Trimethoprim/ Sulfamethoxazole [c]	2	52	≤0.5	≤0.5	≤0.5	100	56	≤0.5 - >4	≤0.5	≤0.5	96.4	43	≤0.5 - >4	≤0.5	≤0.5	97.7	151	≤0.5 - >4	≤0.5	≤0.5	98.0

Supporting Information Item S7. Temporal and cumulative susceptibility data of Pseudomonas spp. isolated from foals with sepsis, UC Davis, USA, 1979-2010

Antimicrobial drug	Break-point	1979-1990					1991-1997					1998-2010					Total (1979-2010)				
		N	Range	MIC_{50}	MIC_{90}	%S	N	Range	MIC_{50}	MIC_{90}	%S	N	Range	MIC_{50}	MIC_{90}	%S	N	Range	MIC_{50}	MIC_{90}	%S
Amikacin	16	13	≤0.5 - >32	8	16	92.3	4	≤0.5 – 16	2	16	100	10	≤0.5 – >32	2	>32	80.0	27	≤0.5 - >32	4	>32	88.9
Ampicillin	8	13	8 - >16	>16	>16	7.7	2	2 - >16	2	>16	50.0	9	>16	>16	>16	0.0	24	2 - >16	>16	>16	8.3
Ceftiofur	2	9	2 - >4	>4*	>4*	11.1	4	4 - >4	>4*	>4*	0	10	>4	>4*	>4*	0.0	23	2 - >4	>4	>4	4.4
Ceftizoxime	8	13	2 - >32	>32	>32	7.7	4	4 - >32	32	>32	25.0	8	32 - >32	32	>32	12.5	25	2 - >32	>32	>32	12.0
Cefalothin	8	11	>16	>16	>16	0	4	8 - >16	>16	>16	25.0	6	>16	>16	>16	0.0	21	8 - >16	>16	>16	4.6
Chloramphenicol	8	13	≤4 - >16	>16	>16	15.4	4	>16	>16	>16	0	9	>16	>16	>16	11.1	26	≤4 - >16	>16	>16	11.5
Enrofloxacin	0.5	12	≤0.25 - >1	>1	>1	16.7	4	≤0.25 – 1	0.5	1	50.0	9	1 - >1	1	>1	33.3	25	≤0.25 - >1	1	>1	28.0
Gentamicin	2	13	≤0.25 - >16	8	>16	23.1*	4	≤0.25 - >16	1	>16	50.0*	10	0.5 - >16	1	>16	70.0*	27	≤0.25 - >16	4	>16	44.4
Imipenem	4	11	≤1 - >8	2	4	90.9	4	≤1	≤1	≤1	100	8	1 - >8	2	>8	62.5	23	≤1 - >8	≤1	>8	82.6
Tetracycline	4	13	≤1 - >8	>8	>8	15.4	4	≤1 - >8	>8	>8	25.0	8	4 - >8	>8	>8	12.5	25	≤1 - >8	>8	>8	16.0
Ticarcillin/ Clavulanic Acid [d]	64	11	≤16 - >64	32	64	90.9	4	≤16 – 64	≤16	64	100	10	32 - >64	32	>64	80.0	25	≤16 - >64	32	>64	88.0
Trimethoprim/ Sulfamethoxazol [c]	2	13	≤0.25 - >4	>4	>4	15.4	4	≤0.25 - >4	>4	>4	25.0	10	4 - >4	>4	>4	10.0	27	≤0.25 - >4	>4	>4	14.8

Supporting Information Item S8. Temporal and cumulative susceptibility data of Enterococcus spp. isolated from foals with sepsis, UC Davis, USA, 1979-2010

Antimicrobial drug	Break-point	1979-1990					1991-1997					1998-2010					Total (1979-2010)				
		N	Range	MIC_{50}	MIC_{90}	%S	N	Range	MIC_{50}	MIC_{90}	%S	N	Range	MIC_{50}	MIC_{90}	%S	N	Range	MIC_{50}	MIC_{90}	%S
Amikacin [e]	NA	18	4 - >32	>32	>32	-	17	32 - >32	>32	>32	-	42	8 - >32	>32	>32	-	77	4 - >32	>32	>32	-
Ampicillin	8	16	≤0.25 - >16	1	>16	81.3	19	≤0.25 - >16	1	>16	68.4	41	≤0.25 - >16	2	>16	70.7	76	≤0.25 - >16	2	>16	72.4
Ceftiofur [e]	NA	14	≤0.25 - >8	>8*	>8*	-	17	≤0.25 - >8	>8*	>8*	-	37	0.5 - >8	>8*	>8*	-	68	≤0.25 - >8	>8	>8	-
Ceftizoxime [e]	NA	17	≤1 - >32	>32	>32	-	17	2 - >32	>32	>32	-	38	2 - >32	>32	>32	-	72	≤1 - >32	>32	>32	-
Cefalothin [e]	NA	14	≤2 - >16	>16	>16	-	17	4 - >16	>16	>16	-	31	2 - >16	>16	>16	-	62	≤2 - >16	>16	>16	-
Chloramphenicol	8	18	≤4 - >16	8	>16	61.1	19	≤4 - >16	8	16	84.2	37	≤4 - >16	8	>16	78.4	74	≤4 - >16	8	>16	75.7
Enrofloxacin	0.5	11	0.5 - >2	1	>2	27.3	19	0.5 - >2	1	>2	31.6	39	≤0.25 - >2	1	>2	30.8	69	≤0.25 - >2	1	>2	30.4
Erythromycin	0.5	15	≤0.25 - >2	>2	>2	13.3	19	≤0.25 - >2	2	>2	36.8	38	≤0.25 - >2	1	>2	28.9	72	≤0.25 - >2	2	>2	27.8
Gentamicin [e]	NA	14	0.5 - >16	16	>16	-	18	4 - >16	>16	>16	-	41	2 - >16	16	>16	-	73	0.5 - >16	16	>16	-
Imipenem	4	14	≤1 - >8	≤1	4	92.9*	17	≤1 - >8	≤1	>8	64.7*	31	≤1 - >8	2	>8	58.1*	62	≤1 - >8	2	>8	67.7
Penicillin [f]	8	16	0.25 - >4	4	>4	-	19	0.25 - >4	4	>4	-	39	0.25 - >4	4	>4	-	74	0.25 - >4	4	>4	-
Rifampicin	1	15	≤1 - >4	4	>4	33.3	19	≤1 - >4	>4	>4	21.1	40	≤1 - >4	2	>4	32.5	74	≤1 - >4	4	>4	29.7
Tetracycline	4	15	≤1 - >8	>8	>8	26.7	19	≤1 - >8	≤1	>8	52.6	39	≤1 - >8	>8	>8	46.2	73	≤1 - >8	>8	>8	43.8
Ticarcillin/ Clavulanic Acid [d,f]	8	15	≤16 - >64	≤16*	>64*	-	18	≤16 - >64	32*	>64*	-	42	≤16 - >64	64*	>64*	-	75	≤16 - >64	32	>64	-
Trimethoprim/ Sulfamethoxazole [c,e]	NA	16	≤0.5 - >4	≤0.5	>4	-	19	≤0.5 - >4	≤0.5	>4	-	41	≤0.5 - >4	≤0.5	>4	-	76	≤0.5 - >4	≤0.5	>4	-

Supporting Information Item S9. Temporal and cumulative susceptibility data of β-haemolytic Streptococcus spp. isolated from foals with sepsis, UC Davis, USA, 1979-2010

Antimicrobial drug	Break-point	1979-1990					1991-1997					1998-2010					Total (1979-2010)				
		N	Range	MIC_{50}	MIC_{90}	%S	N	Range	MIC_{50}	MIC_{90}	%S	N	Range	MIC_{50}	MIC_{90}	%S	N	Range	MIC_{50}	MIC_{90}	%S
Amikacin	16	29	2 - >32	>32*	>32*	34.5*	37	2 - >32	>32*	>32*	16.2*	37	8 - >32	>32*	>32*	5.4*	103	2 - >32	>32	>32	17.5
Ampicillin	0.25	28	≤0.25 - 16	≤0.25	≤0.25	92.9	35	≤0.25 - >32	≤0.25	≤0.25	91.4	30	≤0.25	≤0.25	≤0.25	100	93	≤0.25 - >32	≤0.25	≤0.25	94.6
Ceftiofur	0.25	26	≤0.25	≤0.25	≤0.25	100	36	≤0.25 – 0.5	≤0.25	≤0.25	94.4	37	≤0.25	≤0.25	≤0.25	100	99	≤0.25 - 0.5	≤0.25	≤0.25	98.0
Ceftizoxime	8	28	≤0.5 - >128	≤0.5	≤0.5	96.4	36	≤0.5 – 1	≤0.5	≤0.5	100	36	≤0.5	≤0.5	≤0.5	100	100	≤0.5 - >128	≤0.5	≤0.5	99.0
Cefalothin	8	27	≤2	≤2	≤2	100	36	≤2 – 4	≤2	≤2	100	34	≤2	≤2	≤2	100	97	≤2 - 4	≤2	≤2	100
Chloramphenicol	4	29	≤4	≤4	≤4	100	37	≤4 – 16	≤4	≤4	94.6	37	≤4 – 8	≤4	≤4	94.6	103	≤4 - 16	≤4	≤4	96.1
Enrofloxacin	0.5	26	≤0.25 – 2	0.5	1	57.7	36	≤0.25 - >2	0.5	2	55.6	31	≤0.25 - >2	0.5	1	61.3	93	≤0.25 - >2	0.5	1	58.1
Erythromycin	0.25	28	≤0.25 – 2	≤0.25	≤0.25	92.9	34	≤0.25 - >2	≤0.25	≤0.25	91.2	35	≤0.25 – 2	≤0.25	≤0.25	97.1	97	≤0.25 - >2	≤0.25	≤0.25	93.8
Gentamicin	4	27	≤0.25 - >16	16	>16	40.7*	36	0.5 - >16	16	>16	16.7*	37	1 - >16	16	>16	5.4*	100	≤0.25 - >16	16	>16	15.0
Imipenem	4	27	≤1 – 64	≤1	≤1	96.3	33	≤1	≤1	≤1	100	35	≤1	≤1	≤1	100	95	≤1 - 64	≤1	≤1	99.0
Penicillin	0.12	28	≤0.12 - 0.25	≤0.12	≤0.12	92.9	36	≤0.12 - >8	≤0.12	≤0.12	97.2	37	≤0.12	≤0.12	≤0.12	100	101	≤0.12 - >8	≤0.12	≤0.12	97.0
Rifampicin	1	26	≤1 – 4	≤1	≤1	96.2	36	≤1 - >4	≤1	≤1	97.2	37	≤1	≤1	≤1	100	99	≤1 - >4	≤1	≤1	98.0
Tetracycline	2	28	≤1 - >8	2	>8	71.4	35	≤1 - >8	2	>8	60.0	36	≤1 - >8	2	>8	83.3	99	≤1 - >8	2	>8	81.8
Ticarcillin/ Clavulanic Acid [d,f]	8	28	≤16 - >128	≤16	≤16	-	36	≤16	≤16	≤16	-	37	≤16	≤16	≤16	-	101	≤16 - >128	≤16	≤16	-
Trimethoprim/ Sulfamethoxazole [c,h]	2	28	≤0.25 – 0.5	≤0.25	≤0.25	100	36	≤0.25 - >4	≤0.25	>4	88.9	37	≤0.25 - >4	≤0.25	0.5	91.9	101	≤0.25 - >4	≤0.25	0.5	93.1

Supporting Information Item S10. Temporal and cumulative susceptibility data of Staphylococcus spp. isolated from foals with sepsis, UC Davis, USA, 1979-2010

Antimicrobial drug	Break-point	1979-1990					1991-1997					1998-2010					Total (1979-2010)				
		N	Range	MIC_{50}	MIC_{90}	%S	N	Range	MIC_{50}	MIC_{90}	%S	N	Range	MIC_{50}	MIC_{90}	%S	N	Range	MIC_{50}	MIC_{90}	%S
Amikacin	16	13	≤0.5 – 2	≤0.5	2	100	22	≤0.5 - >64	≤0.5	8	95.5	21	≤0.5 – 16	≤0.5	2	100	56	≤0.5 - >64	≤0.5	4	98.2
Ampicillin [g]	0.25	9	≤0.25 - >16	1	>16	44.4	17	≤0.25 - >16	≤0.25	8	52.9	19	≤0.25 - >16	≤0.25	16	57.9	45	≤0.25 - >16	≤0.25	16	53.3
Ceftiofur [g]	2	11	0.5 - >8	2	>8	54.6	19	≤0.06 - >8	0.5	4	89.5	20	0.25 – 8	1	4	80.0	50	≤0.06 - >8	1	8	78.0
Ceftizoxime [g]	8	12	≤0.5 - >32	2	>32	75.0	22	≤0.5 - >32	1	16	86.4	20	≤0.5 - >32	4	>32	80.0	54	≤0.5 - >32	2	>32	81.5
Cefalothin [g]	8	10	≤2	≤2	≤2	100	21	≤2 – 32	≤2	≤2	90.5	13	≤2	≤2	≤2	100	44	≤2 - 32	≤2	≤2	95.5
Chloramphenicol	8	14	≤4 - >32	≤4	>32	64.3*	22	≤4 - >32	≤4	8	90.9*	19	≤4 – 8	≤4	8	100*	55	≤4 - >32	≤4	16	87.3
Enrofloxacin	0.5	9	≤0.25	≤0.25	≤0.25	100	22	≤0.25 - >2	≤0.25	0.5	90.9	21	≤0.25 - >2	≤0.25	0.5	90.5	52	≤0.25 - >2	≤0.25	0.5	92.3
Erythromycin	0.5	12	≤0.25 - >2	≤0.25	>2	50.0	21	≤0.25 - >2	≤0.25	>2	66.7	20	≤0.25 - >2	≤0.25	>2	70.0	53	≤0.25 - >2	≤0.25	>2	64.2
Gentamicin	4	13	≤0.25 – 16	≤0.25	4	92.3	21	≤0.25 - >16	≤0.25	2	90.5	22	≤0.25 - >16	≤0.25	8	77.3	56	≤0.25 - >16	≤0.25	8	85.7
Imipenem [g]	4	7	≤1	≤1	≤1	100	18	≤1 – 2	≤1	≤1	100	14	≤1	≤1	≤1	100	39	≤1 – 2	≤1	≤1	100
Penicillin [g]	0.12	12	≤0.12 - >4	1	>4	25.0	22	≤0.12 - >4	0.25	>4	31.8	21	≤0.12 - >4	≤0.12	>4	52.4	55	≤0.12 - >4	0.25	>4	38.2
Rifampicin	1	10	≤1 - >4	≤1	≤1	90.0	22	≤1 - >4	≤1	4	86.4	19	≤1 – 2	≤1	2	89.5	51	≤1 - >4	≤1	2	88.2
Tetracycline	4	14	≤2 - >8	≤2	>8	85.7	22	≤2 - >8	≤2	>8	77.3	20	≤2 - >8	≤2	>8	75.0	56	≤2 - >8	≤2	>8	78.6
Ticarcillin/ Clavulanic Acid [d,f]	8	6	≤16	≤16	≤16	-	19	≤16 – 64	≤16	32	-	22	≤16	≤16	≤16	-	47	≤16 – 64	≤16	≤16	-
Trimethoprim/ Sulfamethoxazole [c]	2	14	≤0.25 - >4	0.5	>4	71.4	22	≤0.25 - >4	≤0.25	>4	86.4	21	≤0.25 - >4	≤0.25	>4	71.4	57	≤0.25 - >4	≤0.25	>4	77.2

Supporting Information Item S11. Temporal and cumulative susceptibility data of coagulase-positive Staphylococcus spp. isolated from foals with sepsis, UC Davis, USA, 1979-2010

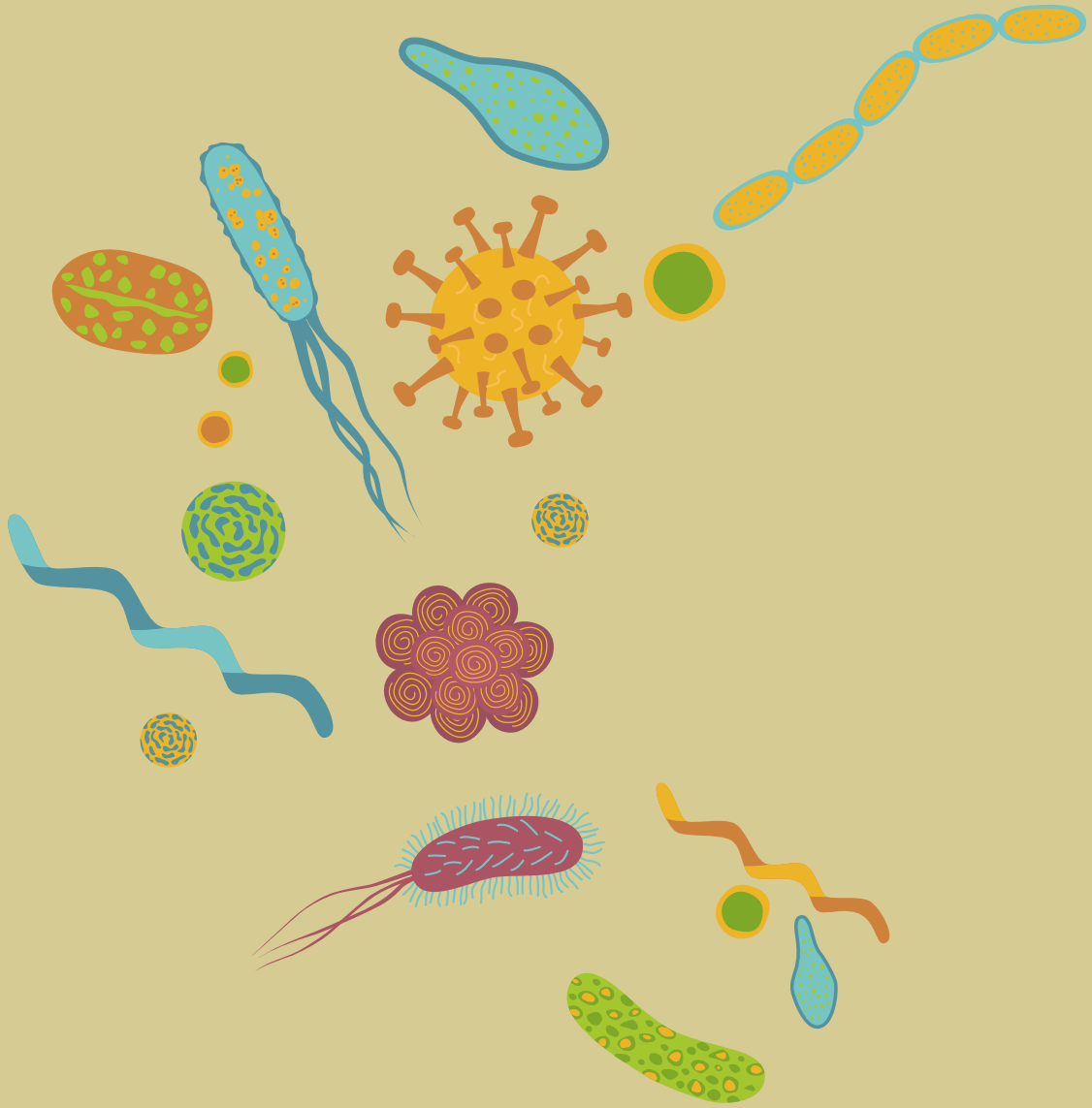
Antimicrobial drug	Break-point	1979-1990					1991-1997					1998-2010					Total (1979-2010)				
		N	Range	MIC_{50}	MIC_{90}	%S	N	Range	MIC_{50}	MIC_{90}	%S	N	Range	MIC_{50}	MIC_{90}	%S	N	Range	MIC_{50}	MIC_{90}	%S
Amikacin	16	7	≤0.5 – 2	≤0.5	2	100	11	≤0.5 – 8	1	8	100	12	≤0.5 – 16	1	8	100	30	≤0.5 – 16	1	8	100
Ampicillin [g]	0.25	5	≤0.25 - >16	1	>16	0.0	7	≤0.25 - >16	≤0.25	>16	71.4	10	≤0.25 - >16	≤0.25	16	50.0	22	≤0.25 - >16	≤0.25	>16	50.0
Ceftiofur [g]	2	7	0.5 – 16	2	16	57.1	11	≤0.25 – 4	1	1	90.9	12	≤0.25- 8	1	8	66.7	30	≤0.25 – 16	1	8	73.3
Ceftizoxime [g]	8	7	≤0.5 - >32	2	>32	85.7	11	≤0.5 - >32	4	4	90.9	12	≤0.5 - >32	8	>32	66.7	30	≤0.5 - >32	4	>32	80.0
Cefalothin [g]	8	7	≤2	≤2	≤2	100	10	≤2 – 16	≤2	≤2	90.0	8	≤2	≤2	≤2	100	25	≤2 – 16	≤2	≤2	96.0
Chloramphenicol	8	8	≤4 - >32	16	>32	37.5*	11	≤4 – 16	≤4	8	90.9*	10	≤4 – 8	≤4	8	100*	29	≤4 - >32	4	>32	79.3
Enrofloxacin	0.5	6	≤0.25	≤0.25	≤0.25	100	11	≤0.25 - >2	≤0.25	0.5	90.9	12	≤0.25 - >2	≤0.25	0.5	91.7	29	≤0.25 - >2	≤0.25	0.5	93.1
Erythromycin	0.5	8	≤0.5 - >2	≤0.5	>2	62.5	11	≤0.5 - >2	≤0.5	>2	63.6	12	≤0.5 - >2	≤0.5	>2	75.0	31	≤0.5 - >2	≤0.5	>2	67.7
Gentamicin	4	7	≤0.25 – 4	≤0.25	4	100	11	≤0.25 - >16	≤0.25	2	90.9	12	≤0.25 - >16	≤0.25	16	75.0	30	≤0.25 - >16	≤0.25	8	86.7
Imipenem [g]	4	5	≤1	≤1	≤1	100	8	≤1	≤1	≤1	100	8	≤1	≤1	≤1	100	21	≤1	≤1	≤1	100
Penicillin [g]	0.12	8	≤0.12 - >4	1	>4	25.0	11	≤0.12 - >4	≤0.12	>4	54.5	12	≤0.12 - >4	1	>4	41.7	31	≤0.12 - >4	1	>4	41.9
Rifampicin	1	6	≤1 - >4	≤1	>4	83.3	11	≤1 - >4	≤1	>4	81.8	10	≤1 – 2	≤1	≤1	90.0	27	≤1 - >4	≤1	>4	85.2
Tetracycline	4	8	≤2 - >8	≤2	>8	75.0	11	≤2 - >8	≤2	>8	81.8	12	≤2 - >8	≤2	>8	75.0	31	≤2 - >8	≤2	>8	77.4
Ticarcillin/ Clavulanic Acid [d,f]	8	4	≤16	≤16	≤16	-	9	≤16 – 64	≤16	64	-	12	≤16	≤16	≤16	-	25	≤16 – 64	≤16	≤16	-
Trimethoprim/ Sulfamethoxazol [c]	2	8	≤0.25 - >4	0.5	>4	75.0	11	≤0.25 - >4	≤0.25	1	90.9	12	≤0.25 - >4	≤0.25	>4	75.0	31	≤0.25 - >4	≤0.25	>4	80.7

Supporting Information Item S12. Temporal and cumulative susceptibility data of coagulase-negative Staphylococcus spp. isolated from foals with sepsis, UC Davis, USA, 1979-2010

Antimicrobial drug	Break-point	1979-1990					1991-1997					1998-2010									
		N	Range	MIC_{50}	MIC_{90}	%S	N	Range	MIC_{50}	MIC_{90}	%S	N	Range	MIC_{50}	MIC_{90}	%S	N	Range	MIC_{50}	MIC_{90}	%S
Amikacin	16	6	≤0.5 – 2	≤0.5	2	100	10	≤0.5 - >64	≤0.5	≤0.5	90.0	8	≤0.5 – 1	≤0.5	1	100	24	≤0.5 - >64	≤0.5	1	95.8
Ampicillin [g]	0.25	4	≤0.25 – 32	≤0.25	32	75.0	9	≤0.25 – 8	1	8	44.4	8	≤0.25 – 8	≤0.25	8	75.0	21	≤0.25 – 32	≤0.25	8	61.9
Ceftiofur [g]	2	4	0.5 - >8	1	>8	50.0*	10	≤0.25 - >8	≤0.25	2	90.0*	8	≤0.25 – 2	1	2	100*	22	≤0.25 - >8	0.5	>8	86.4
Ceftizoxime [g]	8	5	≤0.5 - >64	1	>64	60.0	10	≤0.5 - >64	1	16	80.0	7	≤0.5 – 8	1	8	100	22	≤0.5 - >64	1	>64	81.8
Cefalothin [g]	8	3	≤2	≤2	≤2	100	10	≤2 – 32	≤2	≤2	90.0	4	≤2	≤2	≤2	100	17	≤2 – 32	≤2	≤2	94.1
Chloramphenicol	8	6	≤4	≤4	≤4	100	10	≤4 - >32	≤4	8	90.0	8	≤4	≤4	≤4	100	24	≤4 - >32	≤4	8	95.8
Enrofloxacin	0.5	3	≤0.25	≤0.25	≤0.25	100	10	≤0.25 – 2	≤0.25	≤0.25	90.0	8	≤0.25 – 0.5	≤0.25	0.5	100	21	≤0.25 – 2	≤0.25	≤0.25	95.2
Erythromycin	0.5	6	≤0.25 - >2	≤0.25	>2	50.0	10	≤0.25 - >2	≤0.25	>2	70.0	7	≤0.25 - >2	≤0.25	>2	71.4	23	≤0.25 - >2	≤0.25	>2	65.2
Gentamicin	4	6	≤1 – 16	≤1	16	83.3	10	≤1 - >32	≤1	≤1	90.0	8	≤1 – 8	≤0.25	8	87.5	24	≤1 - >32	≤1	8	87.5
Imipenem [g]	4	2	≤1	≤1	≤1	100	9	≤1 – 2	≤1	2	100	5	≤1	≤1	≤1	100	16	≤1 – 2	≤1	≤1	100
Penicillin [g]	0.12	4	≤0.12 - >8	0.25	>8	25.0*	10	≤0.12 - >8	0.5	>8	10.0*	8	≤0.12 – 4	≤0.12	4	75.0*	22	≤0.12 - >8	0.25	>8	36.4
Rifampicin	1	4	≤1	≤1	≤1	100	10	≤1 – 4	≤1	≤1	90.0	8	≤1	≤1	≤1	100	22	≤1 – 4	≤1	≤1	95.5
Tetracycline	4	6	≤2 – 4	≤2	≤2	100	10	≤2 - >8	≤2	>8	70.0	7	≤2 - >8	≤2	>8	71.4	23	≤2 - >8	≤2	>8	78.3
Ticarcillin/ Clavulanic Acid [d,f]	8	2	≤16	≤16	≤16	-	9	≤16 – 32	≤16	32	-	8	≤16	≤16	≤16	-	19	≤16 – 32	≤16	≤16	-
Trimethoprim/ Sulfamethoxazol [c]	2	6	≤0.25 - >4	≤0.25	>4	66.7	10	≤0.25 - >4	≤0.25	>4	80.0	8	≤0.25 - >4	≤0.25	>4	62.5	24	≤0.25 - >4	≤0.25	>4	70.8

Legend Supporting Information Items S1-S12

N *Number of isolates.*
MIC *Minimum Inhibitory Concentration, expressed in µg/ml.*
MIC_{50} *the MIC at which 50% of the total number of isolates in a certain group of bacteria were inhibited in growth.*
MIC_{90} *the MIC at which 90% of the total number of isolates in a certain group of bacteria were inhibited in growth.*
% S *percentage of isolates that were susceptible to an antimicrobial agent.*
NA *not available.*
* *statistically significant trend $P \leq 0.05$.*
[a] *K. pneumoniae, K. oxytoca, E. coli and P. mirabilis sometimes produce Extended Spectrum Beta Lactamases (ESBLs). These isolates may be clinically resistant to therapy with penicillins and cephalosporins despite in vitro susceptibility. Isolates in this study were not routinely tested on the prevalence of ESBLs.*
[b] *Salmonella spp. sometimes appear to be susceptible to aminoglycosides and first and second generation cephalosporins in vitro, but these results often do not correspond with susceptibility in vivo.*
[c] *The breakpoint for trimethoprim/ sulfamethoxazole consists of two values, one for trimethoprim (2) and one for sulfamethoxazole (38). MIC values for trimethoprim are presented.*
[d] *The breakpoint for ticarcillin/ clavulanic acid consists of two values, one for ticarcillin (16) and one for clavulanic acid (2). MIC values for ticarcillin are presented.*
[e] *Enterococcus spp. sometimes appear to be susceptible to aminoglycosides, cephalosporins and TMS in vitro. These results often do not correspond with susceptibility in vivo.*
[f] *Calculation of a susceptibility percentage is not possible because the breakpoint is outside the tested MIC range.*
[g] *Staphylococcus spp. isolates which are resistant for oxacillin may appear susceptible in vitro to penicillins, cephalosporins and carbapenems, but are not susceptible in vivo. Susceptibility data on oxacillin was not consistently available for all Staphylococcus spp. isolates.*
[h] *Despite in vitro activity of TMS against β-haemolytic streptococci, TMS is not effective in eradicating Streptococcus equi ssp. zooepidemicus in vivo in horses.*



CHAPTER 4

Initial antimicrobial treatment of foals with sepsis: Do our choices make a difference?

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ABSTRACT

The study objectives were to provide cumulative antimicrobial susceptibility data at the patient level and to evaluate the effect of initial antimicrobial treatment on survival in foals with sepsis. Foals below 30 days of age with a diagnosis of sepsis, confirmed by isolation of bacteria from normally sterile sites on the day of hospital admission, were included. Susceptibility testing was performed using the broth microdilution procedure. In total, 213 foals and 306 bacterial isolates were included. The likelihood of survival for foals from which all bacteria were susceptible to the initial antimicrobial treatment was 65.4% (*n* = 106/162; 95% confidence interval (CI) 57.6% to 72.7%) versus 41.7% (*n* = 10/24; 95% CI 22.1% to 63.4%) if one or more isolates were resistant (relative risk 1.57, 95% CI 0.96 to 3.06). Based on this study, amikacin combined with ampicillin remains an appropriate antimicrobial drug combination for initial treatment of foals with sepsis.

***Keywords**: Amikacin; Ampicillin; Antimicrobial resistance; Equine; Neonatal intensive care unit*

SHORT COMMUNICATION

Several studies have reported antimicrobial susceptibility of bacteria isolated from foals with sepsis [1-7]. However, those studies did not consider that sepsis in foals is often polymicrobic, with a reported incidence ranging from 8% to 45% [1,8]. In polymicrobic infection, the antimicrobial susceptibility patterns of the bacteria involved often differ. For clinical decision-making, cumulative antimicrobial susceptibility data at foal level is more useful than data at isolate level. The first objective of this study was therefore to report on cumulative antimicrobial susceptibility data at foal level.

Legislation for prescribing antimicrobials is becoming increasingly restrictive, encouraging veterinarians to minimise use of antimicrobials. Therefore, it is important to evaluate the efficacy of antimicrobial treatment. The second objective of this study was to evaluate potential differences in survival between foals initially treated with antimicrobial drugs to which all of the bacteria isolated at hospital admission were susceptible ('correct' initial antimicrobial therapy) and foals treated with antimicrobial drugs to which at least one of the bacteria was resistant ('incorrect' initial antimicrobial therapy). The third objective was to evaluate the effect of type of infection (single organism versus polymicrobic infection) on survival.

All foals below 30 days of age admitted to the University of California, Davis, USA, between 1 January 1990 and 31 December 2015 with a diagnosis of sepsis, confirmed by isolation of bacteria from normally sterile sites on the day of hospital admission, were included. Only foals for which complete susceptibility data were available for all isolated bacteria were included. Necropsy culture results were included if the necropsy was performed on the day of hospitalisation and all bacteria were isolated from more than one normally sterile site, to minimise the likelihood of including contaminated samples.

Bacterial isolation, identification, classification and antimicrobial susceptibility testing were performed as described previously [6]. Breakpoints published by the Clinical & Laboratory Standards Institute (CLSI) were used to determine susceptibility, occasionally modified based on equine research (See Appendix: Supplementary Table S1) [9].

In total, 213 foals and 306 bacterial isolates were included (See Appendix: Supplementary Table S2). The percentages of foals from which all bacteria isolated at hospital admission were susceptible to the tested antimicrobial drug or combinations are presented in Table 1. Based on these data, the combination of amikacin and ampicillin appears suitable for empirical treatment in foals with sepsis. Based on the WHO list

of critically important antimicrobials[a], and the results of the current study, there is limited justification for the use of enrofloxacin, ceftizoxime or other third generation cephalosporins as initial antimicrobial therapy without bacteriological culture and susceptibility testing, in the absence of contra-indications to use amikacin or ampicillin. Carbapenems such as imipenem should be reserved for use in human patients and should not be used in veterinary species.[b]

Table 1. Cumulative susceptibility at 'foal level' of bacteria isolated from foals with sepsis at hospital admission (UC Davis, USA) between 1 January 1990 and 31 December 2015.

Antimicrobial drug (combination)	Number of foals	Percentage of foals from which all isolates were susceptible	95% confidence interval
Amikacin	213	63.4%	56.5 – 69.9
Amikacin + penicillin	210	88.6%	83.5 – 92.5
Amikacin + ampicillin	213	91.5%	87.0 – 94.9
Gentamicin	213	62.0%	55.1 – 68.5
Gentamicin + penicillin	211	82.0%	76.1 – 86.9
Gentamicin + ampicillin	213	83.6%	77.9 – 88.3
Ceftiofur	211	86.3%	80.9 – 90.6
Ceftiofur + amikacin	211	89.6%	84.6 – 93.4
Ceftizoxime	194	89.7%	84.5 – 93.6
Chloramphenicol	207	81.6%	75.7 – 86.7
Enrofloxacin	211	82.9%	77.2 – 87.8
Imipenem	175	92.6%	87.6 – 96.0
Trimethoprim/sulfamethoxazole	213	59.6%	52.7 – 66.3

To evaluate the effect of initial antimicrobial treatment on outcome, information on initial antimicrobial treatment was required; otherwise foals were excluded from this analysis. Foals that died or were euthanised at hospital admission and did not receive antimicrobial treatment were excluded from this part of the study. Outcome was defined as 'survival' if the foal survived until discharge or 'non-survival' if the foal died or was euthanised during hospitalisation. Commercial software was used for statistical analysis (StatXact Version 11, Cytel Software Corporation). The relative likelihood for survival reported as risk ratio (RR) and 95% confidence intervals (95% CI) are presented.

a See: WHO, 2016, WHO List of Critically Important Antimicrobials (CIA) – 5th Revision. http://www.who.int/foodsafety/publications/antimicrobials-fifth/en/ (accessed 14 November 2017)

b See: British Small Animal Veterinary Association (BSAVA) Medicine Guide - Antibacterials https://www.bsava.com/Resources/Veterinary-resources/Medicines-Guide/Antibacterials (accessed 21 February 2018)

Initial antimicrobial treatment was known for 186 foals. If all bacteria isolated from a single foal were susceptible to the initial antimicrobial treatment, the likelihood of survival was 65.4% (*n* = 106/162; 95% CI 57.6% to 72.7%), compared to 41.7% (*n* = 10/24; 95% CI 22.1% to 63.4%) if one or more bacteria were resistant (RR 1.57; 95% CI 0.96 to 3.06, *P* = 0.054) (Table 2). A similar result was reported in a study in human patients with sepsis [10]. Interestingly, 34.6% (*n* = 56/162; 95% CI 27.3% to 42.4%) of the foals died despite receiving 'correct' antimicrobial therapy and 41.7% (*n* = 10/24; 95% CI 22.1% to 63.4%) of foals survived despite being treated with 'incorrect' antimicrobial drugs initially, highlighting the influence of other factors on outcome.

Table 2. Survival of foals with sepsis in relation to the choice of initial antimicrobial treatment at UC Davis (USA) between 1 January 1990 and 31 December 2015.

Initial antimicrobial therapy	Total number of foals	Survival (%)	Non-survival (%)	RR[a]	95% CI[b]
'Correct'[c]	162	106 (65.4%)	56 (34.6%)	1.57 1.0	0.96 – 3.06
'Incorrect'[d]	24	10 (41.7%)	14 (58.3%)		

a RR, relative risk.
b 95% VI, 95% confidence interval.
c All bacteria isolated at hospital admission were susceptible to the initial antimicrobial therapy.
d At least one of the bacteria isolated at hospital admission was resistant to the initial antimicrobial therapy.

All 213 foals were included in the evaluation of the effect of type of infection on outcome. Thirty per cent of foals (n = 64/213; 95% CI 24.0% to 36.7%) had polymicrobic infection. Foals with single organism infection had a significantly higher likelihood of survival (61.7%; n = 92/149; 95% CI 53.4% to 69.6%) compared with foals with polymicrobic infection (40.6%; n = 26/64; 95% CI 28.5% to 53.6%) (RR 1.52; 95% CI 1.10 to 2.29; *P* = 0.005) (Table 3). This finding is in contrast to previous studies in other geographical regions [3,5].

Table 3. Survival of foals with sepsis in relation to the type of infection at UC Davis (USA) between 1 January 1990 and 31 December 2015.

Type of infection	Total number of foals (%)	Survival (%)	Non-survival (%)	RR[a]	95% CI[b]
All types of infection	213 (100%)	118 (55.4%)	95 (44.6%)		
Single organism infection	149 (70.0%)	92 (61.7%)	57 (38.3%)	1.52	1.10 – 2.29*
Polymicrobic infection	64 (30.0%)	26 (40.6%)	38 (59.4%)	1.0	

a RR, relative risk.
b 95% CI, 95% confidence interval.
** P < 0.05.*

Potential limitations of this study are that findings could be geographically restricted, cases might have been excluded because essential information was missing, and administration of antimicrobial drugs before hospitalisation could have influenced bacterial culture results. Unfortunately, information on other factors potentially affecting outcome was not consistently available. Finally, some foals may have been euthanised based on economic considerations.

Our results indicate that empirical treatment of foals with antimicrobials to which the infecting bacteria are susceptible has a positive effect on outcome and supports the common practise of initiating antimicrobial treatment prior to culture and susceptibility results being available. Nevertheless, it remains important to collect samples for bacteriological culture from these foals to evaluate the potential efficacy of the chosen therapy. Based on this study, the combination of amikacin and ampicillin remains an appropriate choice for initial treatment of foals with sepsis.

Conflict of interest statement

None of the authors of this paper have a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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SUPPLEMENTARY TABLES

Supplementary Table S1. Minimum Inhibitory Concentration (MIC) Breakpoints used to determine antimicrobial susceptibility of bacteria isolated from foals with sepsis (UC Davis, USA) between 1 January 1990 and 31 December 2015.

Antimicrobial drug	MIC Break-point [a]	Special MIC breakpoint (Bacterial species for which this breakpoint was applied)	Bacterial species reported as resistant, regardless of tested MIC [b]
Amikacin	4	-	*Enterococcus* spp. *Streptococcus* spp. *Salmonella* spp.
Ampicillin	8	0.5 (*Actinobacillus* spp.) 0.25 (*Streptococcus* spp.) 0.25 (*Staphylococcus* spp.)	*Pseudomonas* spp.
Ceftiofur	2	0.25 (*Streptococcus* spp.)	*Enterococcus* spp.
Ceftizoxime	8	-	*Enterococcus* spp.
Chloramphenicol	8	-	-
Enrofloxacin	0.5	-	*Enterococcus* spp.
Gentamicin	2	-	*Enterococcus* spp. *Streptococcus* spp. *Salmonella* spp.
Imipenem	1	-	-
Penicillin	0.5	8 (*Enterococcus* spp.)	Enterobacteriaceae *Pseudomonas* spp.
Tetracycline	4	2 (*Streptococcus* spp.)	-
Trimethoprim/ Sulfamethoxazole	0.5	-	*Enterococcus* spp. *Streptococcus* spp. *Pseudomonas* spp,

[a] *Standard breakpoint used for all bacterial species (exceptions are specified in the 3rd column)*
[b] *These include bacterial species that are intrinsically resistant to the tested antimicrobial drug or bacterial species for which it is known that in vitro susceptibility results do not correspond well with in vivo efficacy of the drug and are therefore reported as resistant regardless of in vitro test result*

Breakpoints used for Enterobacteriaceae were also applied to Aeromonas spp. and Moraxella spp. isolates.
Breakpoints used for Pseudomonas spp. were also applied to Acinetobacter spp., Ralstonia spp. and Stenotrophomonas spp. isolates and non-enteric isolates that were not further characterized.
Breakpoints used for Streptococcus spp. were also applied to Aerococcus spp. isolates.
Breakpoints used for Staphylococcus spp. were also applied to Arthrobacter spp., Bacillus spp. and Micrococcus spp. isolates.
Breakpoints used for Actinobacillus spp. were also applied to Pasteurella spp. isolates and non-enteric isolates that were not further characterized beyond the level of 'non-fermenter'.

Supplementary Table S2. Bacterial isolates from foals with sepsis (UC Davis, USA) between 1 January 1990 and 31 December 2015.

Bacterial species	Number of isolates	(% of total isolates)
Gram-negative isolates	**233**	**(76%)**
E. coli	111	(36%)
Actinobacillus spp.	58	(19%)
Klebsiella spp.	17	(6%)
Enterobacter spp.	8	(3%)
Salmonella spp.	7	(2%)
Aeromonas spp.	5	(2%)
Acinetobacter spp.	3	(1%)
Pantoea spp.	3	(1%)
Pasteurella spp.	3	(1%)
Other Gram-negative isolates	18	(6%)
Gram-positive isolates	**73**	**(24%)**
Streptococcus spp.	33	(11%)
Staphylococcus spp.	19	(6%)
Enterococcus spp.	17	(6%)
Other Gram-positive isolates	4	(1%)

CHAPTER 5

Differences in isolation rate and antimicrobial susceptibility of bacteria isolated from foals with sepsis at admission and after ≥48 hours of hospitalization

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ABSTRACT

Background: Antimicrobial treatment protocols for foals with sepsis that do not improve clinically often are adjusted based on bacteriological and antimicrobial susceptibility testing results from samples collected at hospital admission.

Objectives: To evaluate whether hospitalization for ≥48 hours affects bacteriological and antimicrobial susceptibility testing results.

Animals: Two-hundred sixty-seven foals <30 days of age admitted to a neonatal intensive care unit and diagnosed with sepsis.

Methods: Medical records were reviewed retrospectively to identify foals with sepsis and positive bacteriological cultures. Results from samples collected at hospital admission were compared to those collected ≥48 hours after admission. Logistic regression for clustered data and exact logistic regression were used for statistical analysis.

Results: Three-hundred fifty-three unique bacterial isolates were obtained from 231 foals at hospital admission and 92 unique bacterial isolates were obtained from 57 foals after ≥48 hours of hospitalization. Relative isolation frequency after ≥48 hours of hospitalization increased for *Acinetobacter* spp., 0.6% versus 3.3% (odds ratio [OR], 7.63; 95% confidence interval [CI], 1.28–45.45); *Enterococcus* spp., 4.8% versus 19.6% (OR, 5.37; 95% CI, 2.64–10.90); *Klebsiella* spp., 5.1% versus 10.9% (OR, 2.27; 95% CI, 1.05–4.89); *Pseudomonas* spp., 3.0% versus 7.6% (OR, 3.49; 95% CI, 3.49–240.50); and, *Serratia* spp., 3.0% versus 5.4% (OR, 20.23; 95% CI, 2.20–186.14). Bacteria isolated after ≥48 hours of hospitalization were less susceptible to all tested antimicrobial drugs, except for imipenem.

Conclusions and Clinical Importance: Decreased antimicrobial susceptibility of bacteria isolated after ≥48 hours of hospitalization provides a rationale for repeated bacteriological culture and susceptibility testing in hospitalized foals with sepsis.

Keywords: *Amikacin; Ampicillin; Antimicrobial resistance; Ceftiofur; Chloramphenicol; Enrofloxacin; Gentamicin; Healthcare-associated infections; Horse; Hospital-acquired infections; Imipenem; Neonate; Nosocomial infections; Penicillin; Tetracycline; Trimethoprim/sulfamethoxazole*

INTRODUCTION

Up to 60% of foals admitted to an intensive care unit in Florida were considered septic at hospital admission [1]. *Escherichia coli* is the bacterium most commonly isolated from foals with sepsis in most studies [2-8]. Antimicrobial susceptibility of bacteria isolated from foals with sepsis varies among different geographic regions [3,5,6,8-12]. Temporal trends toward increased antimicrobial resistance to frequently used antimicrobial drugs, such as gentamicin, amikacin, and ceftiofur, have been identified [12]. This finding highlights the need to perform bacteriological culture and susceptibility testing in foals suspected of sepsis.

Bacteriological and antimicrobial susceptibility testing is performed routinely on samples collected from foals with suspected sepsis at hospital admission [13]. While awaiting test results, the choice of antimicrobials to initiate treatment typically is based on historical data on antimicrobial susceptibility patterns of pathogens causing sepsis of foals in that geographic location [3,5,6,8-12]. The antimicrobial treatment regimen then is adjusted as necessary based on the results of culture and susceptibility testing of admission samples. Although this approach results in a successful outcome in 65% of affected foals, 35% fail to show clinical improvement despite treatment with antimicrobials that should be effective based on susceptibility testing of bacteria isolated from admission samples [13]. Clinicians then may opt to give the chosen antimicrobial protocol more time to be effective or adjust the treatment protocol to include other antimicrobials to which the bacteria isolated from admission samples were susceptible. Both of these approaches assume that the bacterial species infecting the foal and the antimicrobial susceptibility profile of these bacteria remain the same as those obtained from admission samples.

In adult horses, hospitalization and treatment with antimicrobial drugs create selection pressure on bacteria, leading to the development of antimicrobial resistance [14-17]. Several studies have reported on isolation rate and susceptibility patterns of bacteria isolated from foals with sepsis [2,3,5,6,8-12]. However, the effect of hospitalization and antimicrobial treatment before the time of sampling on culture results has not been investigated in foals with sepsis.

The Centers for Disease Control and Prevention (CDC) defines healthcare-associated infections (HAIs) as localized or systemic conditions resulting from an adverse reaction to the presence of an infectious agent(s) or its toxins in which case there is no evidence that the infection was present or incubating at the time of hospital admission [18]. Critically ill human patients admitted to intensive care units are at risk of devel-

oping HAIs, frequently related to particular surgical and medical procedures, which often involve specific species or strains of bacteria that are resistant to many antimicrobial drugs [19]. The same is likely true for foals admitted to neonatal intensive care facilities, but data to support this assumption currently are lacking. The main purpose of our study was to compare isolation rates and antimicrobial susceptibility patterns of bacteria isolated from foals with sepsis between samples collected on hospital admission and after ≥48 hours of hospitalization. The 2nd aim was to determine if HAIs occurred in foals after ≥48 hours of hospitalization.

The hypotheses were that different bacterial species would be isolated after ≥48 hours of hospitalization compared to samples collected at hospital admission and that these bacteria would be more resistant to antimicrobials. Also, we hypothesized that a large proportion of positive bacterial cultures after ≥48 hours of hospitalization potentially would be the result of HAIs.

MATERIALS AND METHODS

Study design and case selection

A retrospective review of medical records of foals ≤30 days of age admitted to the William R. Pritchard Veterinary Medical Teaching Hospital (VMTH), University of California (Davis, California, USA) between January 1, 1990 and December 31, 2015 was performed. Data recorded in the medical records at admission and during hospitalization of the foal were retrieved from the hospital veterinary medical information system. Records for those foals with a clinical diagnosis of sepsis, confirmed by a positive bacteriological culture from blood or normally sterile internal sites (abdominal fluid, pleural fluid, cerebrospinal fluid, IV catheter tips and joints) before 30 days of age, were selected for further evaluation. For each case, data on year of hospital admission, age, body temperature, results from blood tests (eg, hematology, serum fibrinogen and glucose concentrations, blood pH, pCO_2, and bicarbonate concentration), presence or absence of scleral injection, petechial hemorrhage, anterior uveitis, diarrhea, respiratory distress, neurologic signs or joint swelling, information on initial antimicrobial treatment and results from all bacteriological cultures and susceptibility testing performed during hospitalization (including culture site and time of sampling relative to hospital admission) were collected. Results from culture of samples collected at necropsy also were included. To minimize the likelihood of including contaminated samples, results only were included if isolates were identified from >1 normally sterile site (ie, liver, spleen, kidney, lungs, heart, meninges, body cavity, or joints). Carcasses of foals were kept refrigerated after death or euthanasia until the necropsy was performed on the day of death or the next day.

Cases were included only if foals showed ≥5 clinical or pathologic signs of systemic sepsis at the time of sample collection, such as fever (>38.9 °C), neutropenia, or neutrophilia (< 4000 or > 12000 neutrophils/μL), increased band neutrophil count (>50 band neutrophils/μL), presence of toxic changes in neutrophils, hyperfibrinogenemia (>400 mg/dL), hypoglycemia (<80 mg/dL), metabolic acidosis, scleral injection, petechial hemorrhage, anterior uveitis, diarrhea, respiratory distress, neurologic signs (hypotonia, lethargy, coma, or seizures), or joint swelling. To address the main goal of the study, all samples collected on the day of hospital admission were included in the group of "samples collected at hospital admission". All samples collected after ≥48 hours of hospitalization were included in the group of "samples collected after ≥48 hours of hospitalization". Samples collected after the day of hospital admission but before 48 hours of hospitalization were excluded from the study to prevent overlap. To address the 2nd aim of the study, samples were included only if they were collected after ≥48 hours of hospitalization and, from the same foals, bacteriological cultures also had been performed at hospital admission.

Bacterial isolation, identification, classification, and antimicrobial susceptibility testing

Bacterial isolation, identification, and classification were performed as described previously [12]. The broth microdilution Sensititre procedure (ThermoFisher Scientific, Cleveland, Ohio, USA) was used for antimicrobial susceptibility testing, following Clinical Laboratory Standards Institute (CLSI) protocols [20]. The minimum inhibitory concentration (MIC) was recorded as the lowest concentration of antimicrobial drug that inhibited visible growth of bacteria or 80% inhibition in the case of trimethoprim/sulfamethoxazole (TMS). Breakpoints published in the 3rd edition of the "Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated From Animals" by CLSI were used to determine susceptibility for all isolates included in the study, occasionally modified based on research in horses (see Table S1) [20]. For foals that were treated with combinations of antimicrobial drugs, an isolate was considered to be susceptible to this combination of drugs if its MIC for at least 1 of the drugs in the combination was equal to or less than the breakpoint.

Antimicrobial drugs

The following antimicrobial drugs were evaluated for activity against bacteria isolated from foals with sepsis: amikacin, ampicillin, ceftiofur, chloramphenicol, enrofloxacin, gentamicin, imipenem, penicillin, tetracycline, and TMS. Based on the measured antimicrobial activity of these drugs, the susceptibility of individual isolates to the following combinations of drugs, which frequently are used to treat foals with sepsis,

was predicted: amikacin + penicillin, amikacin + ampicillin, amikacin + ceftiofur, gentamicin + penicillin, and gentamicin + ampicillin.

Positive bacterial cultures after ≥48 hours of hospitalization

Positive bacterial culture results after ≥48 hours of hospitalization were compared to results from samples collected from the same foals at hospital admission. All isolates were classified as belonging to 1 of the 4 categories. The 1st category included isolates that were obtained only from samples collected after ≥48 hours of hospitalization. Samples collected on the day of hospital admission from the same foals were either culture-negative or were positive for other organisms that were no longer isolated after ≥48 hours of hospitalization. The 2nd category included isolates that were obtained from samples collected at both time points and on both occasions were susceptible to the initially administered antimicrobials. The 3rd category included bacteria that also were isolated on both time points, but these isolates were susceptible to the administered antimicrobials on hospital admission and resistant after ≥48 hours of hospitalization. The 4th category included isolates that also were obtained at both time points, but were, on both occasions, resistant to the initial antimicrobial treatment.

Statistical analysis

Logistic regression using cluster robust SE estimation was used to assess the association between isolation frequency and antimicrobial susceptibility of bacteria isolated at hospital admission as compared to those isolated after ≥48 hours of hospitalization (Stata/IC 14.1) [21,22]. Several potential confounders of these differences were identified before analysis of the data: "antemortem versus postmortem culture", "culture site", "year of culture" and for the detection of differences in susceptibility patterns, "bacterial species isolated" also were identified. The potential confounding effects of these variables were assessed by comparing the results of the single-variable analysis to those of the multivariable analysis, including the potential confounder as covariate. A change of ≥10% in the odds ratio (OR) was considered evidence of sufficient confounding to justify retention of the variable in the model regardless of its statistical significance; otherwise, the variable was excluded from the model. Because a strong association was found between time of sampling ("hospital admission" versus "after ≥48 hours of hospitalization") and the variables "antemortem versus postmortem culture" and "culture site" (eg, postmortem cultures were overrepresented in the group of "samples collected after ≥48 hours of hospitalization" and blood cultures were overrepresented in the group of "samples collected at hospital admission"), the method described above could not be applied to "antemortem versus postmortem culture" and "culture site". Therefore, a chi-square test was used to assess the association of these variables with isolation frequency and antimicrobial susceptibility within the group

of "samples collected at hospital admission" only. Because they were not found to be significantly associated with either isolation frequency or antimicrobial susceptibility, both variables were excluded from further analyses.

Inclusion or exclusion of the covariates is shown in Tables 1-3. When some of the cells formed by the outcome and predictor variable had no observations, exact logistic regression was used instead of ordinary logistic regression; correction for within-cluster correlation was maintained. The statistical methods used are noted in the tables presenting the results (see Tables 1-3). Associations are expressed as ORs with 95% confidence intervals (CIs). Statistical significance was defined as $P < .05$.

RESULTS

A total of 445 bacterial isolates from 267 foals were included in this study. Three-hundred fifty-three isolates were obtained from samples collected from 231 foals (median age, 4 days; range, 0-28 days) on the day of hospital admission and therefore were included in the group of "samples collected at hospital admission". Of the isolates included in this group, 286 were obtained from samples collected antemortem and 67 were obtained from samples collected postmortem. The majority of these bacteria were isolated from blood cultures (n = 231), but bacteria also were isolated from various organs at necropsy (n = 67), joint aspirates (n = 30), peritoneal fluid samples (n = 15), IV catheter tips (n = 4), pleural fluid samples (n = 3), and cerebrospinal fluid samples (n = 3). Ninety-two isolates were obtained from samples collected from 57 foals (median age, 6 days; range, 2-29 days) after ≥48 hours of hospitalization (median time of sampling postadmission, 5 days; range, 3-30 days) and therefore were included in the group "samples collected after ≥48 hours of hospitalization". Of the isolates included in this group, 46 were obtained from samples collected antemortem and 46 were obtained from samples collected postmortem. The majority of these bacteria were isolated from various organs at necropsy (n = 46), but bacteria also were isolated from blood cultures (n = 11), joint aspirates (n = 11), peritoneal fluid samples (n = 11), and IV catheter tips (n = 13). Of 57 foals with positive cultures after ≥48 hours of hospitalization, 21 had positive cultures at both hospital admission and after ≥48 hours of hospitalization and therefore were included in both groups, 30 had negative cultures at admission, and 6 had no cultures performed at admission.

Isolation frequency

Escherichia coli was isolated most frequently from samples collected on the day of hospital admission, followed by *Actinobacillus* spp. and *Streptococcus* spp. After ≥48

hours of hospitalization, *E. coli* remained the most frequently isolated bacterium, followed by *Enterococcus* spp. and *Klebsiella* spp.

The odds of *Actinobacillus* spp. (OR, 0.15; 95% CI, 0.00-0.91) and *Streptococcus* spp. (OR, 0.35; 95% CI, 0.13-0.91) being isolated from samples collected after ≥48 hours of hospitalization significantly decreased (Table 1). The odds of *Acinetobacter* spp. (OR, 7.63; 95% CI, 1.28-45.45), *Enterococcus* spp. (OR, 5.37; 95% CI, 2.64-10.90), *Klebsiella* spp. (OR, 2.27; 95% CI, 1.05-4.89), *Pseudomonas* spp. (OR, 3.49; 95% CI, 3.49-240.50), and *Serratia* spp. (OR, 20.23; 95% CI, 2.20-186.14) being isolated from samples after ≥48 hours of hospitalization all significantly increased (Table 1).

Table 1. Isolation frequency of bacteria cultured from foals with sepsis at admission versus after ≥48 hours of hospitalization

	Admission (n=353)		≥48 hours of hospitalization (n=92)			
Bacterial species	**Number of isolates**	**% of total isolates**	**Number of isolates**	**% of total isolates**	**Odds ratio**	**95% confidence interval**
Gram-negative bacteria	266	75.4	65	70.7	1.25[a]	0.74 – 2.11
Escherichia coli	130	36.7	30	32.6	0.83[a]	0.51 – 1.36
Klebsiella spp.	18	5.1	10	10.9	2.27[a]	1.05 – 4.89
Enterobacter spp.	9	2.5	6	6.5	2.67[a]	0.91 – 7.83
Salmonella spp.	6	1.7	0	0	3.00[b]	0.00 – 57.00
Pantoea spp.	4	1.1	1	1.1	0.86[a,c]	0.09 – 8.06
Proteus spp.	4	1.1	2	2.2	1.94[a]	0.33 – 11.51
Serratia spp.	1	0.3	5	5.4	20.23[a]	2.20 – 186.14
Actinobacillus spp.	69	19.5	0	0	0.15[b]	0.00 – 0.91
Aeromonas spp.	6	1.7	0	0	0.33[b]	0.00 – 6.33
Pasteurella spp.	5	1.4	0	0	0.57[d]	0.00 – 3.15
Acinetobacter spp.	2	0.6	3	3.3	7.63[a]	1.28 – 45.45
Pseudomonas spp.	1	0.3	7	7.6	3.49[a]	3.49 – 240.50
Other Gram-negative isolates	11	3.1	1	1.1	-	-
Gram-positive bacteria	87	24.6	27	29.3	1.25[a]	0.74 – 2.11
Streptococcus spp.	41	11.6	4	4.3	0.35[a]	0.13 – 0.91
Enterococcus spp.	17	4.8	18	19.6	5.37[a,c]	2.64 – 10.90
Staphylococcus spp.	19	5.4	5	5.4	1.01[a]	0.31 – 3.26
Bacillus spp.	7	2.0	0	0	0.39[d]	0.00 – 2.04
Other Gram-positive isolates	3	0.8	0	0	-	-

[a] *Cluster robust SE (CRSE).*
[b] *Exact logistic regression (including correction for clustering) (CRSE not possible because of no observations in one of the groups).*
[c] *The variable "year of culture" was included in the model as a co-variable.*
[d] *Exact logistic regression (ignoring clustering, because of sparse data the clustering for those variables could not be evaluated).*

Antimicrobial susceptibility

Susceptibility data, specified by antimicrobial drug or combination of antimicrobial drugs, are presented in Table 2. The following antimicrobial drugs and their combinations were predicted to have an efficacy of >90% against bacteria isolated at hospital admission: amikacin + ampicillin (93.7%), amikacin + ceftiofur (93.7%), amikacin + penicillin (90.9%), ceftiofur (90.9%), and imipenem (94.1%). None of the antimicrobial drugs or their combinations had a predicted efficacy of >90% against bacteria isolated from samples collected after ≥48 hours of hospitalization. The odds of bacteria isolated after ≥48 hours of hospitalization being susceptible to individual antimicrobial drugs or combinations of drugs tested in the study decreased significantly compared to bacteria isolated at hospital admission for all drugs and drug combinations (range of ORs, 0.03 to 0.31), except for imipenem (OR, 0.49; 95% CI, 0.18-1.33).

Table 2. Susceptibility of bacteria cultured from foals with sepsis at admission versus after ≥48 hours of hospitalization

	Admission		≥48 hours of hospitalization			
Antimicrobial drug (combinations)	**Total number of isolates**	**Number of susceptible isolates**	**Total number of isolates**	**Number of susceptible isolates**	**Odds ratio**	**95% confidence interval**
Amikacin	334	229 (68.6%)	85	36 (42.4%)	0.11[a,b]	0.04 – 0.27
Ampicillin	331	229 (69.2%)	84	24 (28.6%)	0.31[a,b]	0.16 – 0.58
Ceftiofur	331	301 (90.9%)	85	42 (49.4%)	0.03[a,b,c]	0.01 – 0.11
Chloramphenicol	326	277 (85.0%)	83	37 (44.6%)	0.22[a,b]	0.12 – 0.41
Enrofloxacin	330	289 (87.6%)	84	56 (66.7%)	0.28[a]	0.16 – 0.51
Gentamicin	334	229 (68.6%)	85	26 (30.6%)	0.17[a,b]	0.08 – 0.34
Imipenem	289	272 (94.1%)	76	59 (77.6%)	0.49[a,b]	0.18 – 1.33
Penicillin	324	128 (39.5%)	76	10 (13.2%)	0.23[a]	0.11 – 0.52
Tetracycline	306	237 (77.5%)	81	27 (33.3%)	0.26[a,b]	0.14 – 0.50
Trimethoprim / Sulfamethoxazole	334	215 (64.4%)	85	19 (22.4%)	0.22[a,b]	0.11 – 0.45
Amikacin + penicillin	331	301 (90.9%)	77	45 (58.4%)	0.15[a,b]	0.07 – 0.32
Amikacin + ampicillin	334	313 (93.7%)	84	48 (57.1%)	0.13[a,b]	0.06 – 0.29
Amikacin + ceftiofur	333	312 (93.7%)	85	53 (62.4%)	0.04[a,b]	0.01 – 0.18
Gentamicin + penicillin	329	285 (86.6%)	77	35 (45.5%)	0.18[a,b]	0.09 – 0.35
Gentamicin + ampicillin	333	290 (87.1%)	84	37 (44.0%)	0.19[a,b]	0.10 – 0.36

[a] *Cluster robust SE.*
[b] *"Bacterial species isolated" was included in the model for the statistical analysis as a co-variable.*
[c] *The variable "year of culture" was included in the model for the statistical analysis as a co-variable.*

Escherichia coli was the only bacterial species for which the number of isolates was high enough to make a meaningful comparison between hospital admission and after ≥48

hours of hospitalization. Thus, we also have included separate susceptibility data for *E. coli* (Table 3). The odds that *E. coli* isolated after ≥48 hours of hospitalization were susceptible to individual antimicrobial drugs or combinations of drugs also decreased significantly compared to *E. coli* isolated at hospital admission for all drugs and drug combinations (range of ORs, 0.02-0.30), except for enrofloxacin (OR, 0.26; 95% CI, 0.03-1.98) and imipenem (OR, 1.28; 95% CI, 0.21 to ∞).

Table 3. Susceptibility of E. coli cultured from foals with sepsis at admission versus after ≥48 hours of hospitalization

	Admission		≥48 hours of hospitalization			
Antimicrobial drug (combinations)	**Total number of *E. coli***	**Number of susceptible *E. coli***	**Total number of *E. coli***	**Number of susceptible *E. coli***	**Odds ratio**	**95% confidence interval**
Amikacin	124	114 (91.9%)	29	17 (58.6%)	0.12[a]	0.04 – 0.35
Ampicillin	124	87 (70.2%)	29	12 (41.4%)	0.30[a]	0.13 – 0.71
Ceftiofur	124	123 (99.2%)	29	23 (79.3%)	0.02[a,b]	<0.01 – 0.39
Chloramphenicol	121	95 (78.5%)	29	11 (37.9%)	0.17[a]	0.07 – 0.38
Enrofloxacin	124	121 (97.6%)	29	27 (93.1%)	0.26[a,b]	0.03 – 1.98
Gentamicin	124	108 (87.1%)	29	12 (41.4%)	0.10[a]	0.04 – 0.25
Imipenem	113	109 (96.5%)	27	27 (100%)	1.28[c]	0.21 – ∞
Penicillin[d]	124	0 (0%)	27	0 (0%)	NA	NA
Tetracycline	115	84 (73.0%)	27	9 (33.3%)	0.18[a]	0.08 – 0.45
Trimethoprim / Sulfamethoxazole	124	86 (69.4%)	29	10 (34.5%)	0.23[a]	0.10 – 0.52
Amikacin + penicillin	124	114 (91.9%)	28	17 (60.7%)	0.14[a]	0.05 – 0.38
Amikacin + ampicillin	124	119 (96.0%)	29	19 (65.5%)	0.08[a]	0.02 – 0.28
Amikacin + ceftiofur	124	123 (99.2%)	29	26 (89.7%)	0.07[a]	0.01 – 0.68
Gentamicin + penicillin	124	108 (87.1%)	28	12 (42.9%)	0.11[a]	0.05 – 0.26
Gentamicin + ampicillin	124	109 (87.9%)	29	13 (44.8%)	0.11[a]	0.05 – 0.27

[a] *Clustered robust SE.*
[b] *The variable "year of culture" was included in the model as a co-variable.*
[c] *Exact logistic regression (ignoring clustering, because of sparse data the clustering for those variables could not be evaluated).*
[d] *E. coli was always classified as resistant to penicillin (regardless of MIC value).*

Positive bacterial cultures after ≥48 hours of hospitalization

Fifty-one foals had positive cultures after ≥48 hours of hospitalization and also had cultures performed at hospital admission, resulting in 82 isolates that were included in this part of the study. Of these 51 foals, 21 foals had positive cultures both at hospital admission and after ≥48 hours of hospitalization. Thirty foals had negative cultures at admission, but positive cultures after ≥48 hours of hospitalization.

For the 82 bacteria isolated after ≥48 hours of hospitalization, we compared the results of bacteriological culture and susceptibility testing after ≥48 hours of hospitalization to the results of samples collected from the same foals at hospital admission.

Seventy (85.3%) of 82 isolates were found only in samples collected after ≥48 hours of hospitalization. Samples collected on the day of hospital admission from the same foals were either culture-negative or positive for other organisms that were no longer isolated after ≥48 hours of hospitalization. Five (6.1%) of 82 isolates were obtained at both time points and on both occasions were susceptible to the antimicrobials initially administered. Four (4.9%) of 82 isolates also were obtained at both time points, but were susceptible to the antimicrobials initially administered on hospital admission and resistant after ≥48 hours of hospitalization. Three (3.7%) of 82 isolates also were obtained at both time points, but were resistant to the initial antimicrobial treatment on both occasions.

DISCUSSION

Several studies have reported on isolation frequency and susceptibility of bacteria obtained from foals with sepsis [2,3,5,6,8-12]. However, none of these studies has examined the association of time of sampling during hospitalization on the results. In adult horses, *E coli* bacteria isolated from fecal samples were more resistant after a period of hospitalization and treatment with antimicrobials, demonstrating the effect on bacteriological culture and susceptibility testing results also seen in our study of foals with sepsis [14-17].

The odds on nonsurvival were 2.26 times higher in bacteremic foals compared to foals with negative blood cultures in a study performed in Florida, emphasizing the necessity of empirical selection of antimicrobial drugs to initiate treatment in foals with sepsis [1]. This initial antimicrobial treatment regime should be reviewed frequently and adjusted as necessary, particularly in cases that fail to improve clinically, because the likelihood of survival for foals with sepsis treated with antimicrobials for which all infecting bacteria are susceptible is 65% compared to 42% in foals for which at least 1 of the infecting bacteria is resistant to initial treatment [13]. It is therefore important to know which bacteria are most likely to be involved and what their expected susceptibility patterns are at different points in time during hospitalization. Our study showed that isolation frequency and antimicrobial susceptibility of bacteria differed significantly between samples collected at hospital admission and after ≥48 hours of hospitalization. This finding indicates that selection of antimicrobial drugs to treat

foals with on-going sepsis during hospitalization cannot be based solely on culture results from samples collected at the time of hospital admission.

Of the drugs included in our study, ceftiofur and enrofloxacin are classified as "highest priority critically important antimicrobials" by the World Health Organization (WHO), which means they are regarded as critically important to human health [23]. Amikacin, ampicillin, gentamicin, and imipenem are classified as "high priority critically important antimicrobials". Chloramphenicol, penicillin, tetracycline, and TMS are "highly important antimicrobials" according to the WHO. The use of "highest priority critically important antimicrobials" in horses should be restricted, according to the WHO, and should be reserved only for cases for which no alternative antimicrobials are effective, and only after appropriate susceptibility testing. In our opinion, this policy also should be applied to imipenem, because it is the only "high priority critically important antimicrobial" we tested that is listed as the sole treatment available for specific diseases in humans.

Isolation frequency

After ≥48 hours of hospitalization, the odds of samples being positive for *Actinobacillus* spp. and *Streptococcus* spp. significantly decreased compared to samples collected at admission. These bacterial species typically are susceptible to antimicrobial drugs commonly used for initial treatment of foals with sepsis, such as the combination of amikacin and ampicillin [12]. Therefore, it is not surprising that in samples collected after ≥48 hours of hospitalization these bacteria were isolated less frequently.

After ≥48 hours of hospitalization, the odds of bacterial cultures being positive for *Acinetobacter* spp., *Enterococcus* spp., *Klebsiella* spp., *Pseudomonas* spp., and *Serratia* spp. all significantly increased. A high proportion of these species of bacteria are known to show intrinsic or acquired resistance to many antimicrobial drugs, including those commonly used in initial treatment protocols for foals with sepsis [24-29]. These bacterial species were responsible for a large proportion of the HAIs in a large study in humans: *Enterococcus* spp., 13.9%; *Klebsiella* spp., 8%; *Pseudomonas* spp., 7.5%; *Serratia* spp., 2.1%; and *Acinetobacter* spp., 1.8% [30]. In equine medicine, there also are several reports on the role of these bacteria in HAIs. A study on *Acinetobacter baumanni* isolates from companion animals and horses in Switzerland found that a majority of these infections were hospital-acquired [31]. In a study of surgical site infection after laparotomy in horses, *Enterococcus* spp. were the 2nd most commonly isolated bacteria [32]. *Klebsiella* spp. were identified as causative organisms of pneumonia in 11 horses that had undergone mechanical ventilation under general anesthesia [33]. The relatively high proportion of bacterial species that are known to frequently cause HAIs isolated in our

study from samples after ≥48 hours of hospitalization suggests that HAIs could also play an important role in equine neonatal care. Further genotypic characterization would be required to confirm this hypothesis.

Antimicrobial susceptibility

Bacteria isolated after ≥48 hours of hospitalization were less susceptible to all antimicrobial drugs and combinations evaluated in our study compared to those isolated at admission. This decreased susceptibility was significant for all drugs and drug combinations, except for imipenem. None of the antimicrobial drugs or their combinations were predicted to have an efficacy of >90% against bacteria isolated after ≥48 hours of hospitalization. Susceptibility patterns of these bacteria were unpredictable. This observation can be explained in part by the different bacterial species that were isolated. Antimicrobial treatment was initiated in all foals included in our study at hospital admission after collection of the 1st sample for bacteriological culture and susceptibility testing. This approach likely led to an antimicrobial selection pressure, favoring growth of resistant bacterial populations, as is also seen in studies in adult horses [14-17]. Therefore, the bacterial species and strains isolated after ≥48 hours of hospitalization frequently were more resistant to multiple antimicrobial drugs compared to the species and strains isolated at hospital admission. However, the results for *E. coli* clearly indicate that, even within the same bacterial species, the odds of being susceptible significantly decreased between admission and ≥48 hours after admission (Table 3), indicating selection of more resistant strains, development of acquired resistance, or both.

Positive bacterial cultures after ≥48 hours of hospitalization

From the time of hospital admission, all foals included in our study were treated with antimicrobial drugs. Therefore, in all cases in which samples were collected during hospitalization, the foals had been treated with antimicrobials before collection of these samples. Negative cultures resulted after ≥48 hours of hospitalization in most foals. However, in 57 foals, samples submitted for bacteriological culture and antimicrobial susceptibility testing after ≥48 hours of hospitalization were positive (n = 92 isolates).

The 2nd aim of our study was to determine if HAIs occurred in foals after ≥48 hours of hospitalization by comparing results from cultures after ≥48 hours of hospitalization to test results from samples collected from the same foals at hospital admission. In 6 foals, no bacteriological culture was performed at hospital admission. Bacteria isolated from these foals (n = 10 isolates) therefore were excluded from this part of the study.

The remaining 82 bacteria that were isolated after ≥48 hours of hospitalization were divided into 4 categories.

The 1st category included bacteria that were isolated only from samples collected after ≥48 hours of hospitalization. Samples collected on the day of hospital admission from the same foals were either culture-negative or were positive for other organisms that were eliminated by the initial antimicrobial treatment and were therefore no longer isolated after ≥48 hours of hospitalization. Two possible explanations exist for inclusion in this category. The 1st explanation is that in these cases the sample collected at hospital admission was a false negative and the infection was not cleared by the initial antimicrobial treatment. The 2nd explanation is that these foals acquired an infection during hospitalization (HAIs). Given the study design, it is impossible to distinguish between these 2 possible explanations. Seventy of 82 isolates belong to this category (85.4%).

The 2nd category of positive samples after ≥48 hours of hospitalization includes presumed treatment failures (n = 5/82; 6.1%). The same bacteria were isolated from samples collected at both time points and were, on both occasions, susceptible to the administered antimicrobials.

The 3rd category includes bacteria that were isolated at both time points and were susceptible to the administered antimicrobials on hospital admission, but resistant after ≥48 hours of hospitalization. These bacteria potentially acquired resistance during hospitalization and antimicrobial treatment (n = 4/82; 4.9%), but further genotypic characterization would be required to confirm this possibility. Two other explanations for inclusion in this category are possible. First, it is possible that >1 morphologically identical strain of a particular bacterial species (and therefore >1 susceptibility pattern) was present in both samples, but only 1 of these strains was selected for susceptibility testing. Selection of different colonies then could have led to different susceptibility results. And 2nd, foals could have become infected with a more resistant strain of the same bacterial species during hospitalization (HAI) resulting in a different antimicrobial susceptibility pattern.

The 4th category of positive samples collected ≥48 hours of hospitalization includes bacteria that were not eliminated because they were resistant to the initial antimicrobial treatment on hospital admission (n = 3/82; 3.7%).

Given the study design, and by using the CDC definition of HAIs [18], it is impossible to determine with 100% certainty if a positive culture after ≥48 hours of hospitalization included in category 1 is the result of an HAI. However, the numbers of positive cultures after ≥48 hours of hospitalization included in category 2 (n = 5) and 4 (n = 3) were very low, suggesting it is rare for susceptible bacteria not to be eliminated by the initial antimicrobial treatment and it is equally rare for bacteria to be resistant to initial antimicrobial treatment. One of these scenarios would need to be the case for isolates that were included in category 1 as a result of a false negative culture at hospital admission. Therefore, we conclude that the majority of samples included in category 1 are most likely the result of HAIs. However, genotypic characterization would be required to confirm this hypothesis with certainty.

These findings support the conclusion that HAIs with resistant strains of bacteria potentially play an important role in equine neonatal medicine.

Limitations

We acknowledge that several aspects of the design of our study could have influenced the results obtained. First, only isolates originating from samples from foals treated at the University of California-Davis VMTH were included, which could have led to geographically restricted findings. Second, our study was largely based on a retrospective review of medical records, and therefore cases for which essential information was missing were excluded. Given the retrospective nature of our study, we did not have follow-up samples for bacteriological culture and susceptibility testing available for all foals included in the study. At hospital admission, blood cultures were collected routinely in foals suspected of sepsis, whereas later sampling during hospitalization was based on the clinical situation (eg. poor treatment response). This situation potentially could have created substantial sampling bias and prevented conclusions being drawn regarding potential mechanisms for the observed differences in isolation frequency and antimicrobial susceptibility. However, this factor does not restrict the clinical value of the data in guiding clinicians who need to decide whether to adjust antimicrobial treatment protocols in foals with on-going sepsis. Not all susceptibility testing was performed at the same time, although the same methods were used throughout the study and the same interpretation criteria regarding antimicrobial susceptibility were applied to all isolates included in the study [20]. Administration of antimicrobial drugs before hospitalization could have influenced susceptibility profiles of bacteria isolated at hospital admission. Data on antimicrobial treatment before admission were not available for all cases and could not be taken into account. Antimicrobial removal devices (ARD) were not consistently used for blood cultures throughout the study period. Without use of an ARD, inhibition of growth of susceptible bacteria in vitro may have occurred and given false negative culture results in some cases. Samples collected postmortem may have a higher risk of contamination

compared to samples collected antemortem. To minimize the risk of this factor influencing our results, isolates collected postmortem only were included if they were isolated from at least 2 normally sterile sites.

CONCLUSIONS AND CLINICAL RELEVANCE

Susceptibility patterns of bacteria isolated after ≥48 hours of hospitalization were less predictable than those of foals tested at the time of admission, and therefore no general guidelines could be formulated regarding the choice of antimicrobial treatment under these circumstances. Considering that results of culture and antimicrobial susceptibility testing typically are not available for at least 48 hours after sample collection, it would be rational to repeat bacteriological culture and susceptibility testing at 48 hours intervals on foals hospitalized in neonatal intensive care units, in order to detect on-going infections or HAIs at an early stage and select effective antimicrobials for treatment. A large proportion of the bacteria isolated after ≥48 hours hospitalization potentially could be the result of HAIs, emphasizing the importance of these infections in foals treated in neonatal intensive care units. Our findings emphasize the need for strategies to prevent and control HAIs in equine hospitals.

List of abbreviations

ARD: antimicrobial removal device
CDC: Centers for Disease Control and Prevention
CI: confidence interval
CLSI: Clinical Laboratory Standards Institute
CRSE: cluster robust standard errors
HAI: healthcare-associated infection
MIC: minimum inhibitory concentration
OR: odds ratio
TMS: trimethoprim-sulfamethoxazole
VMTH: veterinary medical teaching hospital
WHO: World Health Organization

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Conflict of interest declaration

Authors declare no conflict of interest.

Off-label antimicrobial declaration

Authors declare no off-label use of antimicrobials.

Institutional animal care and use committee (IACUC) or other approval declaration

Authors declare no IACUC or other approval was needed.

Human ethics approval declaration

Authors declare human ethics approval was not needed for this study.

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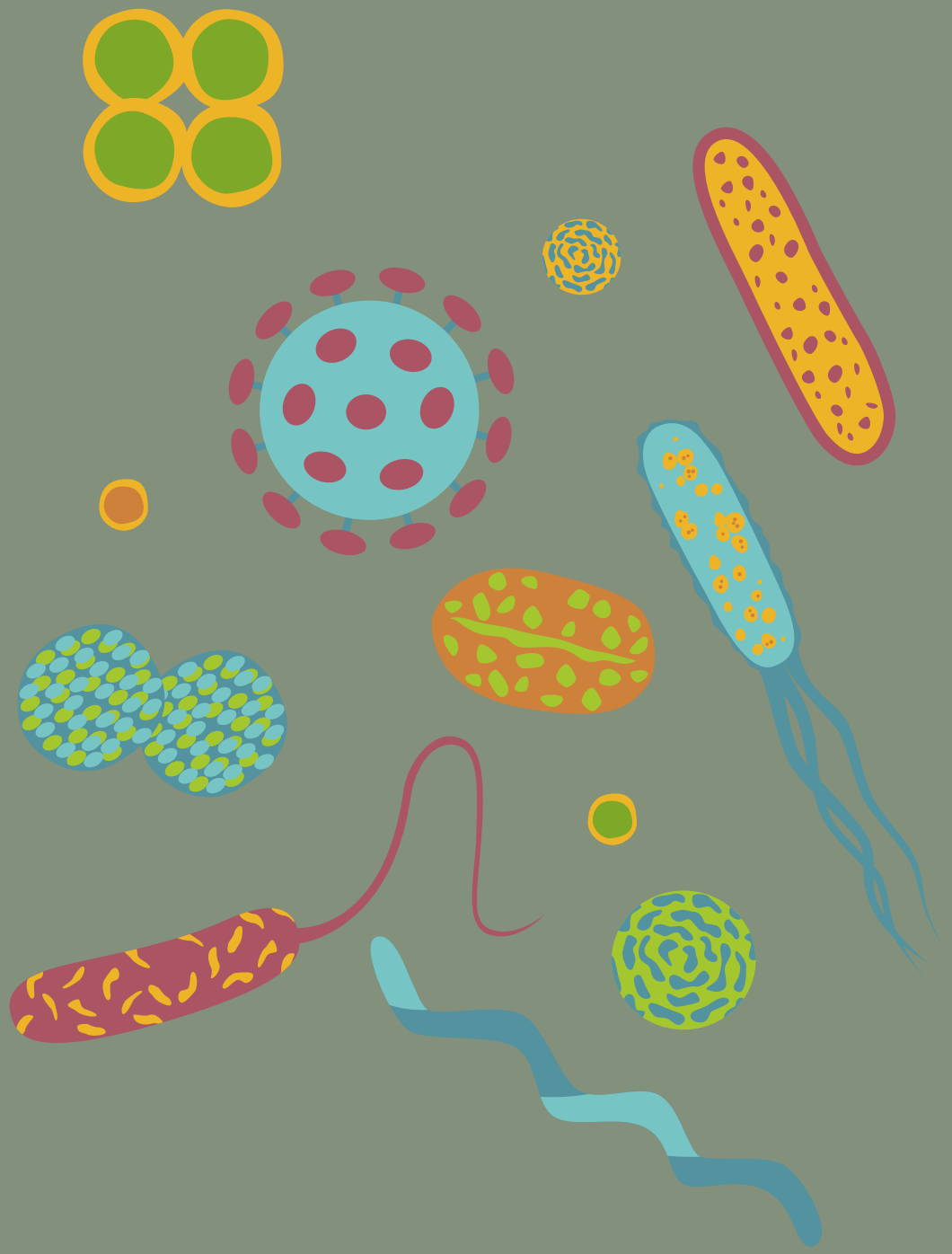
SUPPORTING INFORMATION

Supporting Information Table S1. MIC Breakpoints used to determine antimicrobial susceptibility of bacteria isolated from foals with sepsis

Antimicrobial drug	**MIC Break-point** [a]	**Special MIC breakpoint** (Bacterial species for which this breakpoint was applied)	**Bacterial species reported as resistant, regardless of tested MIC** [b]
Amikacin	4	-	*Enterococcus* spp. *Streptococcus* spp. *Salmonella* spp.
Ampicillin	8	0.5 (*Actinobacillus* spp.) 0.25 (*Streptococcus* spp.) 0.25 (*Staphylococcus* spp.)	*Pseudomonas* spp.
Ceftiofur	2	0.25 (*Streptococcus* spp.)	*Enterococcus* spp.
Chloramphenicol	8	-	-
Enrofloxacin	0.5	-	*Enterococcus* spp.
Gentamicin	2	-	*Enterococcus* spp. *Streptococcus* spp. *Salmonella* spp.
Imipenem	1	-	-
Penicillin	0.5	8 (*Enterococcus* spp.)	Enterobacteriaceae *Pseudomonas* spp.
Tetracycline	4	2 (*Streptococcus* spp.)	-
Trimethoprim/ Sul-famethoxazole	0.5	-	*Enterococcus* spp. *Streptococcus* spp. *Pseudomonas* spp,

[a] Standard breakpoint used for all bacterial species (exceptions are specified in the 3rd column)
[b] These include bacterial species that are intrinsically resistant to the tested antimicrobial drug or bacterial species for which it is known that in vitro susceptibility results do not correspond well with in vivo efficacy of the drug and are therefore reported as resistant regardless of in vitro test result

Breakpoints used for Enterobacteriaceae were also applied to Aeromonas spp. and Moraxella spp. isolates.
Breakpoints used for Pseudomonas spp. were also applied to Acinetobacter spp., Ralstonia spp. and Stenotrophomonas spp. isolates and non-enteric isolates that were not further characterized.
Breakpoints used for Streptococcus spp. were also applied to Aerococcus spp. isolates.
Breakpoints used for Staphylococcus spp. were also applied to Arthrobacter spp., Bacillus spp. and Micrococcus spp. isolates.
Breakpoints used for Actinobacillus spp. were also applied to Pasteurella spp. isolates and non-enteric isolates that were not further characterized beyond the level of "non-fermenter".



CHAPTER 6

The equine faecal microbiota of healthy horses and ponies in the Netherlands: impact of host and environmental factors

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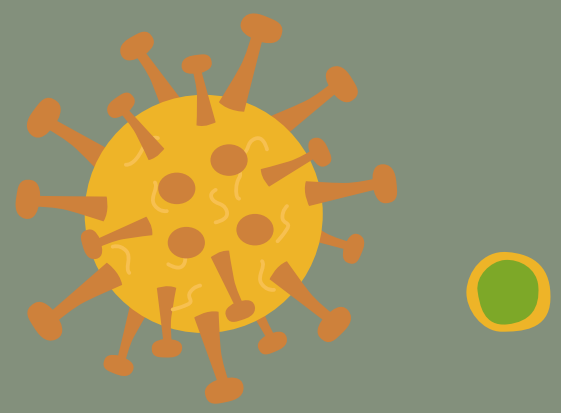

SIMPLE SUMMARY

Several studies have described the bacterial composition in the intestines of horses, and several factors of influence have been detected. Variation in the results between studies, however, is substantial. Therefore, the current study aimed to study the bacterial composition in the faeces of healthy horses and ponies kept under standard housing and management condition in The Netherlands. Seventy-nine horses and ponies originating from two farms were included. Several factors, such as location, age, the season of sampling, horse type (horses vs. ponies) and pasture access significantly affected the bacterial composition. The current study provides important baseline information on variation in the bacterial composition in healthy horses and ponies under standard housing and management conditions. The aforementioned factors identified in this study to affect the bacterial population of the gut should be considered in future studies regarding the bacterial population of the equine gut.

ABSTRACT

Several studies have described the faecal microbiota of horses and the factors that influence its composition, but the variation in results is substantial. This study aimed to investigate the microbiota composition in healthy equids in The Netherlands under standard housing and management conditions and to evaluate the effect of age, gender, horse type, diet, pasture access, the season of sampling and location on it. Spontaneously produced faecal samples were collected from the stall floor of 79 healthy horses and ponies at two farms. The validity of this sampling technique was evaluated in a small pilot study including five ponies showing that the microbiota composition of faecal samples collected up to 6 h after spontaneous defaecation was similar to that of the samples collected rectally. After DNA extraction, Illumina Miseq 16S rRNA sequencing was performed to determine microbiota composition. The effect of host and environmental factors on microbiota composition were determined using several techniques (NMDS, PERMANOVA, DESeq2). Bacteroidetes was the largest phylum found in the faecal microbiota (50.1%), followed by Firmicutes (28.4%). Alpha-diversity and richness decreased significantly with increasing age. Location, age, season, horse type and pasture access had a significant effect on beta-diversity. The current study provides important baseline information on variation in faecal microbiota in healthy horses and ponies under standard housing and management conditions. These results indicate that faecal microbiota composition is affected by several horse-related and environment-related factors, and these factors should be considered in future studies of the equine faecal microbiota.

Keywords: *Equine; Faecal; Microbiota; Age; Gender; Pony; Diet; Pasture; Season; Location*

INTRODUCTION

A well-functioning intestinal tract and intestinal microbiota are considered essential for maintaining health in horses [1]. Disturbances of the microbiota are associated with diseases in horses, such as colitis, equine metabolic syndrome and colic [2-4], although it is difficult to assess what comes first, disease or altered microbiota. Several studies have described the faecal microbiota of healthy horses, but the variation in results is substantial [5-17]. Therefore, our understanding of what can be considered normal variation and truly abnormal is currently limited. Inter-individual variation has been demonstrated to be larger than intra-individual variation [7,18]. The single main predictor of microbiota composition is individual identity, and it was suggested that this explains about 50% of the variation [19], meaning that other factors also affect microbiota composition. Several research groups studied the effects of different factors such as age, breed, band, maternal relationship, social behaviour, environmental conditions (such as diet, pasture access, fasting, transportation, exercise and season) and the use of pre- and probiotics and antimicrobials on the equine gut microbiota [6,8-10,13-15,19-21]. Geographic variation in microbiota composition has been demonstrated in humans [22], and the same might be true for horses, highlighting the need for studies from different geographic regions assessing faecal microbiota composition in horses. So far, most studies have compared relatively homogenous groups of horses exposed to one changing variable. This has the advantage of detecting subtle differences attributable to the tested variable, but at the same time, limits the extent to which results can be extrapolated to other populations of horses managed differently. Therefore, more knowledge about the normal faecal microbiota of horses and ponies kept under standard housing and management conditions is needed, for example, to study potential associations between microbiota composition and disease status. This study aimed to describe the microbiota composition in healthy horses and ponies in The Netherlands kept under standard housing and management conditions and assess which factors influence faecal microbiota composition.

MATERIALS AND METHODS

Study design, population and metadata collection

The horses and ponies (n = 79) included in the study were client-owned animals housed at two locations in The Netherlands and sampled once between April 2015 and February 2016. At the main location, farm I, 61 animals (aged 5-31 years) were included. All animals were kept in individual stables with straw bedding, and some had pasture access. An additional 18 Warmblood horses (age 4-16 years) were sampled at a second farm (II). These horses were also kept in individual stables on straw or sawdust and

some of the horses had pasture access. For each animal, information regarding age (in years), gender (male/female), horse type (horse/pony), diet (type of roughage and type and amount of concentrates), pasture access (yes/no) and season of sampling (summer/winter) was recorded. The minimum and maximum environmental temperatures at the moment of sample collection in the summer were 8 to 18 °C and 3 to 10 °C in the winter. None of the animals included in the study were treated with antimicrobials within the last six months, and none of the horses had any health problems in the past six months, according to the owner.

Ethical considerations

No procedures had to be performed on the animals included in this study. Therefore, ethical approval was not required for this study. Informed consent was obtained from all the owners.

Faecal sampling

Faecal samples were collected from the stall floor of individually housed horses and ponies. To ensure fresh samples were collected, sampling occurred within 6 h after the stables had been cleaned. Faeces were collected from the centre of a faecal ball. The samples were stored at -80°C within 2 h of collection. To evaluate the validity of the sampling technique described above, a pilot study was conducted to investigate the effect of air exposure on the equine faecal microbiota when using this convenience sampling technique. For this purpose, faecal samples were collected rectally (t = 0) from five Shetland ponies (that underwent a rectal exam for reproductive purposes), after which these samples were exposed to room air (temperature: 18.2-23.9 °C; humidity 55.0-68.0%) and aliquots were sampled at 1 h, 3 h, 6 h, 12 h and 24 h.

DNA extraction and 16S rRNA amplicon sequencing

DNA extraction was performed using the QIAamp Fast DNA Stool Mini Kit (Qiagen, Hilden, Germany) following a previously published protocol [23]. However, the samples were not treated in the TissueLyser at 30 Hz for 3 x 30 s with cooling on ice in between treatments but were bead-beaten for 5 min on a Vortex-Genie 2 (Scientific Industries, Bohemia, NY, USA). The variable V3 and V4 regions of the 16S rRNA amplicon were amplified, and libraries were prepared following the 16S Metagenomic Sequencing Library Preparation protocol (Illumina, San Diego, CA, USA). Next, each library was normalised, pooled and loaded onto the Illumina MiSeq platform for paired-end sequencing using the 600 cycles MiSeq Reagent Kit V3 (Illumina, San Diego, CA, USA), generating 2 × 300 base pair paired-end reads.

Bioinformatics processing

Data preparation was performed using Jupyter notebook version 5.7.8, running on Python 3.7.3. utilising R version 3.4.4. Raw reads (250 bp) obtained from Illumina 16S rRNA amplicon sequencing provided input for the denoising pipeline DADA2. DADA2 models and corrects Illumina-sequenced amplicon errors with high precision [24]. First, the forward and reverse reads were sorted, and the quality profile was plotted. Trimming parameters were derived from the quality plots, maintaining a minimum quality score of 20. Forward-reads contained higher quality compared to reverse reads, as is common among Illumina data. Truncations were set at 15-290 for forward, 15-210 for reverse. Post filter and trimming the reads were merged and the merged data was used to create a sequence table. After the removal of chimeras, taxonomy was assigned using v. 132 of the Silva database [25].

Data analysis

The DADA2 object was imported into the Phyloseq package [26]. All samples with less than 5000 reads were excluded. All data analyses and visualisations were performed with R version 4.0.2 [27] using vegan [28] and ggplot [29] packages. For alpha-diversity, data was rarefied to the sample with the lowest read counts (14,665 reads). All analyses were performed on the data of 61 horses and ponies from farm I unless mentioned otherwise.

Relative abundance and alpha-diversity

Relative abundances at the phylum level were assessed for each sample, and phylum and class level bar plots were produced. Alpha-diversity (observed richness and Shannon diversity) was calculated from rarefied data. The effect of potential determinants (age, gender, horse type, roughage, concentrates, pasture access, season and location) on sample alpha-diversity was univariably tested with Wilcoxon rank-sum test (two groups), Kruskal-Wallis test (multiple groups) or linear regression (continuous variable). To evaluate the effect of location (farm) on the faecal microbiota, horses of farm II were compared to a comparable group of 17 Warmblood horses in the same age group from farm I.

Microbiota composition (beta-diversity)

Between sample Bray-Curtis dissimilarity was computed on relative abundance data and used for Non-Metric Dimensional Scaling (NMDS). To determine if significant differences in microbiota composition were present between groups based on the previously mentioned factors, permutational multivariate analysis of variance (PERMANOVA), including beta-dispersion analysis, was performed (vegan package function Adonis2 and betadisper).

Differential abundance analysis

To determine taxa that differ in abundance for the tested factors, the DESeq2 package [30] was used. With a Wald test, the DESeq2 package determines if a significant fold change is present. The p-values were adjusted for the FDR (Benjamini-Hochberg approach [31]) with alpha set at 0.05. For this analysis, only factors associated with a significant difference in beta-diversity were considered. Raw count data was used as input, taxa not seen more than three times in at least 20% of the samples were filtered beforehand. Percentages of interesting taxa were calculated to reveal abundances in the entire microbiome.

Data availability

The datasets supporting this article have been uploaded to the sequence read archive as part of the supplementary electronic material and are available under accession PRJEB44895.

RESULTS

Faecal samples from 79 equids were included in the study. Sixty-one animals located at farm I were included in the analysis to study the effects of age, gender, horse type, roughage, concentrates, pasture access and season on faecal microbiota composition. See Table 1 for descriptive characteristics of the study population. Eighteen horses originating from farm II were compared to 17 horses of the same breed and age from farm I to study the effect of location/farm on faecal microbiota composition. A total of 5,015,145 high quality non-chimeric bacterial 16S reads were generated and annotated to 25,011 OTUs. The distribution of the number of reads across samples was as follows: a minimum of 14,665 reads, a maximum of 87,169 reads, and a median of 32,949 reads.

Table 1. Descriptive data of the study population.

Age	n	Mean (Years)	SD (Years)	Range (Years)
	61	15.6	6.7	5-31
Gender				
Male	37			
Female	24			
Horse type				
Pony	29			
Horse	32			
Roughage				
Hay	10			
Haylage	33			
Mixed	18			
Concentrates				
<2 kg	25			
≥2 kg	36			
Pasture access				
None	20			
Daily	41			
Season of sampling				
Summer	30			
Winter	31			
Location[1]				
Farm I	17			
Farm II	18			

[1] *Only Warmblood horses 4-16 years included.*

Effect of air exposure on the equine faecal microbiota

To evaluate the validity of the used sampling technique of collecting fresh faecal samples from the stall floor, a pilot study was conducted to investigate the effect of air exposure on the equine faecal microbiota. Faecal balls were collected rectally from 5 ponies and exposed to room air for different times. Richness and alpha-diversity were stable up to 12 h, but decreased between 12 and 24 h of air exposure, see Figure 1. Significant shifts in relative abundance for different phyla were observed at t = 12 h and t = 24 h compared to t = 0. Bacteroidetes decreased in relative abundance, while Firmicutes increased. At the class level, Bacilli increased after 12 h of air exposure (Supplementary Figure S1).

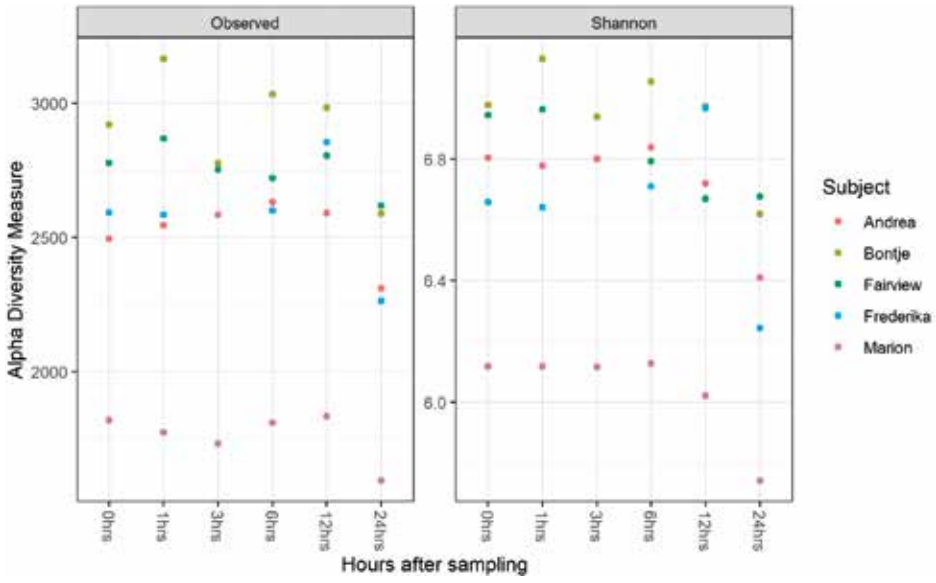

Figure 1. Observed richness and alpha-diversity (Shannon diversity index) of the faecal microbiota over time of faecal samples collected from five ponies after exposure to room air. *A decrease in observed richness alpha-diversity is visible after 12 h of air exposure (p = 0.062).*

Faecal microbiota composition

A relative abundance of taxa in the faecal microbiota of 79 horses and ponies at class level and phylum level is presented in Supplementary Figure S2. Phyla, classes and families with a relative abundance of >1% are presented in Table 2. Bacteroidetes was the phylum with the largest mean relative abundance in our study (50.1%), followed by Firmicutes (28.4%), Spirochaetes (7.1%), Verrucomicrobia (6.5%), Fibrobacteres (5.0%) and Cyanobacteria (1.0%).

Factors affecting faecal microbiota composition

We evaluated the effect of age, gender, horse type, diet (roughage type, concentrates), pasture access, the season of sampling and location on microbiota composition by comparing alpha-diversity, beta-diversity, and differential abundance of taxa between samples.

Table 2. Phyla, classes and families with a relative abundance of >1% in the faecal microbiota.

Phylum	Class	Family	Relative abundance (%)
Bacteroidetes			50.1
	Bacteroidia		50.1
		Rikenellaceae	12.9
		p-251-o5	11.7
		Prevotellaceae	9.6
		F082	4.1
		Bacteroidales_UCG_001	3.4
		Bacteroidales_RF16	2.3
Firmicutes			28.4
	Clostridia		22.8
		Lachnospiraceae	9.8
		Oscillospiraceae	3.5
		UCG-010	2.5
		Ruminococcaceae	1.6
		Anaerovoracaceae	1.2
	Bacilli		3.0
		Erysipelatoclostridiaceae	1.2
	Negativicutes		2.6
		Acidaminococcaceae	2.2
Spirochaetes			7.1
	Spirochaetia		6.7
		Spirochaetaceae	6.7
Verrucomicrobia			6.5
	Kiritimatiellae *		6.0
Fibrobacteres			5.0
	Fibrobacteria		5.0
		Fibrobacteraceae	5.0
Cyanobacteria			1.0
	Vampirivibrionia		1.0

** Further classification down to family level currently unavailable.*

Age

Increasing age led to decreased observed richness ($p < 0.001$) and alpha-diversity ($p = 0.015$), see Figure 2. Age also significantly affected beta-diversity, suggesting that the microbiota composition changes as animals age ($R^2 = 0.031$, $p = 0.002$). Several

amplicon sequence variants (ASVs) were significantly less abundant in older horses, including ASVs assigned to the bacterial families Acidaminococcaceae, Ruminococcaceae, p-251-o5 and an unidentified taxon belonging to the phylum of Bacteroidetes, see Figure 3. Other ASVs were more abundant in older horses. These were assigned to the families Eggerthellaceae, Lactobacillaceae, Selenomonadaceae, Oscillospiraceae, Erysipelatoclostridiaceae, Ruminococcaceae, Anaerovoracaceae, Rikenellaceae, Lachnospiraceae, Prevotellaceae, Spirochaetaceae, Bacteroidales_UCG-001, F082 and UCG-010.

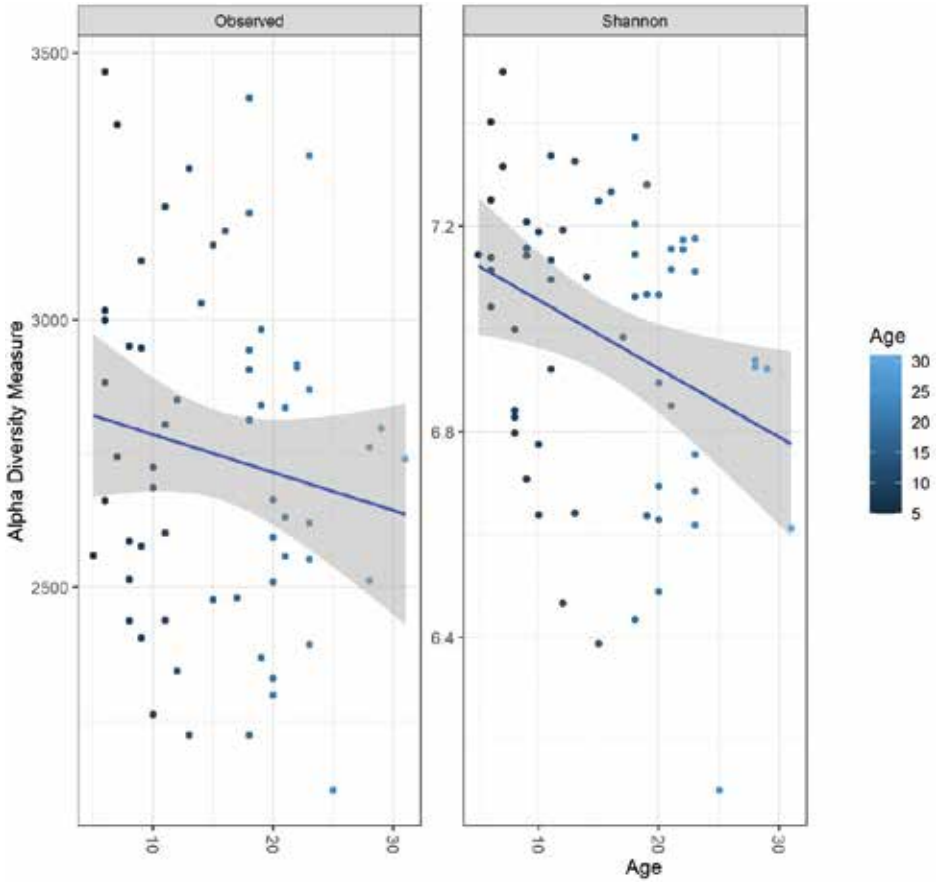

Figure 2. The effect of age on the observed richness and alpha-diversity (Shannon diversity index) on the faecal microbiota of 61 horses and ponies. *A significant decrease, determined with linear modelling, in observed richness and alpha-diversity is visible in older horses.*

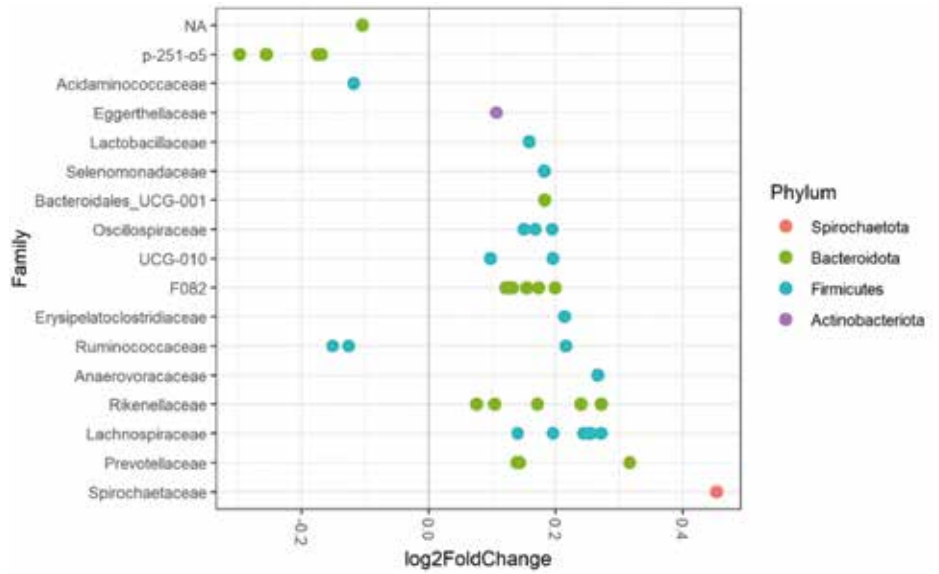

Figure 3. Differentially abundant ASVs grouped by family for increasing age (by year) of the faecal microbiota in 61 horses and ponies. *The log2 fold change (per year) in ASV abundance is shown on the x-axis. ASVs assigned to bacterial families on the left side of the plot are less abundant in horses with increasing age. ASVs assigned to families depicted on the right side of the plot are more abundant in horses with increasing age. NA = ASV belonging to an unknown family (colours indicate the phylum).*

Gender

No significant differences in observed richness, alpha- or beta-diversity were observed for gender in this study.

Horse type

Thirty-two horses (Dutch Warmblood, Rheinlander, Oldenburger and Standardbred horses) and 29 ponies (Haflinger, Tinker, Irish Cobs, Welsh, Appaloosa, New Forest, Fjord, Icelandic, Shetland and mini-Shetland ponies) were included to evaluate for differences in faecal microbiota between horses and ponies. No significant differences in observed richness and alpha-diversity were observed for horses compared to ponies. Beta-diversity was significantly different when horses and ponies were compared. Horse type determined 2.4% of the variation (PERMANOVA p = 0.015 betadisper p = 0.530). ASVs assigned to the following families of bacteria were significantly less abundant in ponies compared to horses: Ruminicoccaceae, Lactobacillaceae, Prevotellaceae, Acidaminococcaceae, Rikenellaceae, p-251-o5 and some unidentified taxa within the Firmicutes phylum. Other ASVs were more abundant in ponies compared to horses. These belonged to the bacterial families of Lachnospiraceae, Acidaminococcaceae, Saccharimonadaceae, Rikenellaceae, Spirochaetaceae and p-251-o5, see Figure 4.

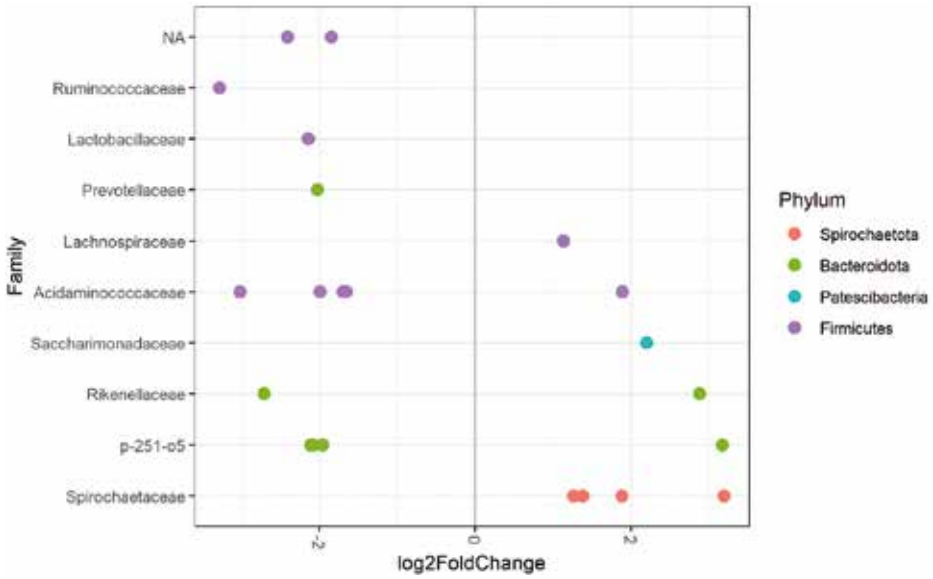

Figure 4. Differentially abundant ASVs grouped by family for horse type (ponies vs. horses) of the faecal microbiota in 61 horses and ponies. *The log2 fold change in species abundance is shown on the x-axis. ASVs assigned to bacterial families on the left side of the plot are less abundant in ponies than horses, ASVs assigned to families depicted on the right side of the plot are more abundant in ponies than horses. NA = ASV belonging to an unknown family (colours indicate the phylum).*

Diet

No significant differences in observed richness, alpha- or beta-diversity were observed for the type of roughage fed. Also, no significant differences in richness, alpha- or beta-diversity were observed for animals fed more than 2 kg of concentrates compared to animals fed less than 2 kg of concentrates.

Pasture access

No significant differences in observed richness and alpha-diversity were observed for pasture access. However, a significant difference in beta-diversity was observed when horses with pasture access were compared to horses that did not have pasture access. Pasture access explained 2.3% of the variation in microbiota composition (PERMANOVA p = 0.035 betadisper p = 0.034). Only a few ASVs were significantly less abundant in animals with pasture access. These belonged to the family of Lachnospiraceae and an unidentified taxon within the phylum of Bacteroidetes. Other ASVs were more abundant in animals with pasture access, belonging to the bacterial families of Prevotellaceae, Rikenellaceae, UCG-010 and two unidentified taxa within the phylum of Bacteriodetes.

Season

No significant differences in observed richness and alpha-diversity were identified for the season of sampling. However, the microbiota composition of samples collected in summer differed from that of samples collected in winter, evidenced by a significant difference in beta-diversity. The season of sampling explained 2.8% of the variation (PERMANOVA p = 0.002, betadisper p = 0.589). Firmicutes were significantly less abundant in samples collected in summer, Bacteroidetes were significantly less abundant in samples collected in winter (Figure 5). On a more detailed level, several ASVs were significantly less abundant in samples collected in the summer than samples collected in the winter. These were assigned to the bacterial families Planococcaceae, Anaerovoraceae, Oscillospiraceae, Rikenellaceae, Lachnospiraceae, Erysipelatoclostridiaceae, F082, Bacteroidales_UCG-001, p-251-o5 and several unidentified taxa within the phyla of Bacteroidetes, Firmicutes and Cyanobacteria. Other ASVs were more abundant in samples collected in the summer than samples collected in the winter, belonging to the bacterial families Ruminococcaceae, Rikenellaceae, Desulfovibrionaceae, Synergistaceae, Lachnospiraceae, Erysipelotrichaceae, Erysipelatoclostridiaceae, Prevotellaceae, Spirochaetaceae and unidentified taxa within the Firmicutes phylum; see Figure 6.

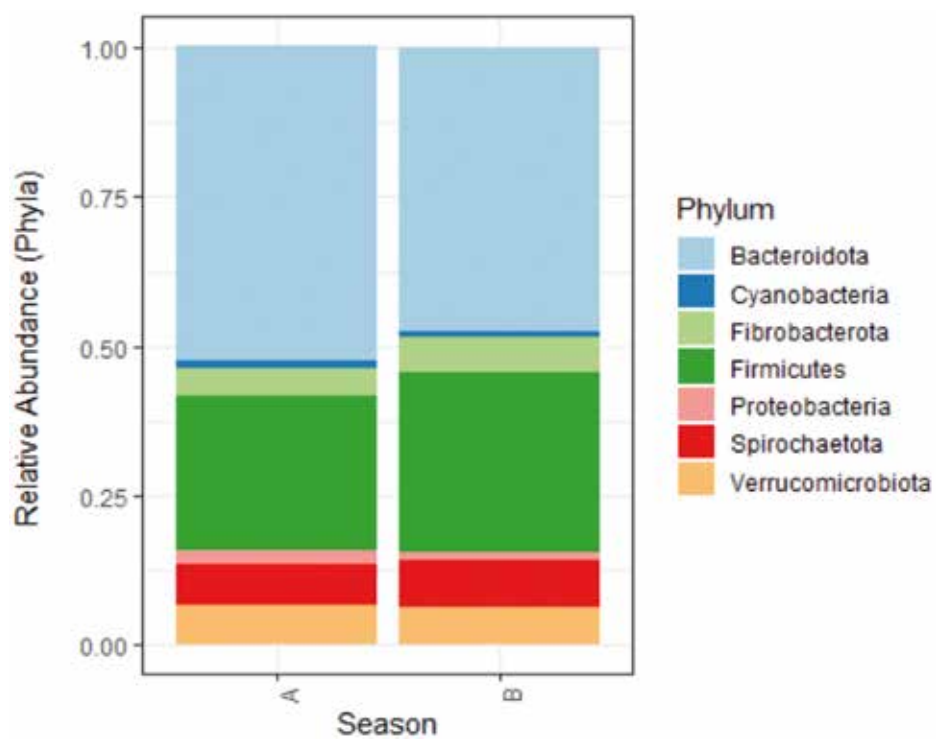

Figure 5. Relative abundance of phyla in the faecal microbiota of 61 horses and ponies in the Netherlands. *A) Summer B) Winter.*

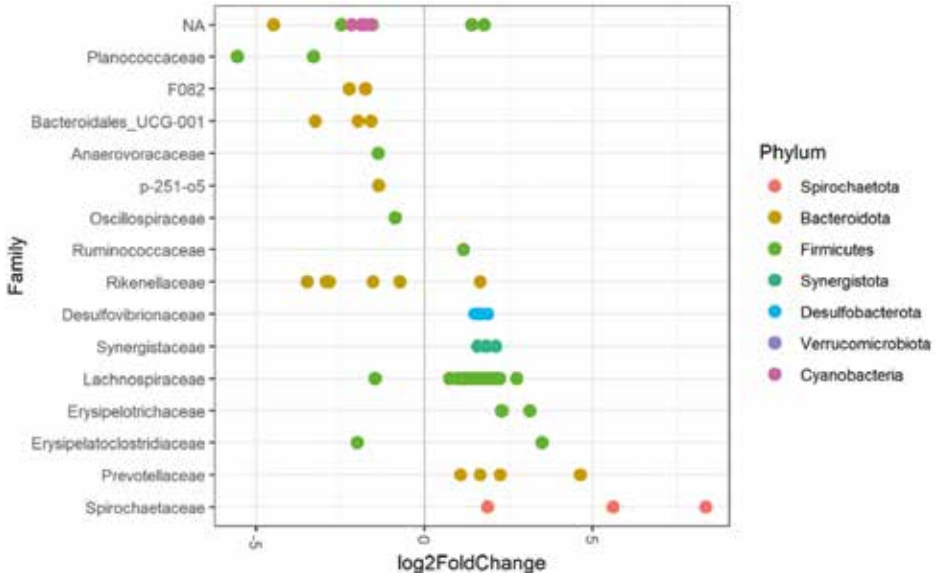

Figure 6. Differentially abundant ASVs grouped by family for season of sampling (winter vs. summer) of the faecal microbiota in 61 horses and ponies. *The log2 fold change in species abundance is shown on the x-axis. ASVs assigned to bacterial families on the left side of the plot are less abundant in samples collected in winter compared to samples collected in summer, ASVs assigned to families depicted on the right side of the plot are more abundant in samples collected in the winter compared to samples collected in the summer. NA = ASV belonging to an unknown family (colours indicate the phylum).*

Location

To evaluate the effect of location (farm) on microbiota composition, data from 17 Warmblood horses aged 4 to 16 years from farm I were compared to data from 18 Warmblood horses of the same age from farm II. No significant differences in observed richness and alpha-diversity were seen for horses from farm I compared to horses from farm II. However, apparent clustering of samples from horses according to farm/location can be observed in the NMDS plot, see Figure 7, explaining 6.4% of the variation (PERMANOVA p = 0.001, betadisper p = 0.779).

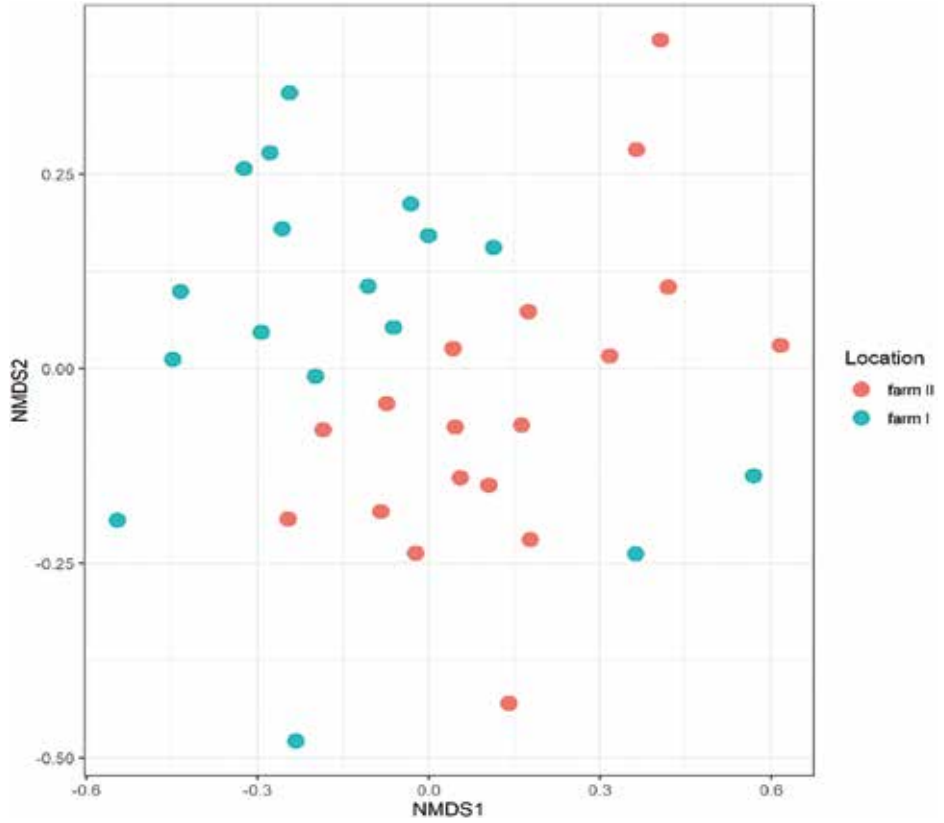

Figure 7. NMDS plot of beta-diversity showing the effect of location (farm) on the faecal microbiota composition of 35 horses. *Red = Farm II, Blue = Farm I. NMDS stress level = 0.17.*

DISCUSSION

The equine faecal microbiota, as well as factors that shape it, have been studied and described in several publications in recent years but the reported results vary significantly [5-17]. These differences might, in part, be a result of methodological differences such as DNA extraction and sequencing techniques used. The large differences in reported results regarding the composition of the intestinal microbiota of healthy horses reduces our ability to draw conclusions from studies evaluating associations between specific equine diseases or physiological factors and the intestinal microbiota. The many uncertainties regarding the intestinal microbiota of healthy horses that still exist today support the publication of new studies evaluating the faecal microbiota of healthy subjects and factors that influence its composition.

Effect of air exposure on the equine faecal microbiota

To validate the use of collecting spontaneously produced faecal samples as a form of convenience sampling in the current study, we studied the effect of air exposure on microbiota composition. Our results show that the observed richness and the alpha-diversity of the microbiota in faecal samples remain stable for up to 6 h, after which they decrease at 12 h of air exposure. The microbiota composition of faecal samples collected up to 6 h after spontaneous defaecation is the same as that of samples collected rectally. Therefore, these samples can be used for faecal microbiota analysis, which validates the faecal sample collection technique applied in the current study. This corresponds to previous reports in which parameters were also reported to be stable up to 6 h after defecation [17,32]. In our study, we found the relative abundance of Bacteroidetes to decrease and Firmicutes to increase over time. At the class level, Bacilli increased significantly in our study, which is in agreement with another study that identified Bacillaceae, Planococcaeae and Enterococcaceae, all families within the class of Bacilli, as bloom taxa [32].

Faecal microbiota composition

In the current study, Bacteroidetes was the most abundant phylum in the faecal microbiota, followed by Firmicutes, Spirochaetes and Fibrobacteres. Our results closely resemble those of some other studies in horses [7,14,17,19]. However, Firmicutes is the main bacterial phylum found in most studies of the faecal microbiota in healthy horses to date [2,3,5,6,8-10,12,13,15,16,21,33-36]. The relative abundance of Bacteroidetes in those studies varies significantly, ranging from 0% to 42% (mean 19%) [3,13]. Of the studies reporting high levels of Bacteroidetes (range 33-52%; mean 43%), similar to our results, almost all used the same DNA extraction kit (QiaAmp Fast DNA Stool Mini Kit) and protocol [7,8,13,14,19,35]. Studies using another commonly used DNA extraction kit and protocol (E.N.Z.A. Stool DNA Kit, Omega Bio-tek, Norcross, GA, USA) consistently report a lower relative abundance of Bacteroidetes ranging from 0% to 25% (mean 7%) [2,3,5,10,15,21]. This indicates that DNA extraction kit and protocol might be a major factor of influence on the results in equine faecal microbiota studies. Several studies have shown significant effects of DNA extraction methods (and sample type) on the results of microbiota analysis [23,37]. However, a study comparing the two DNA extraction kits most commonly used in equine microbiota studies, as mentioned above, is currently lacking. The use of different 16S primers for sequencing might also affect results [38]. Studies evaluating the effect of 16S primer selection on results in equine studies are also currently lacking. The large effect of several critical steps in obtaining microbiota data, as mentioned above, complicates the comparison of taxa abundance and microbiota composition between studies when different protocols are used.

The effect of different factors on faecal microbiota composition

Age

Our results show a reduction in the number of species (observed richness) and alpha-diversity with the increasing age of horses. The current reports in the literature on horses are ambiguous on this topic. Similar results were obtained in a study comparing the faecal microbiota of mature horses (5-12 years) to that of elderly horses (19-28 years) [8]. In other studies, no effect of age on alpha-diversity was observed [11,39] and in specific breeds (Anglo-Arabian horses and wild Prezwalski horses), the opposite trend was observed where alpha-diversity increased with age [10,40]. In adult humans, alpha-diversity decreases with age, similar to the trend observed in our study in horses [41]. In our study, we identified several ASVs whose relative abundance changed with increasing age. Little is known about the role these taxa play in the gut and how this might affect host health. Of interest is the decrease of two taxa within the family of Ruminococcaceae with increasing age in our study, as Ruminococcaceae have been associated with gut health in mammals and lower relative abundance has been associated with colitis, diarrhoea, colic and equine metabolic syndrome in equids [2,4,36,42]. Older horses, therefore, might be more prone to the development of specific diseases. In humans, increasing age, especially in the group of centenarians, leads to a compromised microbiota with increased levels of pathobionts and a heightened inflammatory state, known as inflammageing, and an increase in peripheral blood inflammatory markers can be observed [41]. If the same process occurs in horses is currently unknown. A marked decrease in *Faecalibacterium prauznitzii* (a member of the Ruminococcaceae family) was observed in humans. Our study also observed a decrease of specific ASVs assigned to two families of Ruminococcaceae in horses with increasing age, but we were unable to further specify genus/species.

Gender

In the current study, no effect of gender was found on the faecal microbiota. This is in line with reports from other studies in horses and ponies [5,19]. However, one study did report gender differences in Przewalski horses kept under similar circumstances [43]. Gender differences in faecal microbiota are also observed in humans [44]. Gender differences in microbiota composition in horses might be subtle.

Horse type

Horses and ponies differ in metabolism, and as a result, ponies are more prone to obesity and related disorders such as laminitis and equine metabolic syndrome [45-47]. Differences in the prevalence of several gastrointestinal disorders have also been described for horses and ponies [48]. Therefore, we evaluated differences in microbiota

composition between horses and ponies in our study. We found no differences in alpha-diversity between horses and ponies, which is in line with a recently published study [49]. However, a significant difference in beta-diversity was observed when horses and ponies were compared, demonstrating significant differences in microbiota composition. Ruminococcaceae (fibrolytic bacteria), Lactobacillaceae (carbohydrate fermentation, production of lactic acid) and Prevotellaceae (breakdown of carbohydrates and protein) were less abundant in ponies compared to horses. Lachnospiraceae (plant polysaccharide fermentation and short-chain fatty acid production), Sacchari-monadaceae and Spirochaetaceae were more abundant in ponies. For Acidaminococcaceae, Rikenellaceae and P-251-o5, we observed that specific genera/species within these families increase or decrease according to horse type, suggesting that bacterial shifts occur within these families. In the study by Langner et al. (2020), Ruminococcaceae, Rikenellaceae, F082 and Bacteroidales UCG-001 were found to be more abundant in ponies compared to horses, but only after all subjects included in the study were kept on a 200% net energy requirement diet for two years [49]. The differences, therefore, not only reflect differences between ponies and horses, as they were not present at the start of the study but are heavily influenced by the high energy diet. Breed differences are also described in other studies comparing the microbiota of several European sport horse breeds and Thoroughbred horses and Mongolian horses [10,35]. Most of the identified differences were subtle or were seen only in low abundant phyla and genera. A study comparing the microbiota composition of Quarter, Morgan, Paint and Tennessee Walking horses found no differences [50]. The differences in microbiota composition between horses and ponies observed in the current study indicate that horse type should be considered in experimental studies of the faecal microbiota.

Diet

Our study did not observe a significant effect of diet (roughage and/or concentrates) on microbiota composition. This contrasts with previous studies in which dietary effects on the intestinal microbiota have been demonstrated [51-53]. However, in these studies, larger contrasts in diet between studied groups were tested (e.g., no concentrate feeding vs. high level of concentrate feeding). In our study, horses and ponies living under normal circumstances at a farm were sampled and, therefore, for example, received varying amounts and varying types of concentrates (with varying levels of nutrients) leading to less contrast when groups were compared and limiting our ability to detect differences. Also, the studies mentioned above evaluated the effect of a sudden change in diet instead of assessing the microbiota from equids on a long-term stable diet. Sudden changes in diet might disrupt the microbiota composition and therefore lead to more pronounced differences that are not present while on a steady diet, as was the case for the horses and ponies included in our study.

Pasture access

In contrast to roughage and concentrates, pasture access did significantly affect microbiota composition in the current study. Lachnospiraceae were less abundant in horses with pasture access in our study, whereas Prevotellaceae, Rikenellaceae and UCG-010 were more abundant. In a study performed in New Zealand, specific taxa were identified to be more abundant in horses abruptly transitioned to pasture: an unclassified genus within the order of Clostridiales, an unclassified genus within the order of Lachnospiraceae, CF231 and BF311 [9]. Another recent study reported an increase in Lactobacillaceae in horses that were abruptly changed from a hay-only diet to a grass diet [54]. The horses included in our study did not undergo abrupt dietary changes and had the same level of pasture access over a prolonged period prior to sampling, which might explain the differences observed when the results of our study are compared to that of previous reports. A recent study from Switzerland also detected significant differences in faecal microbiota composition depending on the number of hours horses had pasture access [55]. Based on the current study and the previously published data, pasture access seems to affect microbiota composition in equids.

Season

We observed a seasonal effect on the faecal microbiota in our study, as evidenced by differences in beta-diversity. Genera/species within the same families increased or decreased with the season of sampling, suggesting that shifts occur within bacterial families. We observed this trend for Rikenellaceae, Lachnospiraceae and members of the family of Erysipelatoclostridiaceae. In a longitudinal study by Salem et al., a biphasic change in microbiota composition was observed over a 12-month period, and alpha-diversity showed a significant non-linear trend [14]. In our study, we did not observe any differences in alpha-diversity. In our study and the study by Salem et al., pasture access varied according to season and was higher in the summer months. Therefore, it is difficult to determine which of the observed differences resulted from dietary changes because of pasture access and which of the differences resulted from the change in season. Nevertheless, our results suggest that season should be included as a co-variant in future studies.

Location

We demonstrated an effect of farm/location on microbiota composition in our study. A previously published study showed relatively low variation in microbiota composition between horses housed in the same environment, indicating interaction patterns across horses housed in the same environment [51]. The effects of location on microbiota composition have been reported previously in domesticated and (semi-) feral horses [19,34,39,56,57]. However, these differences might also in part be explained by dietary or

management differences. The location effect should be included in future studies concerning the equine faecal microbiota.

Strenghts and limitations

As demonstrated in the current study and previous studies, many factors contribute to shaping the gut microbiota in individual animals. However, explained variation by individual factors is generally low, in our study up to 6.4%, suggesting that other (unidentified) factors also play a role. This complexity of involved (and potentially interrelated) determinants is a challenge for suitable study designs. The current broad assessment of the faecal microbiota in horses and ponies living under standard housing conditions at equine farms has the advantage that the results represent the actual situation in the field. The difficulty with a non-homogenous study population is that differences between groups of horses might be less pronounced than in experimental studies in a more homogenous study population with only one variable being studied. By including a more significant number of equids than most equine studies, we have tried to overcome this limitation. In the current study, horses from only two farms were included, limiting the extent to which results can be extrapolated to other equine populations. However, given the relatively large number of equids included in the study and the variation in horse characteristics and management conditions it does provide important baseline information on variation in faecal microbiota in healthy horses and ponies under field conditions. Larger, and preferably international, population-based studies are needed to further investigate and understand the complexity of the equine faecal microbiota and the factors that shape it.

CONCLUSIONS

The current study provides essential baseline information on variation in faecal microbiota in a group of horses and ponies managed under normal management circumstances and the factors that influence its composition. Bacteroidetes is the largest phylum found in the faecal microbiota of the horses and ponies included in this study, followed by Firmicutes. Alpha-diversity and richness decreased significantly with increasing age. Location, age, season, horse type and pasture access had a significant effect on the composition of the microbiome, explaining 2.3% to 6.4% of the observed variation. These results indicate that faecal microbiota composition is affected by several horse-related and environment-related factors. Studies investigating potential relationships between environmental factors or disease status and faecal microbiota should take these factors into account when interpreting observed differences to avoid the risk of overinterpretation.

Author contributions

MJPT, ALZ, JAW, and MMSv00 designed the study. MJPT performed the sample collection and the DNA extraction. JWAR performed the 16s rRNA sequencing. ALZ and RECL analysed the sequencing data and performed the statistical analysis and data visualization. All authors contributed to data interpretation. MJPT, ALZ and RECL prepared the manuscript with input and contributions from all authors. All authors have read and agreed to the published version of the manuscript.

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Institutional review board statement

Ethical review and approval were waived for this study, as only spontaneous produced faecal samples were used for this study and therefore no procedures had to be performed on the animals included in this study. Informed consent was obtained from all owners.

Informed Consent Statement

Informed consent was obtained from all horse owners of the subjects involved in the study.

Data availability statement

The datasets supporting this article have been uploaded to the sequence read archive as part of the supplementary electronic material and are available under accession PRJEB44895.

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Conflict of interest

JWAR is currently employed by IDbyDNA Inc. The other authors declare no conflict of interest.

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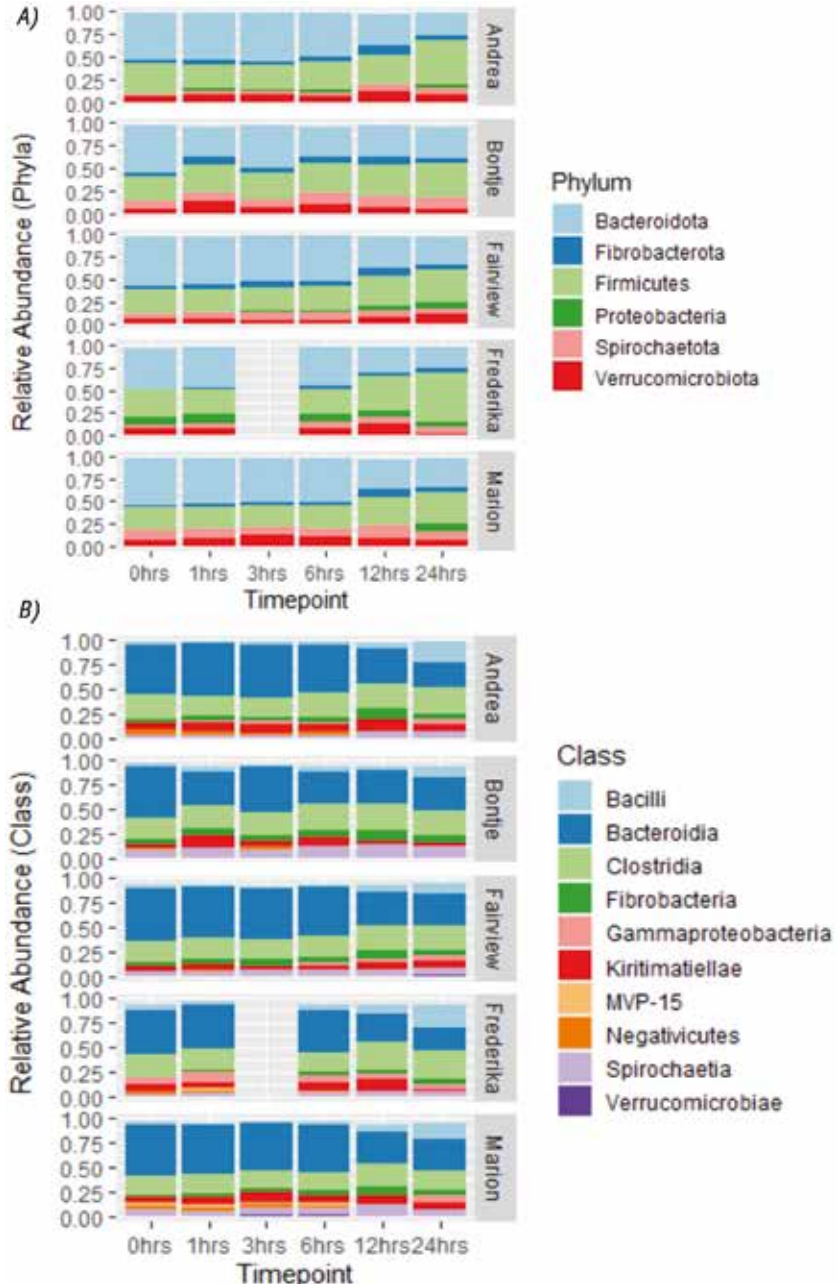
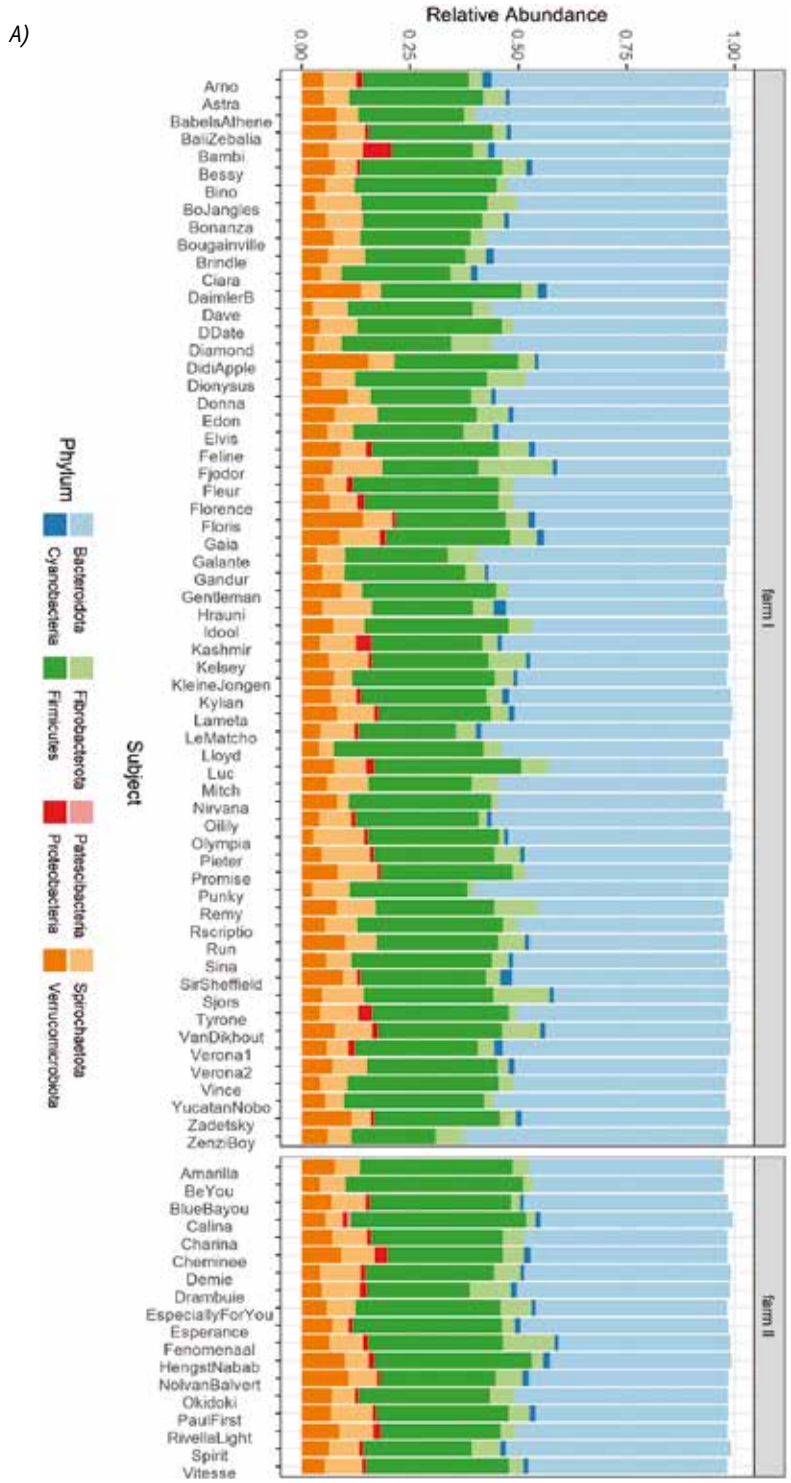
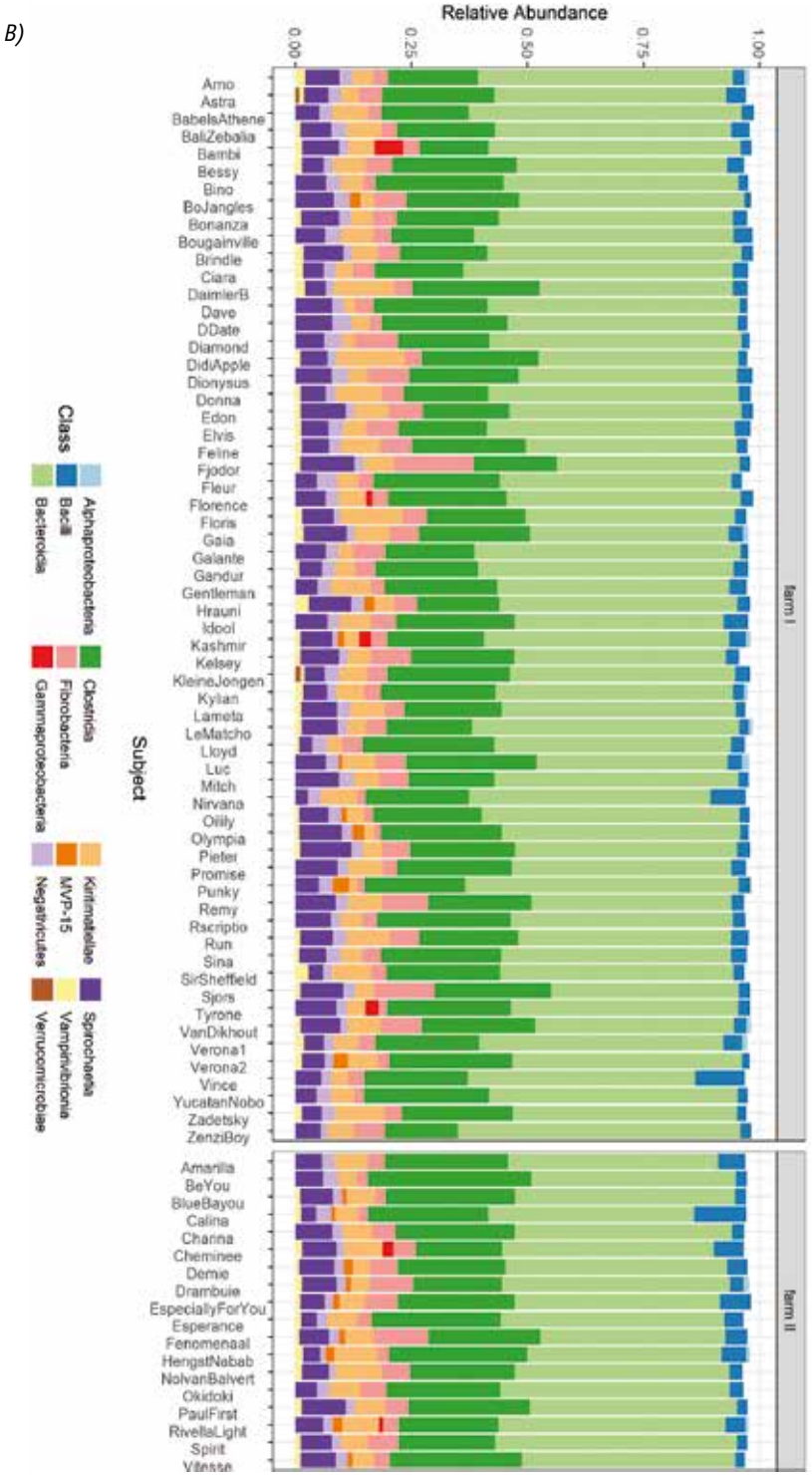
SUPPLEMENTARY FIGURES



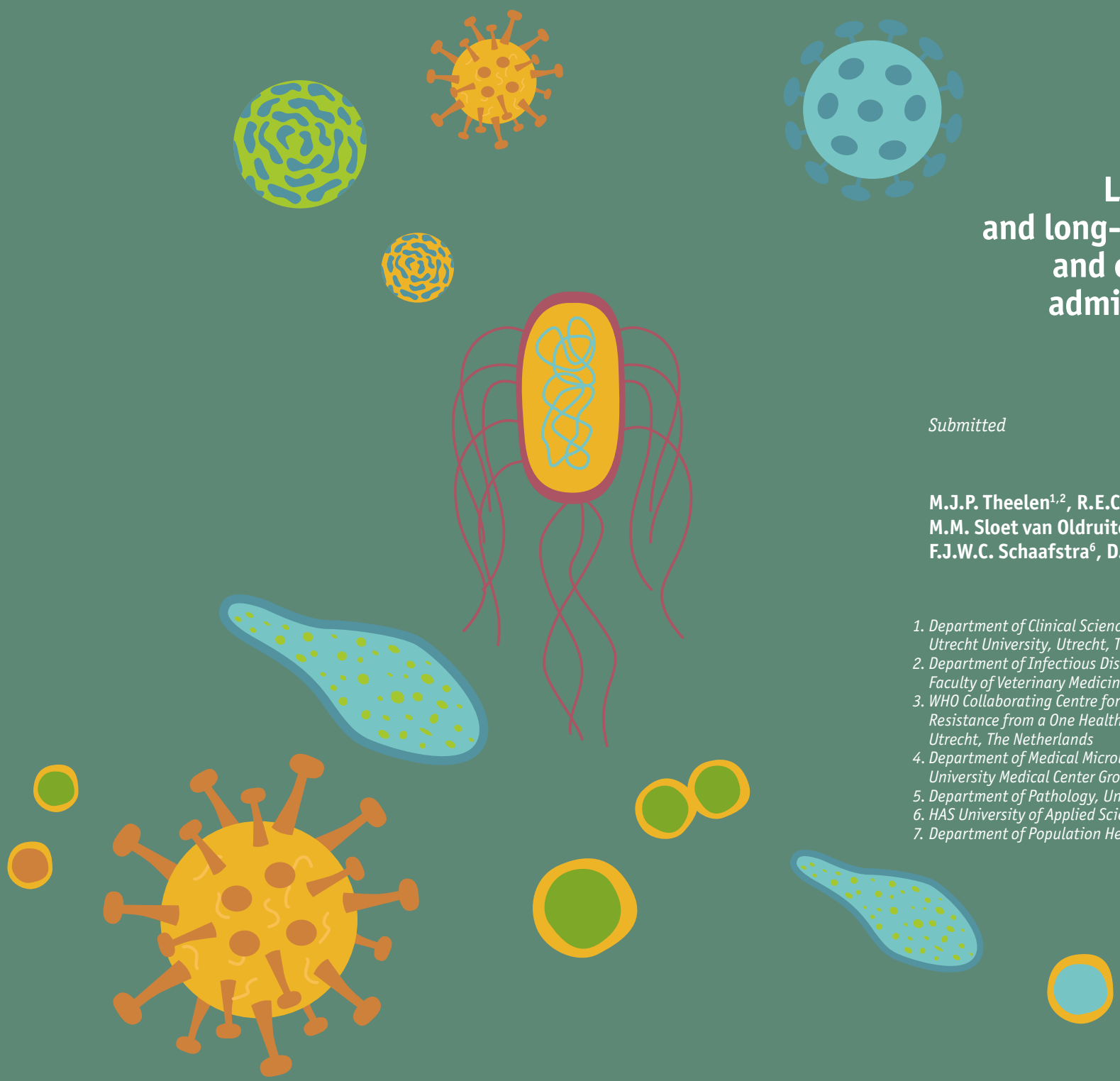
Supplementary Figure S1 *Relative abundance of bacterial communities at (A) phylum- and (B) class level in the microbiota of fecal samples collected from ponies at different time points after exposure to air. The phylum of Bacteroidetes decreased in relative abundance at t = 12 h and t = 24 h compared to t = 0, while Firmicutes increased. At class level Bacilli increased after 12 h of air exposure.*



B)



Supplementary Figure S2. Relative abundance of most abundant phyla and classes in the fecal microbiota of 79 horses and ponies in the Netherlands. *A) 61 equids at farm I and 18 equids at farm II - phylum level B) 61 equids at farm II and 18 equids at farm II – class level*



CHAPTER 7

Longitudinal study of the short- and long-term effects of hospitalisation and oral trimethoprim-sulfadiazine administration on the equine faecal microbiome and resistome

Submitted

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ABSTRACT

Background: Hospitalisation and antimicrobial treatment are common in horses and significantly impact the intestinal microbiota. This may affect host health as horses depend on hindgut fermentation. Antimicrobial treatment might also increase levels of resistant bacteria in faeces, which could spread to other ecological compartments. In this study, we aimed to characterize the short- and long-term effects of transportation, hospitalisation, and trimethoprim-sulfadiazine (TMS) administration on the faecal microbiota and resistome of healthy equids.

Methods: In a longitudinal experimental study design, in which the ponies served as their own control, faecal samples were collected from six healthy Welsh ponies at the farm (D0 – D13-1), immediately following transportation to the hospital (D13-2), during seven days of hospitalisation without treatment (D14 – D21), during five days of oral TMS treatment (D22 – D26) and after discharge from the hospital up to six months later (D27 – D211). 16S rRNA gene sequencing was performed on all samples. For resistome analysis, shotgun metagenomic sequencing was performed on selected samples.

Results: Hospitalisation without antimicrobial treatment did not affect microbiota composition. Oral TMS treatment reduced alpha-diversity significantly. Spirochaetes, Kiritimatiellaeota, Fibrobacteres and Verrucomicrobia decreased in relative abundance, whereas Firmicutes increased. A gradual and partial recovery of the faecal microbiota composition was observed two weeks after discontinuation of TMS treatment and discharge from the hospital. Long-term effects were, however, apparent. The relative abundance of Spirochaetes and Verrucomicrobia remained lower and microbiota composition still differed significantly from that at the start of the study. TMS administration led to a significant (up to 32-fold) and rapid increase in the relative abundance of resistance genes *sul2, tetQ*, *ant6-1a* and *aph(3")-lb*. *lnuC* significantly decreased directly after treatment. Resistance genes *sul2* (15-fold) and *tetQ* (six-fold) remained significantly increased six months later.

Conclusions: Oral treatment with TMS has a rapid and long-lasting effect on faecal microbiota composition and resistome, making the equine hindgut a reservoir and potential source of resistant bacteria posing a risk to animal and human health through transmission. These findings support the judicious use of antimicrobials to minimize long-term faecal presence, excretion and the spread of antimicrobial resistance in the environment.

***Keywords**: Microbiota; Horse; Antimicrobial resistance; Shotgun metagenomic sequencing; Antimicrobial resistance genes; sul2; tetQ; ant6-1a; aph(3")-lb; lnuC*

BACKGROUND

The intestinal microbiota and a well-functioning gastrointestinal tract are essential to equine health [1]. Several host- and environmental factors have been identified to affect the equine faecal microbiota composition [2]. Disturbances of the intestinal microbiota are associated with significant health problems in horses, such as colitis [3]. The administration of antimicrobial drugs profoundly affects the intestinal microbiota composition in horses, especially drugs administered orally, and can lead to dysbiosis and clinical disease [4-6]. The short-term effect of antimicrobials on the composition of the faecal microbiota has been studied and microbiota composition seems to recover to a large extent within 25 days post-treatment, although subtle differences could still be observed at this time [5]. How long these changes in microbiota composition persist and whether these have clinical implications is currently unknown.

Antimicrobial resistance is a growing problem in human and veterinary medicine [7-9]. Exposure to antimicrobials might affect the relative abundance of specific genes within the faecal microbiome, especially antimicrobial resistance genes (ARGs). All the ARGs in a certain environment, of both pathogenic and non-pathogenic bacteria, are called the resistome [10]. The use of antimicrobials provides a selection pressure for resistance genes to emerge and potentially persist in bacterial populations and change the resistome [7, 8]. ARGs are often located on mobile genetic elements such as plasmids, facilitating the spread of resistance among bacteria by horizontal gene transfer [11]. Furthermore, bacteria and resistance genes are not restricted to specific ecological compartments (e.g., animals, humans, soil, etc.) and can spread easily from one compartment to another [7, 9, 11]. Therefore, a One Health approach is needed to address this problem [8, 9]. In the equine hindgut, the resistome can potentially harm the host's health in case of infection but can also lead to the spread of resistance genes in the environment by faecal excretion. This is especially relevant as horses' manure is also used in agriculture [12]. Moreover, horses are kept in close contact with humans, and many of the antimicrobials used to treat infections in horses are also used in human medicine [13]. Therefore, the equine hindgut is a potentially significant reservoir of ARGs, which could be a source of antimicrobial-resistant infections in animals and humans.

Every year large numbers of horses are hospitalised and even more are treated with antimicrobials in equine hospitals as well as in the field. Trimethoprim-sulfadiazine (TMS) is one of the most widely used antimicrobials in horses. Transportation alone and treatment with antimicrobial drugs have shown to affect faecal microbiota composition [5, 14], but the cumulative effect of transportation to the hospital, hospitalisa-

tion and antimicrobial treatment is currently unknown, and existing work has only focused on short-term effects. Up to date, studies in horses reporting information on the faecal resistome are scarce [15, 16], and the knowledge about the effect of hospitalisation and antimicrobial treatment on the faecal resistome is limited. In the current study, we aimed to characterize the effects of hospitalisation and TMS administration on the faecal microbiota and resistome of healthy ponies with a follow-up period of six months to assess long-term effects and determine the potential relevance of the equine hindgut as a reservoir for ARGs.

MATERIALS AND METHODS

Study design

This longitudinal study was performed on six clinically healthy Welsh ponies (aged 10 to 11 years; all geldings) on a single farm and was carried out between February and September 2017. The ponies had no diet changes and no history of antimicrobial treatment for at least seven years. All ponies were housed individually at the farm, received ad libitum hay, and were kept on pasture. The ponies were also housed individually and fed hay during hospitalisation, but had no pasture access. Behaviour and appetite were monitored daily and rectal temperature was collected on sampling days. Faecal samples were collected at the following time points. Initial samples were collected at the farm on day 0 (D0) and again on day 13 (D13-1). Immediately after group transportation by horse truck (total duration 90 minutes) to Utrecht University Equine Hospital, the ponies were resampled (D13-2). The ponies were hospitalised without treatment for seven days and sampled daily in the morning (D14 - D21). At D21 (after collection of the D21 sample), oral treatment with trimethoprim-sulfadiazine (TMS) 5/25 mg/kg BID (Sulfatrim®, AST Farma, Oudewater, the Netherlands) was started for five consecutive days and samples were collected daily (D22 - D26; TMS treatment samples). On D27, the ponies were transported back to the farm as a group by horse truck and kept on individual pastures for the remainder of the study period (six months). The ponies were again sampled immediately before and after transportation (D27-1, D27-2). The first week after discharge from the hospital, follow-up faecal samples were collected from the ponies daily (D28 - D34) and subsequently weekly on D41, D48 and D58, after which the sampling continued monthly on D88, D119, D149, D180 and D211.

Sampling

Faecal samples were collected rectally from individual ponies using a rectal glove. Four aliquots of two gram per sample were stored at -80°C within two hours of collection for subsequent microbiome and resistome analyses.

DNA extraction, 16S rRNA gene sequencing and shotgun metagenomic sequencing

DNA extraction was performed using the QIAamp Fast DNA Stool Mini Kit (Qiagen, Hilden, Germany) following a previously published protocol [17], with the minor modification of replacing the step of 'treatment of samples in the TissueLyser at 30Hz for 3 x 30 seconds with cooling on ice in between treatments' by bead-beating the samples for five minutes on a Vortex-Genie 2 (Scientific Industries, Bohemia, New York, USA). For 16S rRNA gene sequencing, the variable V3 and V4 regions of the 16S rRNA gene were amplified, and libraries were prepared following the 16S rRNA gene Metagenomic Sequencing Library Preparation protocol (Illumina, San Diego, California, USA). Next, each library was normalized, pooled and loaded onto the Illumina MiSeq platform for paired-end sequencing using the 600 cycles MiSeq Reagent Kit V3 (Illumina, San Diego, California, USA), generating 2 × 300 basepair paired-end reads. For shotgun metagenomic sequencing libraries were prepared with the Illumina Nextera XT kit. Multiplexing and sequencing were performed using the Illumina NextSeq platform (150 bp paired-end sequencing) targeted at 42 million reads per sample.

Bioinformatics processing

Data preparation was performed using Jupyter notebook version 5.7.8, running on Python 3.7.3. utilizing R version 3.4.4. To process the 16S rRNA gene sequencing data, raw reads (250 bp) obtained from Illumina 16S rRNA gene sequencing provided input for the denoising pipeline DADA2. DADA2 models and corrects Illumina-sequenced amplicon errors with high precision [18]. First, the forward and reverse reads were sorted, and the quality profile was plotted. Trimming parameters were derived from the quality plots, maintaining a minimum quality score of 20. Forward-reads contained higher quality than reverse reads, common among Illumina data. Truncations were set at 15-290 for forward, and 15-210 for reverse reads. Post filter and trimming the reads were merged. Merged data was used to create a sequence table. Reads were grouped into amplicon sequence variants (ASVs). After removing chimera's, taxonomy was assigned using v. 132 of the Silva database [19]. DNA reads from shotgun metagenomic sequencing were processed using FastDeME with default settings (https://github.com/aldertzomer/FastDeMe). Reads were processed using FastP to remove poor quality reads and sequencing adapters and barcodes [20]. The tool KMA [21] was used to detect hits to known antimicrobial resistance genes with the default Resfinder database [22] to investigate the resistome. Bacterial, archaeal and eukaryotic composition was determined by counting matching shotgun metagenomic reads to the 16S/18S SILVA database v.132 using Kraken2 [23]. Resistance gene abundances were transformed using Additive Log-Ratio (ALR) by dividing by the total 16S rRNA gene counts per sample, times one hundred thousand for better readability and expressed on a log (ln) scale.

The resistome was clustered at the AMR class and the 90% identity cluster level (ARG cluster, CD-HIT-EST) [24, 25]. Resistance classes were obtained from the Resfinder database [22] and hits to each resistance gene were summed per antimicrobial class.

Data analysis

All data analysis and visualization were performed with R version 4.0.2 [26] using Phyloseq [27] vegan [28] and ggplot [29] packages. All samples had at least 10503 reads and no samples were excluded.

Microbiota composition

Relative abundances of bacterial taxa at the phylum level were assessed for each sample, and phylum-, class- and, for the major phyla also family-level, bar plots were produced. Alpha-diversity (observed richness and Shannon diversity) was calculated from rarefied data. Data was rarefied to the sample with the lowest read counts (10503 reads). Between-sample Bray-Curtis dissimilarity was computed on relative abundance data and used for Non-Metric Dimensional Scaling (NMDS). To determine if significant differences in microbiota composition (beta-diversity) were present between samples collected at different time points, permutational multivariate analysis of variance (PERMANOVA), including beta-dispersion analysis, was performed (vegan package function Adonis2 and betadisper). For this analysis samples were clustered in groups: Start of study (D0 - D13-1; n=12), Hospitalisation without treatment (D14 - D21; n=48), TMS treatment (D22 - D26; n=30), Short-term follow-up (D27 - D34; n=48) and Long-term follow-up (D41 - D211; n=48). The samples collected immediately after transportation (D13-2 and D27-2) were excluded from this analysis to avoid interference of transportation effects. The permutation matrix was stratified per individual ('strata = horse'). Data were rarefied for random forest analysis, and the top 200 taxa were used. The analysis was performed with 'start of study' and 'TMS treatment' samples as classes and the microbial density data as classifiers using the 'randomForest' package in Bioconductor [30]. The resulting random forest model was used to predict when microbiota composition was more similar to microbiota composition at the start of the study or during TMS treatment on the samples not used for training. To determine ASVs that differed in abundance between different time points, the DESeq2 package [31] was used. The DESeq2 package determines if a significant fold change is present with a Wald test. P-values were adjusted for the false discovery rate (Benjamini Hochberg approach [32]) with alpha set at 0.01. Raw count data was used as input. ASVs not seen more than three times in at least 20% of the samples were filtered beforehand. Percentages of interesting taxa were calculated to reveal abundances in the entire microbiome. Specific time points were chosen for comparison to assess the effect of the different interventions (transportation D13-1 vs. D13-2; hospitalisation D13-1 vs. D21; TMS treatment D21 vs. D26 and the cumulative effect of all interventions D0-13 vs. D180-211).

Resistome

Shotgun metagenomic sequencing was performed on D13-1, D16, D20, D22, D24, D26, D28, D31, D34, D41, D58, D88 and D211 samples to study the resistome. The relative abundance of ARGs was calculated for each sample. The computed ALR data were visualized with bar plots at the antimicrobial class level. Statistical differences of ARGs between samples were computed (Wilcoxon signed rank), followed by adjustments for multiple testing (Benjamini-Hochberg procedure). The significantly different ARGs were visualized over time with scatterplots.

Metagenomic assembly

The short reads obtained from shotgun metagenomic sequencing were assembled into longer contiguous sequence stretches (contigs), using SPAdes [33], to obtain information on the location of the ARGs in genomes present in the microbiome. Taxonomic classification of the assembled contigs was performed using CAT/BAT [34]. We aimed to identify which species of bacteria were harbouring the ARGs and if the identified ARGs were located on mobile genetic elements, such as plasmids.

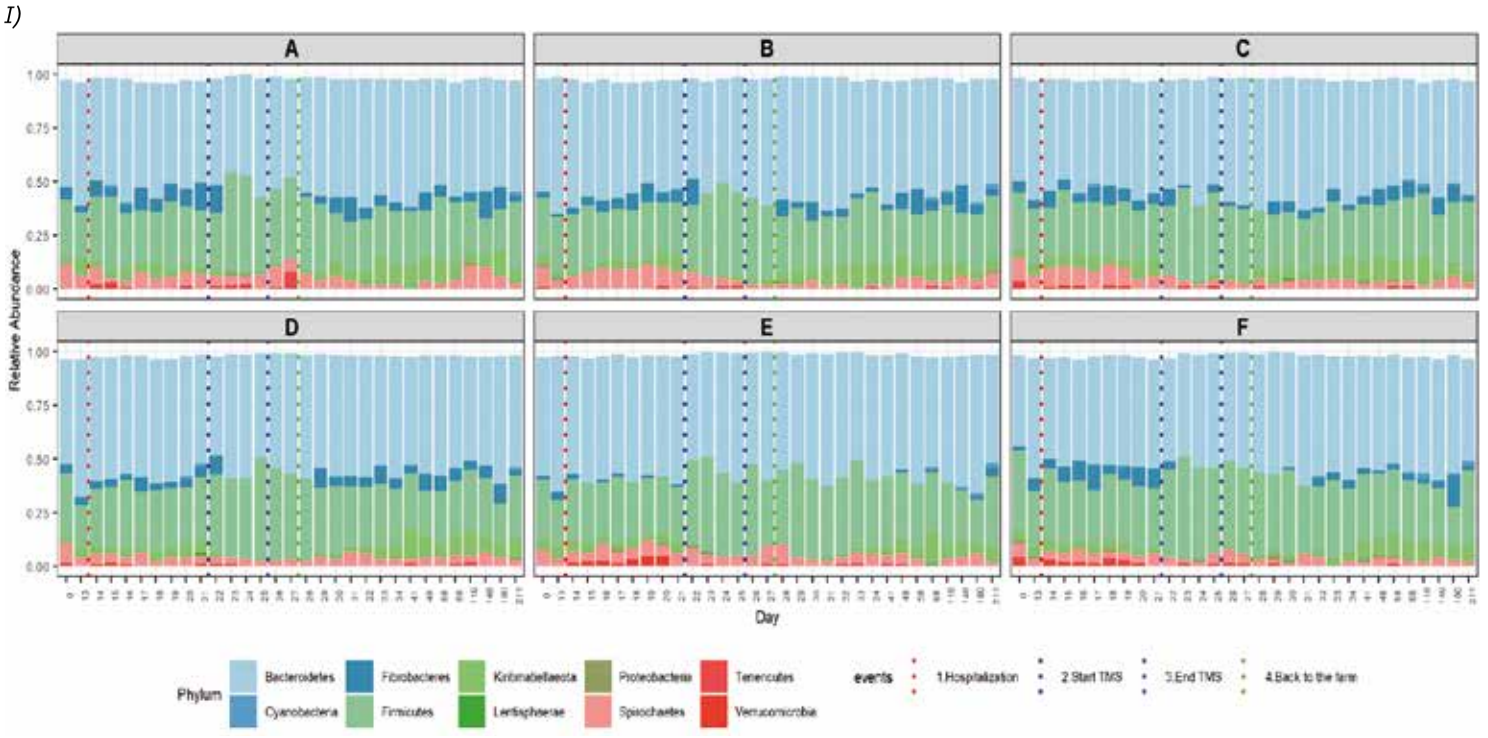
RESULTS

All six ponies remained clinically healthy throughout the study period with no changes in rectal temperature, behaviour and appetite.

Faecal microbiota composition

The composition of the faecal microbiota at phylum- and class level and the relative abundance of the specified phyla over time are presented in Figures 1 and 2. For the two largest phyla, Bacteroidetes and Firmicutes, the relative abundance of families belonging to these phyla over time is presented in Additional file 1. At the start of the study, Bacteroidetes was the phylum with the largest relative abundance (54.7%), followed by Firmicutes (26.6%), Spirochaetes (6.7%), Kiritimatiellaeota (4.4%), Fibrobacteres (3.7%) and Verrucomicrobia (1.0%). All other phyla had a relative abundance of <1%. Mean alpha-diversity (Shannon) was 5.614, ranging from 5.394 to 5.799 (Figure 3).

I)



II)

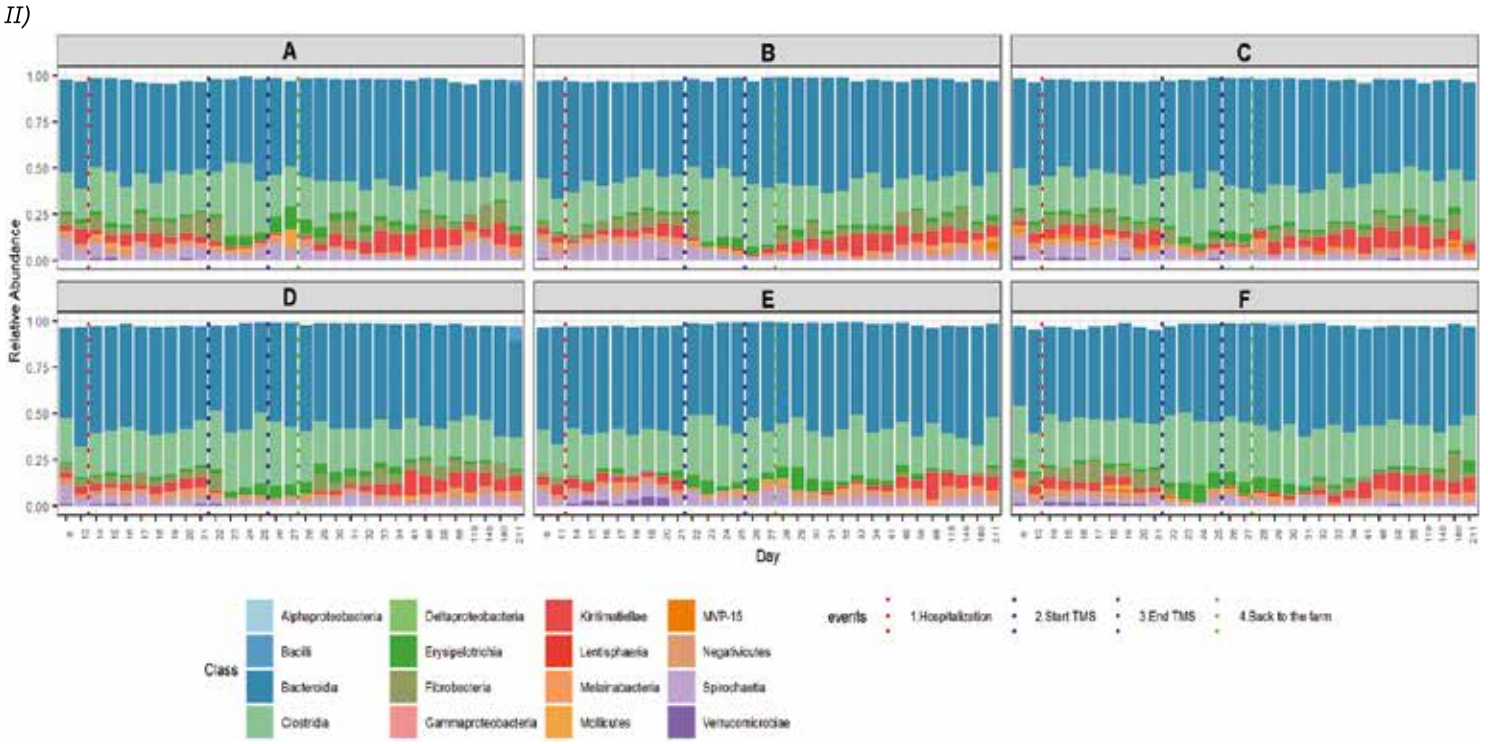


Figure 1. Relative abundance of the most abundant bacterial communities at (I) phylum- and (II) class level *in faecal samples collected from Welsh ponies (A – F) at the farm (D0 - D13-1), during hospitalisation without treatment (D14 - D21), during hospitalisation and treatment with TMS (D22 - D26) and after discharge from the hospital up until six months after hospitalisation and antimicrobial treatment (D27 - D211).*

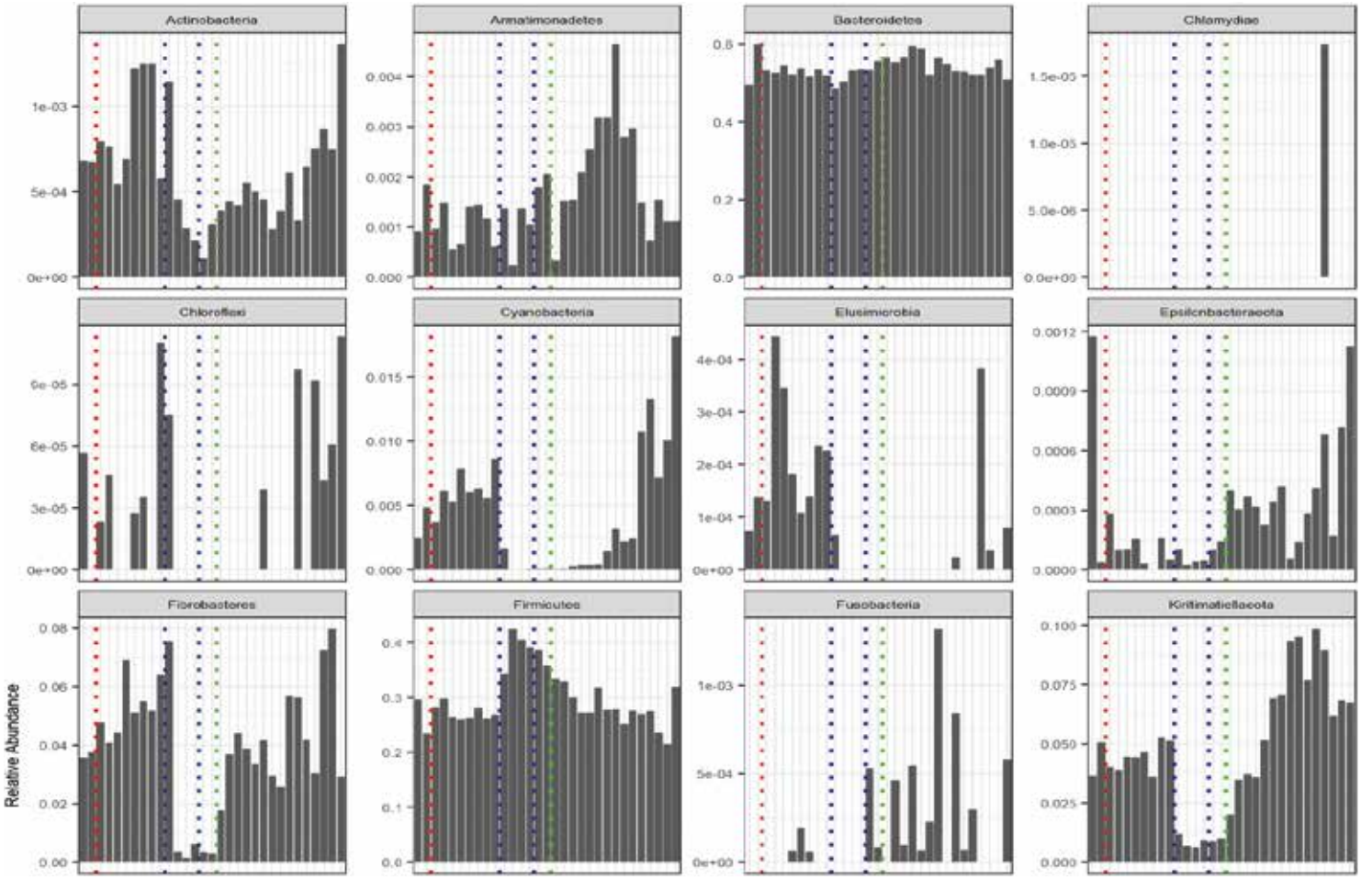


Figure 2. Mean relative abundance of specified phyla over time. *Mean relative abundance of phyla in the faecal microbiota of Welsh ponies at the farm (D0 - D13-1), during hospitalisation without treatment (D14 - D21), during hospitalisation and treatment with TMS (D22 - D26) and after discharge from the hospital up until six months after hospitalisation and antimicrobial treatment (D27 - D211). Scaling on the y-axis varies according to relative abundance.*

Transportation from the farm to the hospital of 90 minutes duration resulted in a sharp increase in alpha-diversity (Shannon) from 5.678 immediately before transport (D13-1) to 6.089 immediately after transport (D13-2; not shown). On the phylum level, an increased relative abundance of Firmicutes (23.5% vs. 28.5%) and a decreased relative abundance of Bacteroidetes were observed (59.8% vs. 54.9%). Differentially abundant ASVs grouped by family before and after transportation to the hospital (DESeq plots) are presented in Additional file 2. The effects of transportation on faecal microbiota composition were no longer present the day after hospital admission (D14).

Hospitalisation without antimicrobial treatment for seven days did not cause any significant changes in the relative abundance of phyla in the faecal microbiota (Figures 1-2) and alpha-diversity (Figure 3). Also, no compositional differences in the faecal microbiota (beta-diversity) were observed (Figure 4). However, some ASVs belonging to different bacterial families did differ significantly between D13-1 and D21 (Additional file 2).

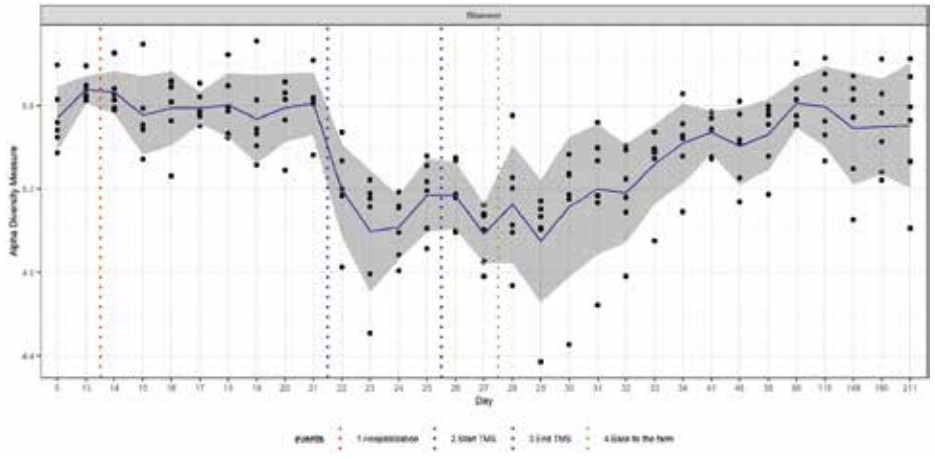


Figure 3. Alpha-diversity (Shannon) over time. *Alpha-diversity of the faecal microbiota of Welsh ponies at the farm (D0 - D13-1), during hospitalisation without treatment (D14 - D21), during hospitalisation and treatment with TMS (D22 - D26) and after discharge from the hospital up until six months after hospitalisation and antimicrobial treatment (D27 - D211). The blue solid line is the mean, and the grey area represents one standard deviation.*

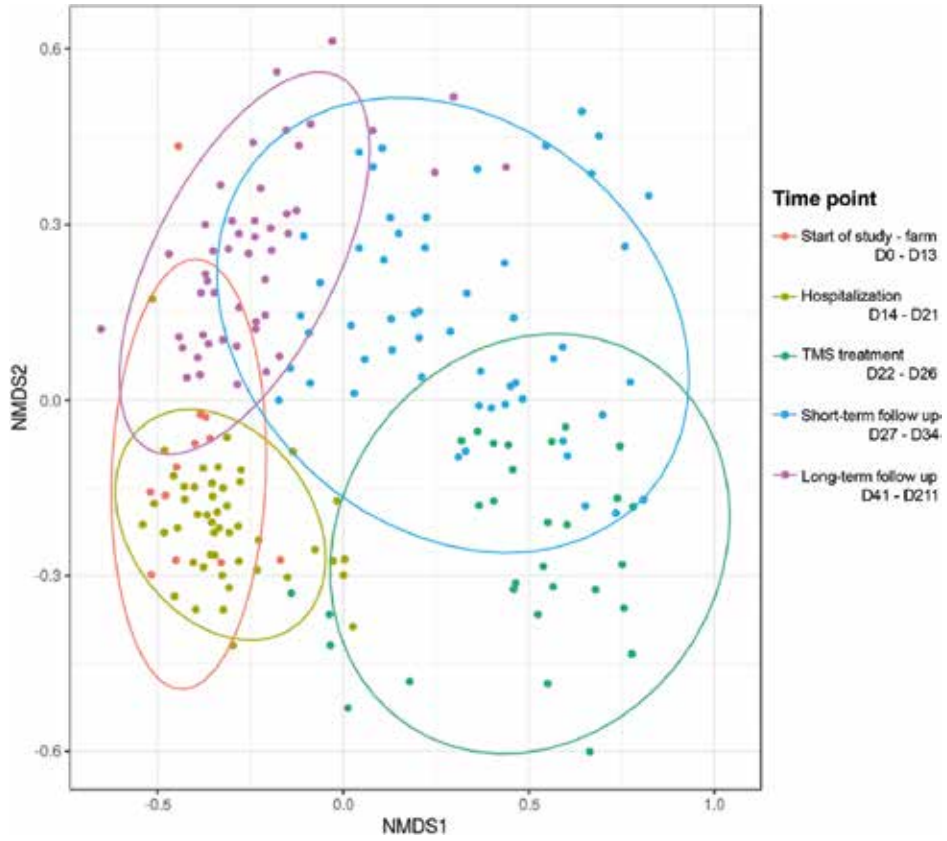


Figure 4. Compositional differences in faecal microbiota over time. *Bray Curtis non-metric multi-dimensional scaling (NMDS) plot of beta-diversity of the faecal microbiota of Welsh ponies at the farm (D0 - D13-1), during hospitalisation without treatment (D14 - D21), during hospitalisation and treatment with TMS (D22 - D26) and after discharge from the hospital (short-term follow-up D27 - D34) up until six months after hospitalisation and antimicrobial treatment (long-term follow-up D41 - D211). Overall, PERMANOVA and pairwise PERMANOVA indicate significant differences between all groups ($p<0.001$), with six out of ten comparisons having significant differences in beta dispersion $p<0.05$).*

Oral treatment with TMS for five consecutive days led to a significant decrease in alpha-diversity (Shannon) from 5.614 on D21 to 5.171 on D26 (Figure 3). Relative abundance of several of the main phyla decreased rapidly after TMS treatment was started (Figures 1-2): Spirochaetes (D21: 6.3% vs. D26: 5.0%), Kiritimatiellaeota (D21: 5.7% vs. D26: 0.9%), Fibrobacteres (D21: 5.7% vs. D26: 0.4%) and Verrucomicrobia (D21: 0.7% vs. D26: 0.1%). Also, many of the smaller phyla decreased rapidly in relative abundance after TMS treatment: Lentisphaerae, Cyanobacteria, Patescibacteria, Actinobacteria, Synergistetes, Elusimicrobia and Planctomycetes. The relative abundance of the large phylum of Firmicutes increased (D21: 26.4% vs. D26: 38.6%) due to TMS treatment. This was also observed for the smaller phyla of Tenericutes, Armatimonadetes, Epsilonbac-

teraeota and Fusobacteria. Within the phylum of Firmicutes, the increase in relative abundance was caused by an increase in the relative abundance of the bacterial families of Ruminococcaceae, Erysipelotrichaceae and Clostridiales_vadinBB60_group (Additional files 1 and 2). Relative abundance of the bacterial family of Veillonellaceae, also belonging to the Firmicutes phylum, decreased after TMS treatment. The relative abundance of the large phylum of Bacteroidetes was unaffected (D21: 52.3% vs. D26: 53.4%); however, changes in the relative abundance of families belonging to this phylum were observed (Additional files 1 and 2). The bacterial families Bacteroidales_UCG-001, Bacteroidales_BS11_gut_group, F082, Rikenellaceae, Bacteroidetes_BD2-2 and COB_p4-1_termite_group all decreased in relative abundance, whereas the bacterial families p-251-o5, Paludibacteraceae and Prevotellaceae increased in relative abundance. No changes in the relative abundance of Proteobacteria were observed. The observed trends were similar in all subjects. PERMANOVA indicates significant differences in beta diversity before and after treatment with TMS ($p<0.001$), demonstrating clear compositional changes in the faecal microbiota (Figure 4).

No transport-related changes in faecal microbiota composition were observed when the ponies were discharged from the hospital and brought back to the farm. This is in contrast to the changes observed after transportation from the farm to the hospital two weeks earlier under identical conditions.

After discharge and discontinuation of antimicrobial treatment, alpha-diversity (Shannon) increased again, approaching levels similar to that of the start of the study two weeks later (D0 - D13-1: 5.614 vs. D41: 5.470) (Figure 3) and faecal microbiota composition was more similar to pre-treatment composition than to composition during TMS treatment at D41 for five out of six ponies, as can be observed in the random forest plots (Figure 5). At D211, six months post-hospitalisation and TMS treatment, the mean alpha-diversity (Shannon) was 5.512, comparable to the mean alpha-diversity at the start of the study (D0 - D13-1: 5.614) (Figure 3). However, the relative abundance of some phyla did not fully recover and remained lower in the six-month follow-up period compared to the start of the study (Figures 1-2). This was the case for Spirochaetes (D0 - D13-1: 6.7% vs. D211: 3.2%), Verrucomicrobia (D0 - D13-1: 1.0% vs. D211: 0.3%) and the smaller phyla of Lentisphaerae and Patescibacteria. The relative abundance of other phyla significantly increased during the follow-up period compared to the start of the study. This was observed for the phyla Kiritimatiellaeota (D0 - 13-1: 4.4% vs. D211: 6.5%), Cyanobacteria (D0 - D13-1: 0.3% vs. D211: 1.8%) and the more minor phyla of Armatimonadetes, Epsilonbacteraeota and Fusobacteria. Looking at beta-diversity, a gradual and partial recovery of the faecal microbiota composition was observed. However, microbiota composition at D211 differed from that at the start

of the study, as indicated in the NMDS plot (Figure 4) and the random forest plots (Figure 5). PERMANOVA between 'start of the study' and 'long term follow-up' indicated significant differences between groups, as well as significant differences in beta dispersion. Differentially abundant ASVs grouped by family at the start of the study vs. six months after hospitalisation and TMS treatment (DESeq plots) are presented in Additional file 2.

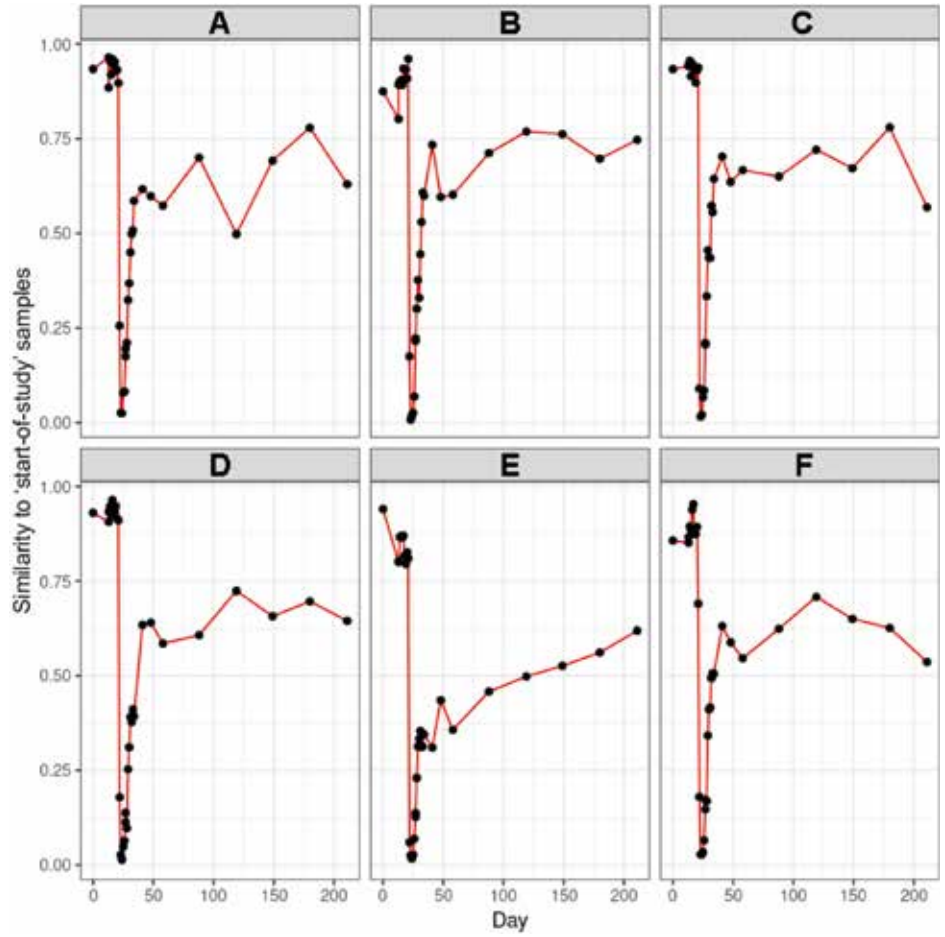


Figure 5. Random forest analysis of microbiota composition over time. *Random forest analysis of faecal microbiota composition of Welsh ponies (A – F) at the farm (D0 - D13-1), during hospitalisation without treatment (D14 - D21), during hospitalisation and treatment with TMS (D22 - D26) and after discharge from the hospital up until six months after hospitalisation and antimicrobial treatment (D27 - D211). 'Start of study' samples (D0-D13-1) and 'TMS treatment' samples (D22-D26) were used as classes for training. 1 = 100% similarity with samples at the start of the study.*

Resistome

The relative abundance of resistance genes in the faecal microbiome in all study subjects is presented in Figure 6. At the start of the study, the relative abundance of resistance genes was low. Resistance genes *lnuC* (lincomycin resistance), *sul2* (sulfonamide resistance), *tet40*, *tetQ* and *tetW* (tetracycline resistance) were detected in faecal samples from all six ponies. Resistance genes *ant(6)-Ia* (aminoglycoside resistance), *mef(A)* (macrolide resistance) and *tet(O/32/0)* (tetracycline resistance) were identified in faecal samples from some, but not all, ponies.

Figure 6. Resistome abundance in the faecal microbiome *Stacked bar chart of the relative abundance of resistance genes clustered at the antimicrobial class level observed in the faecal microbiome of Welsh ponies (A – F) at the farm (D0 - D13-1), during hospitalisation without treatment (D14 - D21), during hospitalisation and treatment with TMS (D22 - D26) and after discharge from the hospital up until six months after hospitalisation and antimicrobial treatment (D27 - D211).*

Hospitalisation without antimicrobial treatment for seven days did not significantly affect the relative abundance of resistance genes in the faeces of the ponies included in this study (Figures 6-7).

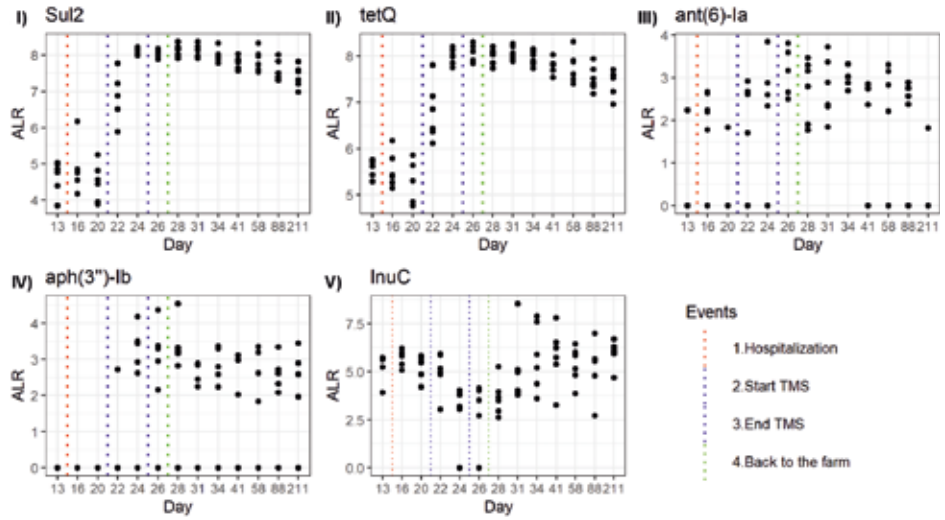


Figure 7. Relative abundance of resistance genes over time. *Relative abundance of resistance genes in the faeces of Welsh ponies at the farm (D0 - D13-1), during hospitalisation without treatment (D14 - D21), during hospitalisation and treatment with TMS (D22 - D26) and after discharge from the hospital up until six months after hospitalisation and antimicrobial treatment (long-term D27 - D211). I) sul2, II) tetQ, III) ant(6)-Ia, IV) w(3")-Ib, V) lnuC*

After oral TMS treatment, a significant increase in the relative abundance of several resistance genes was observed (D20 vs. D26): a 32-fold increase in sulphonamide resistance gene *sul2* and a 16-fold increase in tetracycline resistance gene *tetQ* (Figures 6-7). Aminoglycoside resistance genes *ant6-1a* and *aph(3")-lb* also increased in all subjects after treatment with TMS, but these genes were below the detection limit prior to treatment in most subjects, hindering the calculation of a fold increase. The relative abundance of lincomycin resistance gene *lnuC* significantly decreased six-fold directly after treatment. Differences between individual ponies were present, demonstrated by the relatively large increase in macrolide resistance genes in ponies A and B, whereas this increase was limited in ponies C, D, E and F. Six months after antimicrobial treatment, *sul2* (15-fold) and *tetQ* (six-fold) resistance genes remained significantly increased at D211 compared to D0 (Figures 6-7). Three new types of ARGs were observed in the faeces of some subjects at D211, which were not detected at the start of the study: *aph(3")-lb*, *blaACI* and *cfxA6*.

Metagenomic assembly

By assembling the short reads into longer contigs, we aimed to identify which species of bacteria were harbouring the ARGs and evaluate if the observed ARGs were located on mobile genetic elements, such as plasmids. We observed that resistance genes *sul2* and *tet(Q)* were located on the same contig observed in the genome of an uniden-

tified bacterial species belonging to the class of Bacteroidales (phylum Bacteroidetes). Furthermore, resistance genes *aph(3")-lb* and *aph(6)-ld* (and sometimes also *strA_1*) were located on the same contig observed in the genome of a bacterial species belonging to the genus of *Desulvibrio* (phylum Proteobacteria).

DISCUSSION

Faecal microbiota composition

Faecal microbiota composition at the phylum level was similar to previously reported in healthy equids from the same geographic area [2] and other geographic areas [35-37]. Bacteroidetes was the phylum with the largest relative abundance, followed by Firmicutes. However, in other studies, Firmicutes are reported to be the largest phylum [38-42]. These large differences in microbiota composition between studies might result from technical differences in protocols for DNA extraction, sequencing and data analyses, limiting the comparison of results from different studies.

We noticed a sharp increase in alpha-diversity and richness of the faecal microbiota directly after transport to the hospital compared to samples collected 90 minutes earlier, prior to transportation. We also observed shifts in the relative abundance of major phyla, such as Firmicutes and Bacteroidetes. These changes were of short duration and were no longer observed in samples collected the following day. This contrasts with a previously published study reporting no differences in alpha-diversity and relative abundance of phyla after transportation [42] and another study describing changes in faecal microbiota only to be observed three days later [14]. Transportation can cause significant stress in horses [43]. A study in rats showed that stress significantly increased gastrointestinal transit time [44]. The ponies in the current study were not used to transportation and may have experienced stress. This might have led to increased gastrointestinal transit time and the changes observed in faecal microbiota composition. Interestingly, we did not observe any changes when the ponies were transported back to the farm under identical circumstances. The ponies might have been more accustomed to new situations and less susceptible to stress after their two-week stay at the hospital. We can conclude that transportation can have a significant short-term effect on faecal microbiota composition, and this should be considered when designing future microbiota studies in horses.

In the current study, we did not observe any effect of hospitalisation without treatment on microbiota composition, but this might result from the small number of animals making it difficult to observe subtle changes. To our knowledge, no next-generation

sequencing studies have been performed on horses to evaluate the effect of hospitalisation without antimicrobial treatment on faecal microbiota composition.

Generally, in all animals, irrespective of the type of antimicrobial administered, a decrease in species richness and alpha-diversity is observed [45], as was also the case in our study. The most profound effects of TMS on the intestinal microbiota were observed immediately after treatment, which is in line with previous reports that also demonstrated a rapid, significant decrease in alpha-diversity [5, 35, 46]. Relative abundance of Spirochaetes, Kiritimatiellaeota, Fibrobacteres and Verrucomicrobia decreased significantly, suggesting that TMS has a strong action against bacteria in these phyla. As a result, a relative increase in the abundance of taxa belonging to Firmicutes was observed after TMS treatment. The decrease of Fibrobacteres (degradation of plant-based cellulose [47]) and Verrucomicrobia (mucin degradation [48]) are of interest as they are thought to have a positive effect on equine intestinal health [49, 50]. The observed trends were similar in all study subjects, which corresponds to another study reporting the effect of TMS on the equine faecal microbiota to be predictive [5] but contrast with a recent study reporting high inter-individual variability [35]. In that last study, the authors did not specify horse characteristics such as age, gender and breed, which might affect response to treatment [35]. Also, the horses in that study had not received antimicrobials for only three months prior to the study, so potential previous antimicrobial administration might still have affected microbiota composition [35]. However, by using long-read sequencing, the authors of that study observed that species *Phascolarctobacterium* (phylum Firmicutes) and *Subdivision 5* (former phylum Verrucomicrobia, currently Kiritimatiellaeota [51]) decreased after TMS administration, whereas *Paraprevotella* (phylum Bacteroidetes) increased [35]. Due to the use of short-read sequencing in the current study, we could not detect changes in relative abundance at the species level, limiting direct comparisons between the two studies. However, we also observed a significant decrease in taxa belonging to the phylum Kiritimatiellaeota, anaerobic saccharolytic bacteria found in the mucous layer of the intestine of horses [51]. The same decrease has also been observed in another study assessing the effect of TMS on the faecal microbiota [5]. In that study, they also observed that the relative abundance of Firmicutes increased in response to TMS treatment, similar to what we observed in our study [5]. TMS has a more pronounced effect on the faecal microbiota than other antimicrobials, such as ceftiofur and penicillin [5]. This might result from the administration route (oral vs. parenteral). In a study in swine, oral oxytetracycline resulted in more pronounced changes in the faecal microbial community and a higher relative abundance of ARGs than parenteral injection with the same drug [52]. Future studies comparing oral versus intravenous administration of

TMS in horses could provide more insight into the effect of the route of administration on microbiota and resistome composition.

Both random forest analysis and alpha-diversity analyses show that roughly two weeks after cessation of TMS treatment and discharge from the hospital (D41), microbiota composition was more similar to pre-treatment composition than composition during TMS treatment and thus indicates partial recovery of faecal microbiota composition. However, significant differences in beta-diversity could still be observed, indicating that recovery of microbiota composition is incomplete. In another study assessing the effect of oral TMS on faecal microbiota composition, the authors also observed recovery of microbiota composition to a large extent on day 25 after TMS treatment [5]. In that study, some differences in faecal microbiota composition were still evident 25 days after treatment, but no additional follow up samples were collected to assess long-term effects. In the current study, the relative abundance of several main phyla, such as Spirochaetes, Fibrobacteres and Verrucomicrobia, was still significantly lower two weeks after discharge from the hospital compared to pre-treatment abundance. Kiritimatiellaeota increased at D41 (4.4% vs. 9.3%), indicating rebound 'overgrowth' in the absence of TMS after the microbiota was significantly disturbed. The same trend was observed for some less dominant phyla, such as Cyanobacteria, Armatimonadetes, Epsilonbacteraeota and Fusobacteria, indicating microbiota recovery after antimicrobial treatment is a slow and dynamic process. Six months after discontinuation of TMS treatment and discharge from the hospital (D211), significant differences in faecal microbiota composition could still be observed. The relative abundance of Spirochaetes, Verrucomicrobia and the less abundant phyla of Lentisphaerae and Patescibacteria was still lower than in start-of-study samples. In contrast, the relative abundance of Kiritimatiellaeota, Cyanobacteria and the less abundant phyla of Armatimonadetes, Epsilonbacteraeota and Fusobacteria was higher six months after hospitalisation and TMS treatment. Subtle yet long-lasting (up to 180 days) effects of antimicrobial exposure were also observed in a study on human subjects receiving a cocktail of meropenem, gentamicin and vancomycin [53]. Long-lasting changes in microbiota composition in horses might be relevant as, for example, a decrease in Verrucomicrobia has been associated with diarrhoea in horses [4]. We can conclude that from the moment TMS treatment was stopped and the ponies were discharged from the hospital, a gradual recovery of faecal microbiota composition could be observed. However, microbiota composition never completely returned to pre-treatment composition in the study period.

Resistome

At the start of the study, eight different ARGs could be detected in the faeces of our study subjects. Only the resistance genes *lnuC*, *sul2*, *tet40*, *tetQ* and *tetW* were present in the faeces of all six ponies. Resistance genes *ant(6)-la*, *mefA* and *tet0* were identified in some, but not all, ponies. These findings correspond with a recent study describing ARGs in faeces of healthy foals less than 30 days of age in Australia, in which *tetQ*, *tet0* and *tetW* were most abundant, followed by *aphA3*, *sat4* and *mefA* [15]. Studies of the resistome of pigs and poultry in intensive livestock farming systems [25] and healthy humans [54] seem to indicate a much higher number of different resistance genes. However, comparisons are difficult due to large differences in sample size, geographic location and housing conditions and interaction with the environment and other individuals. Nevertheless, similar to our results in equids, tetracycline resistance genes were most abundant in human and pig faeces [25, 54]. In the current study, the animals were housed extensively and had no contact with other animals (although the pasture was injected with cow manure 12 months prior to the study), limiting their exposure to ARGs from their environment. They remained in their own closed ecological compartment, potentially explaining the low numbers of ARGs present in the faeces at the start of the study. This situation differs significantly from animals kept under intensive livestock farming conditions in which large numbers of animals are kept in close contact. Human individuals also do not live in a closed ecological compartment as they interact with their environment and other humans and animals, most likely leading to higher exposure to ARGs and, therefore, a more extensive resistome. Geographic location also affects the resistome. When the faecal resistome from humans, pigs and poultry from different countries were compared, clear geographic differences in prevalence of ARGs were observed [25, 54]. Subjects from Denmark, a country with a restrictive antimicrobial use policy, showed the lowest ARG levels in both animals and humans. The low prevalence of ARGs in equine faecal samples collected at the start of the current study in the Netherlands could also be a geographically restricted finding, as a result of the strict antimicrobial use policy in the Netherlands. This should be considered when extrapolating this information to other equine populations.

In the current study, we did not observe an effect of hospitalisation without treatment on the faecal resistome. Implementation of an effective infection prevention protocol in the equine hospital in the current study might have prevented or reduced the dissemination of antimicrobial-resistant bacteria and ARGs to hospitalised horses. However, previous studies using culture-based or PCR techniques have shown that antimicrobial-resistant *E. coli* bacteria increase in the faeces of horses, independent of antimicrobial administration, within days of hospitalisation [55-60]. One study compared the duration of faecal shedding of resistant *E. coli* after antimicrobial treatment

between hospitalised horses and non-hospitalised horses [61]. Two weeks following antimicrobial treatment, the odds of detecting resistant isolates in faeces of hospitalised horses were still increased. In contrast, in the non-hospitalised group, detection of resistant isolates returned to pre-treatment levels, suggesting that hospitalisation in that study affected the persistence of ARGs in the faeces after antimicrobial treatment. Culture and PCR techniques, as used in the studies described above, are very sensitive in detecting target isolates and resistance genes; however, no information is collected on other bacteria and ARGs than the targeted ones in the gut microbiota. Shotgun metagenomic sequencing, as used in the current study, does provide information on all bacteria and their full genetic potential (including ARGs) in the microbiota. However, it is less sensitive to detect subtle changes in the prevalence of ARGs due to limitations in sequencing depth. This might, in part, also explain the differences in results. To evaluate the actual effect of hospitalisation on the (duration of) faecal shedding of ARGs, more extensive shotgun metagenomic sequencing studies, including a non-hospitalised control group, are warranted.

Under antimicrobial selection pressure, only ARG-containing bacteria can grow and colonize the gut. The relative abundance of ARGs present at the start of the study before TMS treatment did increase after TMS treatment (enrichment of the intrinsic resistome). We also detected new ARGs in the faeces after antimicrobial treatment that were absent or below the limit of detection at the start of the study, indicating an increase in the diversity of the faecal resistome. A significant increase in the relative abundance of several ARGs could be observed within 24h after treatment with TMS. *Sul2*, *tetQ*, *ant6-la* and *aph(3")-lb* all increased in response to TMS treatment. Unfortunately, we could not observe trimethoprim resistance, as this is generally caused by a point mutation in the *dfr* gene, and the number of *dfr* genes in the databases is minimal. The rapid increase in ARGs shows that selection for (bacteria containing) ARGs occurs even after only one day of treatment. Antimicrobial resistance gene *lnuC* decreased in relative abundance after TMS treatment, indicating that this gene was most likely present in bacteria susceptible to TMS. Up until now, only one study using shotgun metagenomic sequencing has been performed to evaluate the effect of antimicrobial treatment on the excretion of ARGs in faeces of horses [16]. This study demonstrated an increase in the faecal presence of ARGs to several antimicrobial classes (tetracyclines, phenicol, macrolides, glycopeptides, aminoglycosides and bacitracin) in foals from farms with endemic *Rhodococcus equi* infections after oral treatment with a combination of a macrolide and rifampin. Similar to our findings, ARGs encoding for resistance to other classes of antimicrobials than the drugs used for treatment increased significantly. This might be explained by co-selection due to the presence of multiple ARGs on mobile genetic elements, such as plasmids. This is supported by

our finding that sulphonamide and tetracycline resistance genes *sul2* and *tetQ* were located on the same contig as well as aminoglycoside resistance genes *aph(3")-lb* and *aph(6)-ld* (and sometimes also *strA_1*). Many ARGs seem to be located on mobile genetic elements, demonstrating the high risk of horizontal gene transfer between bacteria. This provides a cautionary example of the potential consequences of (injudicious) use of antimicrobials in horses.

The relative abundance of ARGs decreased slowly after TMS treatment was stopped and the ponies were discharged from the hospital. Using culture-dependent techniques, it has been demonstrated before that administration of antimicrobials in horses increases resistant bacteria in the faeces for at least two weeks, but it is unknown how long these effects last [61, 62]. In cattle, subtherapeutic concentrations of antimicrobials led to higher faecal excretion of sulphonamide-, tetracycline- and erythromycin resistance genes, and for *sul1,* excretion levels remained increased for 175 days [63]. In contrast, in a small study involving human subjects, six months post antimicrobial treatment, no significant differences in the relative abundance of resistance genes were observed [53]. In our study in equids, six months after hospitalisation and TMS treatment (D211), two ARGs (*sul2* and *tetQ*) were still detected in significantly higher numbers than pre-treatment, whereas faecal microbiota composition returned to pre-treatment composition to a large extent. Therefore, we can conclude that ARGs are present in a larger proportion of the microbiota, either in more species of bacteria, as a result of horizontal gene transfer between different species or by a shift from susceptible to resistant strains within bacterial species that persist. The former has been demonstrated in a study in mice in which a plasmid conferring multidrug resistance transferred from pathogenic *Salmonella enterica* isolates to commensal *E. coli* isolates in the gut microbiota [64]. The latter was recently observed in the previously mentioned study in foals treated for *Rhodococcus equi* infection, in which the relative abundance of a non-target organism, *Enterococcus spp*., did not change after antimicrobial treatment. However, the proportion of resistant isolates increased significantly [16]. Long read and Hi-C metagenomic assembly would be needed to assess to what level the processes mentioned above contributed to our findings in the equine gut.

The prolonged significant increase in ARGs in equine faeces after antimicrobial treatment demonstrated in this study highlights the potential relevance of the equine hindgut from a One-Health perspective. Spread of ARGs between ecological compartments might be further facilitated by the rich hindgut microbiome of horses, the close contact between horses and humans, the overlap in microbiome components between horses and humans, the agricultural use of equine manure and the fact that many antimicrobials used in horses are also used in human medicine.

Strengths and limitations

While consistent with many other equine microbiome studies, the number of subjects included in the current study was low, which may have limited our ability to detect subtle differences in faecal microbiota composition and relative abundance of ARGs through limitations in statistical power. However, the effect of oral TMS on the faecal microbiota is substantial, and all subjects showed consistent trends in the changes observed, allowing us to draw conclusions based on a limited number of ponies. Also, given the longitudinal study design, and by including sampling before the interventions, the ponies served as their own controls. It is possible that the three successive interventions (transportation, hospitalisation and antimicrobial treatment with TMS) affected one another, limiting our ability to study the effect of each intervention separately. However, in a clinical situation, these interventions also often occur in the same sequence, justifying studying the cumulative 'real-world' effect. Our subjects were limited to one geographic region, dictating caution when extrapolating these results to other equine populations. In the current study, we used healthy ponies on a stable diet without antimicrobial exposure for >7 years. It remains to be determined if the same trends will be observed in (critically) ill patients treated in a hospital setting, especially those treated for gastrointestinal diseases. Due to the limited availability of *dfr* genes in the ARGs database, trimethoprim resistance was not identified and included in our analysis. Lastly, the assembly of metagenomic data in our study was challenging as multiple genomes in varying levels of abundance were present in each sample. Also, many of the bacteria in the equine gut microbiome belong to currently unidentified taxonomic lineages, limiting the ability to identify individual bacterial species that harbour ARGs. A follow-up study using long-read and Hi-C metagenomic assembly would possibly produce more detailed information.

CONCLUSIONS

Successive transportation, hospitalisation and oral TMS treatment led to large and consistent changes in the equine faecal microbiota and a rapid and significant increase in the relative abundance of several ARGs. A gradual, relatively fast, but incomplete recovery of the faecal microbiota composition was observed after cessation of treatment and discharge from the hospital. However, the relative abundance of ARGs decreased very slowly and did not return to pre-treatment levels six months later. The prolonged significant increase in ARGs in equine faeces after antimicrobial treatment demonstrated in this study highlights the potential relevance of the equine hindgut from a One-Health perspective. Therefore, judicious use of antimicrobials in horses is warranted to prevent the spread of resistance genes in the environment

and minimize the potential dissemination of antimicrobial-resistant bacteria from horses to humans.

List of abbreviations

ALR: Additive Log-Ratio
ARGs: Antimicrobial resistance genes
ASVs: Amplicon sequence variants
TMS: trimethoprim-sulfadiazine

Ethics approval and consent to participate

The Animal Welfare Body Utrecht at Utrecht University has reviewed the study protocol and waived the need for official ethics approval for this study as no discomfort was caused to the animals included in this study. However, in line with Utrecht University policy, the work protocol was submitted for detailed review to the Animal Welfare Body Utrecht at Utrecht University, and this was approved on December 5th 2016.

Consent for publication

Not applicable.

Availability of data and materials

The datasets supporting this article are available in the Sequence Read Archive repository as part of the supplementary electronic material and are available under accession PRJEB52712 (https://www.ncbi.nlm.nih.gov/bioproject/PRJEB52712/).

Competing interests

JR is consulting for IDbyDNA Inc. The other authors declare that they have no competing interests.

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Authors' contributions

MT, JW, MS and AZ designed the study. MT and FS performed the sample collection and took care of the animals during the experiment. MT performed the DNA extraction. JR performed the 16s rRNA gene sequencing and the shotgun metagenomic sequencing. AZ and RL analysed the sequencing data and performed statistical analyses and data visualization. All authors contributed to data interpretation. MT, RL and AZ prepared the manuscript with input and contributions from all authors. All authors have read and agreed to the published version of the manuscript.

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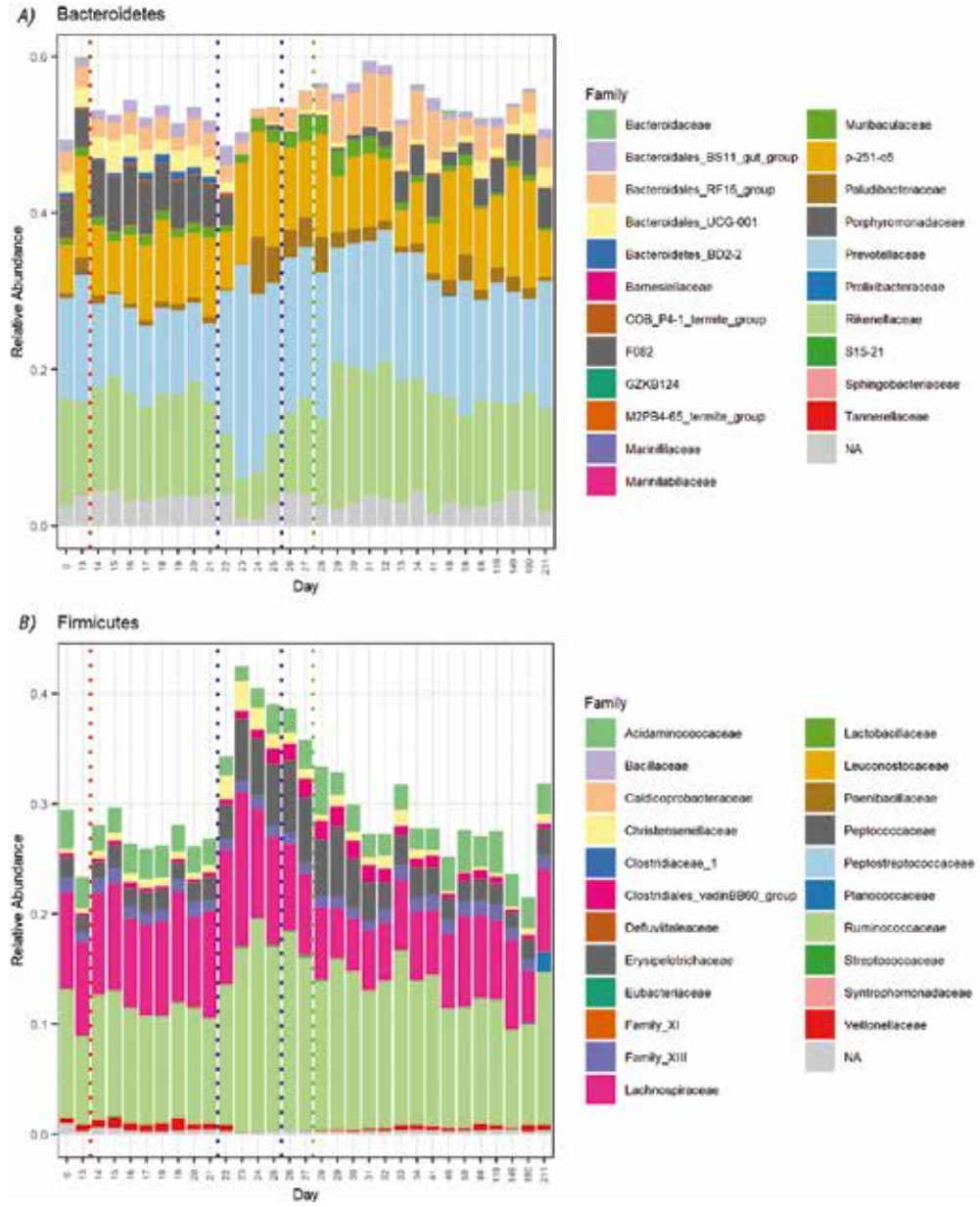
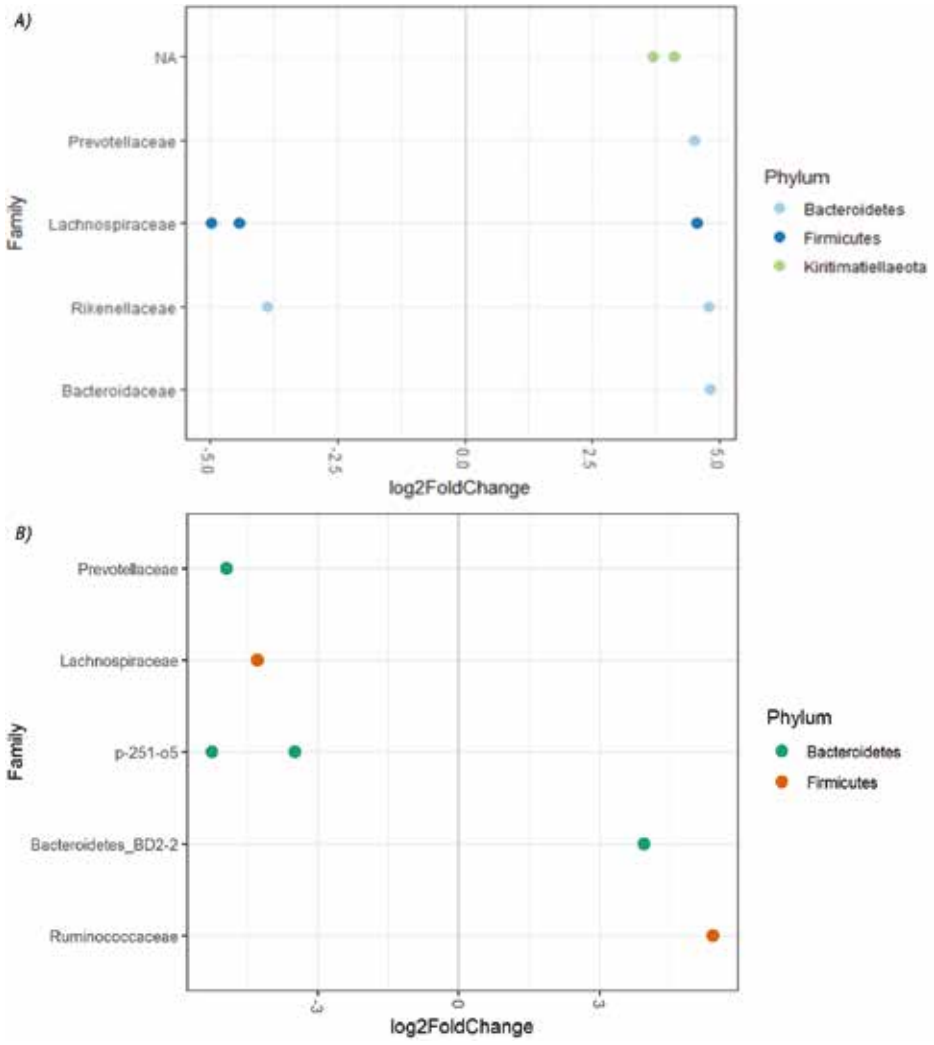
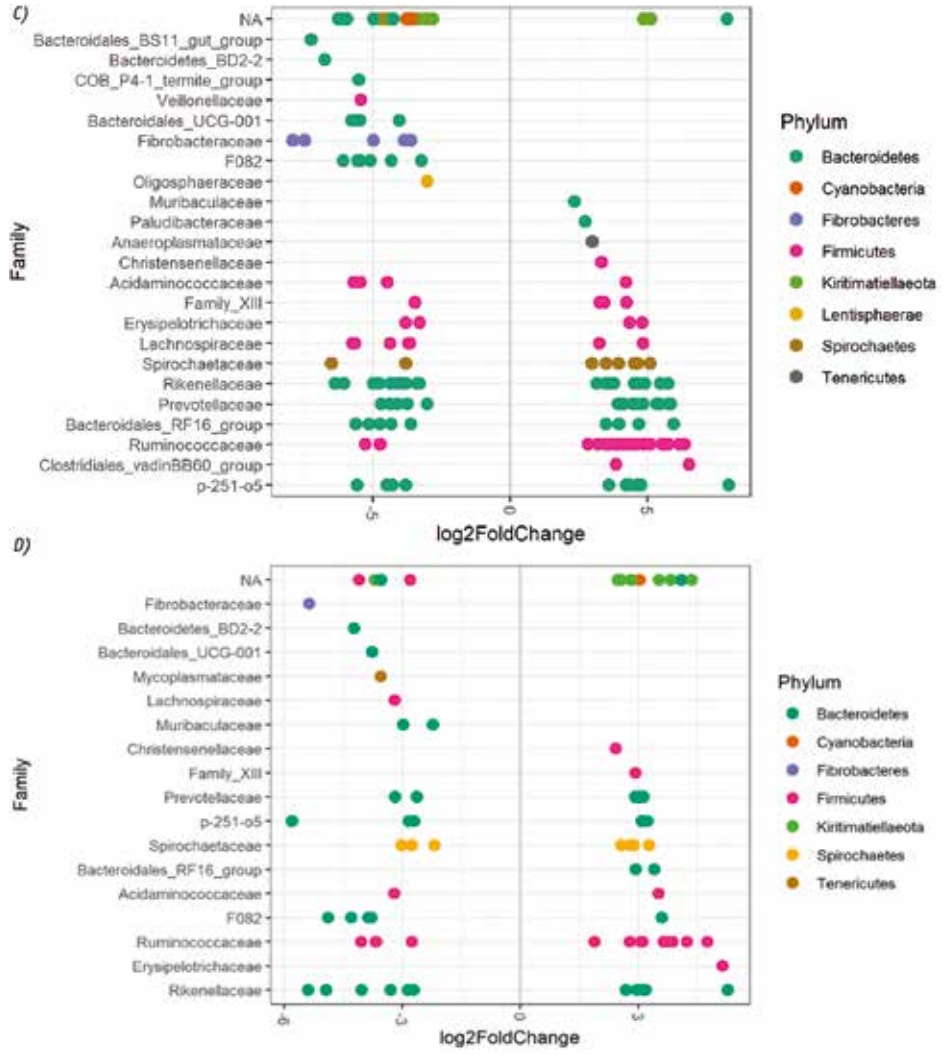
ADDITIONAL FILES

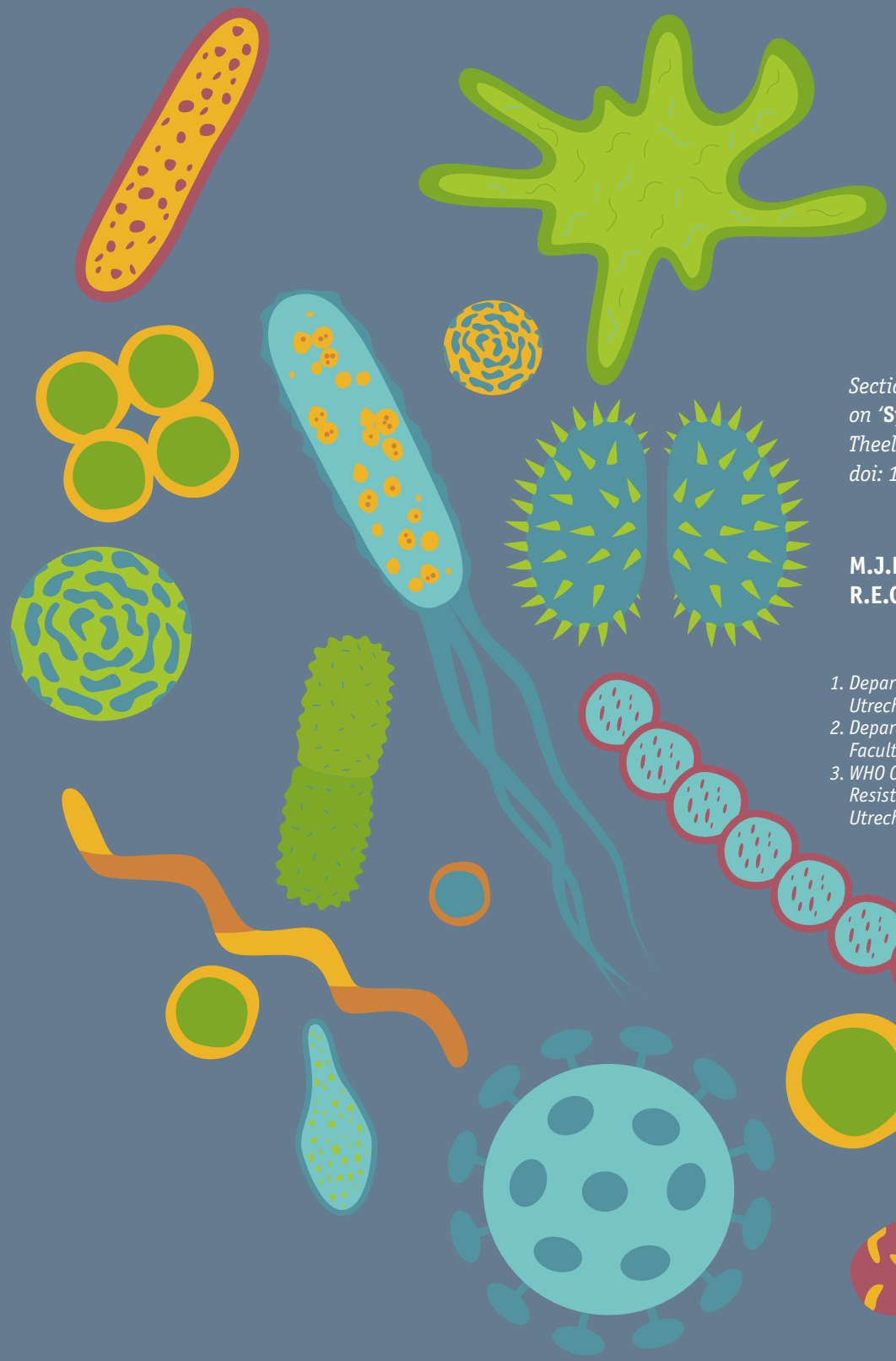


Additional file S1. Relative abundance of bacterial families of the Bacteroidetes (A) and Firmicutes (B) *in faecal samples collected from healthy Welsh ponies at the farm (D0-D13), during hospitalization without treatment (D14-D21), during hospitalization and treatment with trimethoprim-sulfadiazine (TMS) (D22-D26) and after discharge from the hospital up until 6 months after hospitalization and antimicrobial treatment (D27-D211). NA = not applicable, indicating no family level name is yet available.*





Additional file S2. Differentially abundant ASVs at different time points during the study. *Differentially abundant ASVs grouped by family: A) before (D13-1) and after transportation (D13-2) of 1,5h duration to the hospital B) before hospitalization (D13-1) and after one week of hospitalization without antimicrobial treatment (D21), C) before (D21) and after five days of treatment with TMS (D26) and D) at the start (D0 -13) and the end (D180-211) of the study. For this last comparison (D) the variable 'horse' was included as covariate in the model since two samples were included in the start and end category instead of one (as was the case for all other comparisons). The log2 fold change in ASV abundance is shown on the x-axis. ASVs assigned to bacterial families on the left side of the plot are less abundant in samples collected at the later time point compared to earlier time point of sample collection. ASVs assigned to families depicted on the right side of the plot are more abundant in samples collected at the later time point compared to the earlier time point of sample collection. NA = ASV belonging to an unknown family (colours indicate the phylum).*



CHAPTER 8

Antimicrobial stewardship in equine (neonatal) medicine

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INTRODUCTION

Antimicrobial stewardship involves the judicious use of antimicrobials balanced against the requirement to treat the presenting clinical condition [1]. The same principles apply to human medicine as well as veterinary medicine, including equine (neonatal) medicine. A recent study on antimicrobial stewardship in small animal medicine clearly demonstrated the positive effect of implementing an antimicrobial stewardship programme by reducing total antimicrobial use and also demonstrating a shift in type of drugs used from 'restricted' antimicrobials such as cephalosporins and fluoroquinolones to 'first-line' antimicrobials such as penicillins [2]. Furthermore, implementation of antimicrobial stewardship programmes in human medicine has led to a significant healthcare cost reduction and even improved patient outcome [3, 4]. Antimicrobial use in equine practice varies significantly between practices, veterinarians and geographic locations [5]. In some regions, restricted antimicrobials are still commonly used in equine practice, while in other studies a decrease in their use is noted [5-8]. In a recent study regarding antimicrobial use in equine ambulatory practice in the USA, cephalosporins were administered in 13% of the visits in which antimicrobials were prescribed [5]. Developing and incorporating an antimicrobial stewardship strategy are therefore important for all equine practices in order to reduce the use of antimicrobial drugs. Several papers have published guidelines for antimicrobial stewardship in equine medicine [1, 9, 10], however, papers evaluating the effectiveness of implementation of these programmes in equine practice are currently lacking. In this chapter, we will provide practical guidelines for implementation of antimicrobial stewardship in equine practice with an extra focus on equine neonatal medicine as appropriate.

Antimicrobial stewardship requires a multifaceted approach that combines several components, such as reduction of resistance reservoirs, improved clinical diagnosis of (bacterial) infections, improved infection control measures, improved use of preventative health measures, monitoring of culture and susceptibility testing results and monitoring of antimicrobial use, education/creating awareness of antimicrobial resistance and improved communication within the treatment team [1, 11]. A summary of practical action points regarding antimicrobial stewardship that can be applied in equine practice is presented in Table 1.

Table 1. Practical action points for antimicrobial stewardship in equine practice

Reduction of resistance reservoirs	1.	Individual case by case evidence-based decision whether or not antimicrobial treatment is necessary
	2.	Formulation of antimicrobial use protocols based on the latest scientific evidence
	3.	Consider alternative treatment options (e.g. removal of infected tissue)
	4.	Use of local and representative data on culture and susceptibility testing results to select antimicrobials that are most likely effective
	5.	Appropriate dose and route of administration (consider local treatment if possible)
	6.	Use narrow-spectrum antimicrobials instead of broad-spectrum drugs if possible
	7.	Evaluate effectiveness of treatment and modify treatment if indicated
	8.	Appropriate duration of treatment based on clinical improvement
	9.	Appropriate duration of prophylactic treatment (e.g. peri-operative use of antimicrobials) based on recent scientific evidence
	10.	Classification of antimicrobials into the categories 'first-line', 'alternative' and 'restricted'
	11.	Only use 'restricted' antimicrobials after culture and susceptibility testing has proven no alternative options exist
	12.	Antimicrobials should not be used for other potentially beneficial effects aside from treating bacterial infection (e.g. polymyxin B in case of systemic inflammatory response syndrome (SIRS))
	13.	Minimize self-exposure to antimicrobials while handling antimicrobials
Improved clinical diagnosis of (bacterial) infections	14.	Use of appropriate aseptic sample collection techniques to avoid contamination
	15.	Routine submission of samples for culture and susceptibility testing in patients suspected of bacterial infections
	16.	Repeated collection of samples for culture and susceptibility testing in patients that fail to improve (in foals hospitalized in a NICU routine re-culturing at 48h intervals is advisable)
	17.	Make use of the latest diagnostic techniques to detect bacterial disease, if proven to be more sensitive or with shorter turnaround times
Improved infection control measures	18.	Isolation of patients with suspected contagious bacterial infections
	19.	Improved hygiene protocols at farms during disease outbreak
	20.	Isolation of patients with multidrug resistant (MDR) infections and improved hospital hygiene protocols to prevent spread of MDR bacteria

[continued on next page]

Table 1. [continued]

Improved preventative health measures	21.	Vaccination against bacterial disease if possible
	22.	Improved hygiene protocols at hospitals to prevent healthcare-associated infections: including hand hygiene, maintaining a clean environment, ensuring disinfectant availability, adequate ventilation and appropriate waste disposal
	23.	Avoid long work hours and stress for staff to improve protocol compliance
	24.	Improve peripartum management (pre-partum vaccination of mares, clean environment)
	25.	Ensure adequate colostrum intake for neonatal foals (routine IgG measurement in all foals at 24h after birth)
Monitoring of culture and susceptibility testing results and monitoring antimicrobial use	26.	Continuous surveillance of antimicrobial resistance at practice level
	27.	Monitoring and benchmarking antimicrobial drug use within the practice and/or between practices
	28.	Monitoring compliance with practice guidelines
	29.	Discuss trends observed in monitoring within the team to improve awareness and compliance
Education/ creating awareness of antimicrobial resistance	30.	Post-educational training for veterinarians and technicians on antimicrobial resistance
	31.	Active client education on antimicrobial resistance
	32.	Transparent communication about potential negative side effects on antimicrobials to horse owners
Improved communication within the treatment team	33.	Adequate transfer of information regarding referral patients
	34.	Interdisciplinary collaboration involving veterinary specialists from different fields to ensure optimal treatment, providing the best chances for positive outcome, while minimizing the risk for development and spread of resistance.

REDUCTION OF RESISTANCE RESERVOIRS

Several factors contribute to the aim of reducing resistance reservoirs. All are aimed at minimizing antimicrobial drug use and avoiding unnecessary or inappropriate use in order to reduce selection pressure on bacteria, as this is the main driver for development of antimicrobial resistance [12]. Relative abundance of antimicrobial resistance genes remains high in the faeces of horses for at least six months following antimicrobial treatment [13]. Two main reasons for antimicrobial treatment can be identified: first, antimicrobial treatment for suspected or proven bacterial infection and second, for prevention of bacterial infection in patients at risk, such as patients undergoing surgery or neonatal foals born under suboptimal conditions. In case of a suspected bacterial infection, it is important to make an evidence-based decision whether or not a patient should be treated with antimicrobials. Not all patients with bacterial infections need antimicrobial treatment. Recently, a study demonstrated that a policy change limiting treatment of foals with *Rhodococcus equi* pneumonia to only

those with more advanced disease and leave mildly affected foals untreated, reduced antimicrobial usage without increasing mortality [14]. That study provides an excellent example of an opportunity to decrease antimicrobial drug use without negatively affecting patient outcome. Formulating antimicrobial use protocols can be helpful in synchronizing veterinarians within practices/hospitals to work according to the latest evidence-based guidelines regarding antimicrobial use. However, veterinarians should be allowed to deviate from protocols under specific circumstances, based on their professional judgement in individual cases. Removal of the source of infection is another possibility to reduce the need for long-term antimicrobials. For example, abscess drainage or surgical removal of infected tissue can take away the need to treat a patient with antimicrobials. Selection of antimicrobial drugs for treatment should ideally be based on culture and susceptibility testing results. In some cases, for example in foals suspected of sepsis, empirical antimicrobial treatment is indicated while awaiting culture and susceptibility testing results as a delay in treatment is potentially life threatening [15]. In those cases, data regarding potential causative organisms and their antimicrobial susceptibility should be used to select antimicrobial drugs for initial treatment. These data should preferably be local and representative of the same working environment (hospital vs. ambulatory practice). Studies in human medicine have showed that prevalence of bacterial isolates and their susceptibility patterns (especially those involved in healthcare-associated infections) can differ between geographic regions and even between hospitals that are located in the same area [16, 17]. Furthermore, culture and susceptibility results also differ between samples collected in equine hospitals and those collected in equine ambulatory practice [18]. Dose and route of administration should be based on pharmacokinetic studies, with consideration of drug penetration into the infection site and likely efficacy in the local environment. Local delivery methods (e.g. intra-articular administration in a septic joint) can be used to allow for high antimicrobial concentrations at the site of infection, which is especially useful in cases where concentration-dependent antimicrobials are used as bacterial killing is directly proportional to the ratio of peak drug concentration to the MIC of the infecting bacteria. Effectiveness of antimicrobial treatment should be evaluated and treatment should be modified based on culture and susceptibility results. If possible, narrow-spectrum drugs should be used instead of broad-spectrum antimicrobials. Once antimicrobial treatment has been started, it is important to determine appropriate duration of treatment. Disease resolution can be monitored by repeated clinical examination, diagnostic imaging and/or follow up blood work, such as haematology and clinical chemistry. In patients at risk of developing an infection, for example peri-operatively, appropriate treatment duration is also important. A recent pilot study has demonstrated that short duration antimicrobial treatment (pre-operatively + 1 day post-operatively) in colic horses undergoing

abdominal surgery is non-inferior to longer duration antimicrobial treatment (pre-operatively + 5 days post-operatively) in regards to risk of surgical site infections, colitis and also had no significant effect on inflammatory markers such as white blood count, serum amyloid A and fibrinogen [19]. In general, antimicrobials must only be used with veterinary oversight. Owners should not have access to antimicrobial drugs without consulting a veterinarian. Antimicrobials should be classified as 'first-line', 'alternative' and 'restricted' options to assist veterinarians in selecting antimicrobials with the least risk for driving development of antimicrobial resistance. Documents ranking antimicrobials based on risk management of antimicrobial resistance, such as the World Health Organization 'List of Critically Important Antimicrobials for Human Medicine' [20], should be used as guidance for selection of antimicrobial drugs for treatment in veterinary medicine. Antimicrobials that are of critical importance to human health, such as 3rd, 4th and 5th generation cephalosporins and fluoroquinolones [20] should be classified as 'restricted' and their use should always be justified by bacteriological culture and susceptibility testing results demonstrating no alternative treatment options. Other classes of antimicrobials, such as carbapenems, should not be used in veterinary medicine at all given their importance to human health and the risk on development of resistance. Furthermore, antimicrobials should only be used for treating bacterial infections and not for other potentially beneficial effects, such as for example the use of polymyxin B at a low anti-endotoxic dose for treatment of systemic inflammatory response syndrome as this might drive development of antimicrobial resistance [21]. Lastly, equine veterinarians should be aware of the likely consequences of self-exposure to subtherapeutic doses of antimicrobials. Therefore, adequate handling of these drugs is important to minimize self-exposure.

IMPROVED CLINICAL DIAGNOSIS OF BACTERIAL INFECTIONS

Appropriate aseptic sample collection technique is important to avoid sample contamination and overtreatment [21]. In cases that fail to improve, collection of follow-up samples for bacteriological culture and susceptibility testing can be useful. Bacterial pathogens and antimicrobial susceptibility profiles cultured from foals with sepsis differ significantly after >48 hours of hospitalisation from those cultured at hospital admission [22]. This demonstrates that previous test results obtained from samples collected at case presentation should not be used for selection of alternative drugs for treatment if initial treatment is unsuccessful. In cases that fail to improve, repeated bacteriological culture and susceptibility testing are necessary to make an informed decision on treatment. In foals hospitalized in a NICU setting it is therefore advised to collect repeated samples for bacterial culture and susceptibility testing at 48 hour

intervals. Furthermore, new laboratory techniques, such as molecular diagnostics, matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry and microbial cell free DNA, may reduce turnaround times and improve sensitivity of pathogen and antimicrobial resistance detection, thereby contributing to reducing unnecessary or ineffective antimicrobial treatment [23].

IMPROVED INFECTION CONTROL MEASURES

Isolation of equine patients suffering from contagious bacterial infections, such as *Salmonella* or *Clostridium* infections, is key in order to minimize exposure of healthy animals. Hygiene protocols, both at farms and in the hospital, should be optimised to prevent spread of infections, thereby decreasing the number of horses that need antimicrobial treatment. Isolation of patients infected by multi-drug resistant bacteria is of utmost importance to reduce the risk of spread of resistance. This is especially important when infections with the so-called ESKAPE pathogens are detected, as they are often multidrug resistant and are associated with healthcare-associated infections [24]. ESKAPE pathogens include *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumanni, Pseudomonas aeruginosa* and *Enterobacter* species. Healthcare-associated infections also occur in the equine neonatal intensive care setting [22], underlining the need for effective and strict infection control protocols at the NICU in equine hospitals.

IMPROVED USE OF PREVENTATIVE HEALTH MEASURES

Prevention of disease can have a significant effect on reducing the need for antimicrobial drug use. Vaccination against bacterial infections can contribute to prevention of disease. This also includes, for example, tetanus vaccination in pregnant mares six weeks prior to parturition to assure adequate immunoglobin levels in the colostrum to prevent disease in foals. Furthermore, effective hygiene protocols at farms and in hospitals can prevent disease and decrease the need to use antimicrobials to treat infections. Hygiene protocols should include hand hygiene, a clean environment, clean medical equipment, routine disinfection of the hospital environment (including the stables), continuous and readily available disinfectants for cleaning and hand hygiene, adequate ventilation, and appropriate waste disposal [25, 26]. Interestingly, long working hours, high work stress and poor collaboration among staff were associated with healthcare-associated infections in human medicine, potentially as a result of less compliance with hygiene protocols [27]. High workload was also cited as barrier for compliance with infection protocols in a study in equine hospitals [26], and this should be taken into account when aiming to improve hospital hygiene. As foals are very

susceptible to sepsis, it is especially important to take preventative measures, such as excellent peripartum management and assuring adequate colostrum intake to provide the needed antibodies to prevent subsequent infections [28, 29].

MONITORING OF CULTURE AND SUSCEPTIBILITY TESTING RESULTS AND MONITORING ANTIMICROBIAL USE

Surveillance of development of resistance and also monitoring antimicrobial drug use are key concepts in any antimicrobial stewardship strategy. Prevalence of antimicrobial resistance varies between different geographic regions. Utilising studies obtained from different countries with different antimicrobial usages and over different time periods to make decisions regarding antimicrobial treatment in individual patients may therefore result in inappropriate treatment and potentially poor outcome. This highlights the importance of incorporating continuous surveillance of antimicrobial resistance in each practice or hospital. Monitoring development of antimicrobial resistance starts with implementing the policy of collecting samples for bacteriological culture and susceptibility testing in each case and not only in refractory cases. Bacteriological culture and susceptibility testing in individual cases not only benefit the individual patient, but also provide the basis for empirical drug selection in future cases. A designated person within the practice or hospital should be responsible for monitoring antimicrobial resistance. Trends should be discussed on a regular basis within the entire team to increase awareness and engagement. Systematic collection of data on antimicrobial use within the practice/hospital can also contribute to increased awareness. Procurement or prescription data can be retrieved from patient management systems and this allows for benchmarking between practices or even individual veterinarians [7]. Benchmarking can have a positive effect in reducing the total amount of antimicrobials used [2]. Monitoring compliance of individual veterinarians with practice guidelines might result in better implementation of these guidelines. Furthermore, monitoring antimicrobial resistance and antimicrobial drug use can also be used as tools to evaluate the effectiveness of the implementation of antimicrobial stewardship programmes.

EDUCATION/CREATING AWARENESS OF ANTIMICROBIAL RESISTANCE

Post-educational training for veterinarians and technicians on antimicrobial resistance and practice-wide discussions on guidelines should be organised in order to improve awareness and ensure alignment. Horse owner education on antimicrobial

resistance can assist in reducing client pressure on veterinarians to prescribe antimicrobials. This can be achieved through active information campaigns aimed at horse owners. Veterinarians should also encourage horse owners to adopt good hygiene practices. Furthermore, transparent and more elaborate communication about potential negative side effects of antimicrobial treatment on the individual animal might also create client awareness that antimicrobials are not harmless. This could then also result in reduced pressure by owners on veterinarians to prescribe antimicrobials.

IMPROVED COMMUNICATION WITHIN THE TREATMENT TEAM

Adequate transfer of information regarding referral patients is essential in order to prevent unnecessary antimicrobial treatment or use of multiple different types of antimicrobial drugs within a single patient. Communication is also essential when designing the optimal antimicrobial treatment regime for an individual patient [11, 21]. The increasing level of specialization in veterinary medicine has expanded the level of knowledge significantly. Especially in equine neonatal medicine, several veterinary specialists work together to provide the best level of care. When determining the optimal treatment strategy for a critically ill foal, the equine internist, veterinary microbiologist, veterinary pharmacologist and veterinary hospital hygiene specialist should work together in an interdisciplinary and collaborative manner. Bringing together the extensive in-depth knowledge from their diverse backgrounds will make sure the foal receives the best possible treatment, with the greatest chance on a positive outcome, while taking into account minimizing the risk for development and spread of antimicrobial resistant bacteria.

CONCLUSIONS

It is important to realize that a multidisciplinary approach is needed in order to fight the One Health problem of antimicrobial resistance. However, it all starts with making changes, and small changes can potentially have a big effect. In order for the veterinary profession to take responsibility and contribute to the worldwide battle against antimicrobial resistance, all veterinary practices should develop and implement a practice-wide antimicrobial stewardship strategy. (Inter)national professional bodies for veterinarians should provide support to practices in order to enable implementation of antimicrobial stewardship programmes. However, strategies for antimicrobial stewardship are not one-size-fits-all and therefore may vary between practices/hospitals. Each equine practice can start with implementing some of the practical action points listed in Table 1 to create an antimicrobial stewardship programme tailored to the

individual centre. Rather than an all-or-none approach, implementation of specific action points will help focus efforts to improve compliance and gradually bring about the needed institutional cultural change. Monitoring antimicrobial resistance and antimicrobial drug use within the practice can be used as tools to evaluate the effectiveness of the implementation of the antimicrobial stewardship programme.

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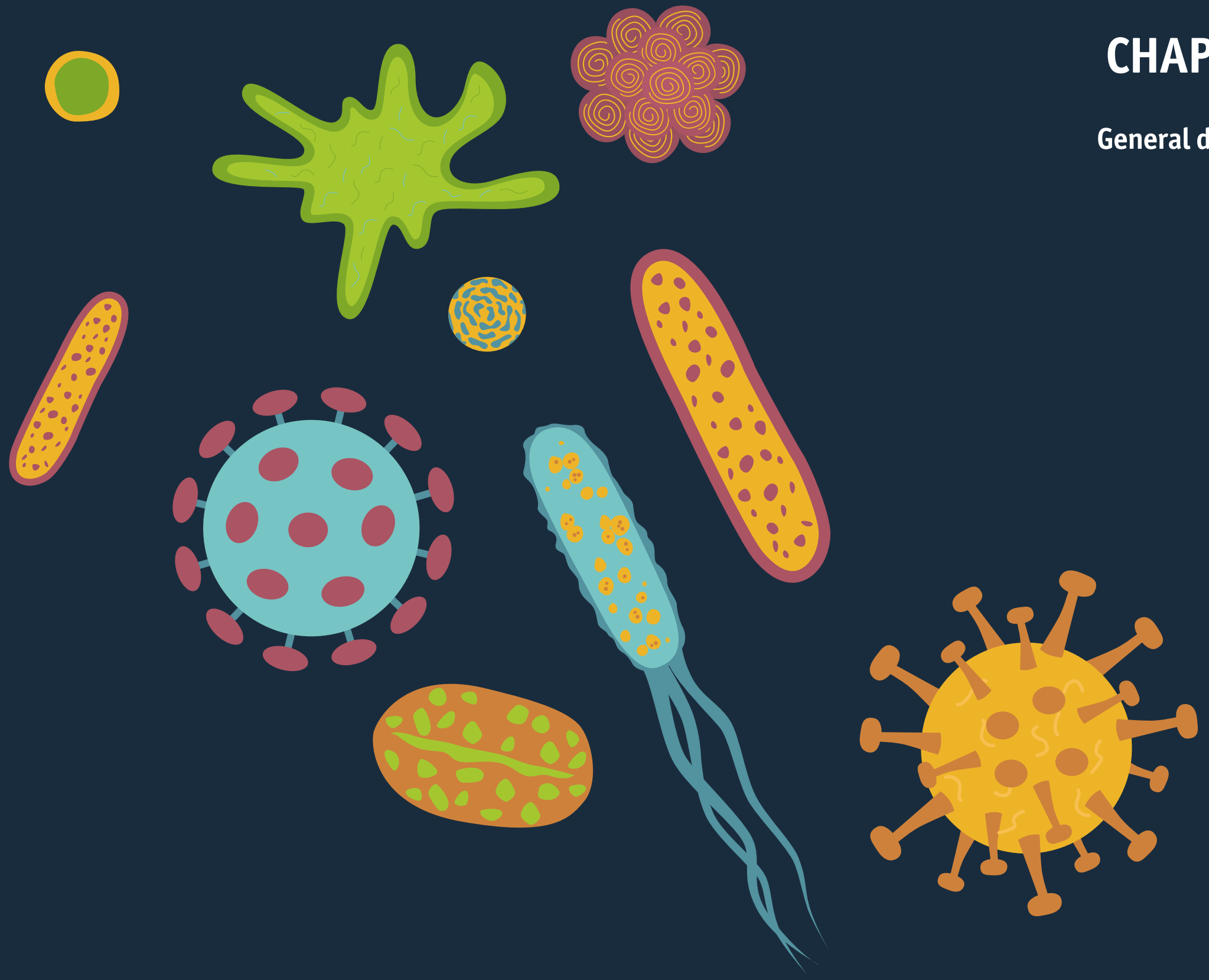

CHAPTER 9

General discussion

In this thesis, several aspects of antimicrobial drug use in horses and specifically in foals with sepsis have been studied. The first objective of this thesis was to study antimicrobial susceptibility and emergence of resistance in bacteria isolated from foals with sepsis in order to provide guidance to clinicians in selecting antimicrobial drugs for initial treatment in these challenging patients. The second objective was to evaluate the role of the intestinal microbiome and resistome as a reservoir of antimicrobial resistance. All studies included in this thesis aimed to contribute to the scientific knowledge that can be used to design or further improve existing antimicrobial stewardship programmes for equine (neonatal) medicine.

FOAL SEPSIS: SELECTING ANTIMICROBIALS FOR TREATMENT AND THE THREAT OF EMERGING ANTIMICROBIAL RESISTANCE

Sepsis is the most common cause of foal death during the first 7 days of life [1]. A recent study showed that the majority of foals admitted to an equine neonatal intensive care unit (NICU) in Florida presented with signs of sepsis [2]. Sepsis results from the dysregulation of the systemic host response to cascading inflammatory and anti-inflammatory mediators induced by infecting organisms and can lead to downstream sequelae including conditions such as septic arthritis, pneumonia, diarrhoea, physitis, osteomyelitis, meningitis and umbilical infections [3].

Antimicrobial therapy is central to the treatment of sepsis along with anti-inflammatory therapy, cardiovascular support, respiratory support, nutritional support and other supportive therapy [4-6]. When initially selecting antimicrobials for the treatment of sepsis, it is important to select broad spectrum, intravenously administered, bactericidal drugs [7]. Empirical selection of broad-spectrum antimicrobials is necessary for foals with sepsis as there is a time delay of several days until blood culture results are available. Bactericidal drugs are advised as foals have a naive immune system compared to adults. Intravenous administration is recommended as critically ill foals are often haemodynamically compromised leading to reduced perfusion of the gut and muscles and thus reduced absorption of drugs administered via the oral or intramuscular routes. Intravenous administration also assures rapid high plasma concentrations for effective treatment. The importance of timely antimicrobial administration in sepsis is well recognised in humans, with earlier administration of antimicrobials associated with an improved outcome [8]. As described in Chapter 4 of this thesis, survival rates of foals were also higher when the empirically selected antimicrobial regime included antimicrobials to which all isolated bacteria at hospital admission

were susceptible [9]. Selection of antimicrobial drugs should be based on local knowledge of the bacterial agents most likely involved, and local resistance patterns.

A number of studies, including the studies presented in Chapters 2 and 3 of this thesis, summarised the most common bacterial isolates in neonatal foals with sepsis and their susceptibility patterns [10-21]. It is important to realize that our data, as well as most publications on this topic, originate from the United States and that there is a lack of data from Europe. Prevalence of bacteria and susceptibility patterns may differ between geographical regions, and even between hospitals in the same country, as previously has been demonstrated in bacteria isolated from critically ill intensive care patients in human medicine [22, 23]. Therefore, caution is advised when extrapolating data from these studies to other parts of the world. Furthermore, prevalence of bacteria and their susceptibility profiles differ between samples collected from patients in equine hospitals compared to samples collected from horses in ambulatory practice, demonstrating the need for using representative data applicable to the working situation of the veterinarian for selection of antimicrobials for initial treatment when culture and susceptibility results are not yet available [24]. In reality, these data, unfortunately, are often not available to veterinarians. Systematic (national and international) surveillance and reporting of culture and susceptibility results would greatly improve the ability of veterinarians to choose appropriate antimicrobials for initial treatment in horses and foals. Both Gram-negative and Gram-positive bacteria are often isolated from foals with sepsis. In Chapter 2, we observed an increase in the proportion Gram-positive isolates in foals with sepsis in recent years [12]. The same trend has been observed in human medicine [25]. The most common bacterial species obtained from foals with sepsis in our study is *Escherichia coli*. Other Gram-negative bacteria that are often identified include *Actinobacillus* spp., *Klebsiella* spp., *Enterobacter* spp., *Pseudomonas* spp. and *Salmonella* spp. Gram-positive bacteria that are frequently reported in septic foals include *Streptococcus* spp., *Enterococcus* spp. and *Staphylococcus* spp. We have observed an increase in isolation rate of *Enterococcus* spp. from foals with sepsis over the years [12]. This is concerning as enterococci are known to be intrinsically resistant to many antimicrobial drugs and also readily accumulate mutations and exogenous genes that confer additional resistance through mobile genetic elements that can potentially be transferred to other bacteria [26].

We have observed polymicrobic infections in 30% of the foals with sepsis included in our study presented in Chapter 4, which is in line with previously published literature [9, 10, 15]. Foals with single-organism infections included in our study had a significantly higher likelihood of survival compared to foals with polymicrobic infections [9].

In the study described in Chapter 3 we observed temporal trends in antimicrobial susceptibility of bacteria to several antimicrobial drugs over a period of more than three decades [11]. These include emergence of antimicrobial resistance and increased minimum inhibitory concentration (MIC) values in important groups of bacteria, such as Enterobacteriaceae, *Actinobacillus* spp. and *Streptococcus* spp. to antimicrobial drugs that are frequently used in equine neonatal medicine such as gentamicin, amikacin and ceftiofur. We also observed development of resistance to antimicrobial drugs that are not used in equine medicine, such as imipenem. Therefore, continuous local monitoring of culture and susceptibility results is of utmost importance to ensure that empirical selection of drugs for treatment is based on contemporaneous and locally applicable susceptibility results. In our study described in Chapter 3, we also noticed trends in increased susceptibility of bacteria to antimicrobial drugs that are no longer used frequently in equine medicine, such as tetracyclines [11]. Similar trends in increasing antimicrobial susceptibility were observed in a study in broilers, pigs and veal calves assessing the effects of a policy change resulting in a significant decrease in the use of antimicrobial drugs in livestock [27]. These observations, support the implementation of antimicrobial stewardship programmes to reduce the use of antimicrobials in order to prevent further development of resistance or even repress prevalence of antimicrobial resistance. Based on the current literature and the results of the studies included in this thesis, the combination of ampicillin with amikacin appears to be an appropriate choice for initial treatment of foals suspected of sepsis at the University of California in Davis, USA. In areas where gentamicin resistance is less commonly encountered, such as parts of Europe [19], gentamicin should be used instead of amikacin in order to preserve amikacin for cases that are gentamicin resistant.

When foals do not improve over the course of a few days after initial antimicrobial treatment has been started, many clinicians opt to select other antimicrobials for treatment based on culture and susceptibility testing results from samples collected at hospital admission. In Chapter 5 we studied the effect of hospitalization on bacterial culture and susceptibility testing results by comparing culture results from samples collected at hospital admission to those collected after more than 48 hours of hospitalization [28]. We observed that different species of bacteria were isolated after 48 hours of hospitalization and susceptibility profiles of those bacteria were unpredictable. Furthermore, 85% of the positive cultures collected after 48 hours of hospitalization met the criteria for suspected healthcare-associated infections and were more often positive for *Acinetobacter* spp., *Enterococcus* spp., *Klebsiella* spp., *Pseudomonas* spp. and *Serratia* spp. All of these bacteria are known to be associated with healthcare-associated infections in human as well as equine medicine [29-32]. Based on the results presented in Chapter 5, no general guidelines could be formulated regarding

the choice of antimicrobial treatment in foals with sepsis that do not respond well to initial treatment. These results provide a rationale for repeating bacteriological culture and susceptibility testing in hospitalised foals at 48 hour intervals and emphasise the importance of hospital hygiene in equine neonatal intensive care units [28].

Based on the studies included in this thesis we can conclude that antimicrobial resistance is emerging and should be taken into account when developing or updating treatment protocols in equine neonatal medicine [11, 12]. Early and correct antimicrobial treatment increases the likelihood of survival in foals with sepsis [9]. Choosing antimicrobials for treatment of equine neonates is complex. Selecting antimicrobial drugs is more complicated than matching 'drugs and bugs'. Knowledge about the drugs and their pharmacokinetics, the microbiological aspects including local representative data regarding potential causative organisms and their susceptibility patterns, the clinical situation of the patient and the potential side effects are all necessary to make an appropriate decision and improve the chance of a positive outcome for the foal. Since antimicrobial treatment also carries the potential 'community cost' of antimicrobial resistance, the challenge for veterinarians is not to always play it safe, just because it is easy. Horse owners rather have their ill horse treated than worry about the impact of one single course of antimicrobial treatment on human and animal health, and veterinarians generally agree. However, these community costs should be consciously taken into consideration by veterinarians when prescribing antimicrobial treatment to make a well-considered decision in each individual case. Antimicrobial use protocols can be helpful to assure alignment of veterinarians with antimicrobial stewardship policies.

THE EFFECT OF ANTIMICROBIALS ON THE FAECAL MICROBIOME

The intestinal microbiome is considered essential for equine health [33]. Several studies have described the faecal microbiota of healthy horses, including our study presented in Chapter 6, but the variation in results is substantial [34-40]. The single main predictor of microbiota composition is individual identity, and it was suggested that this explains about 50% of the variation [41]. Other factors such as age, horse type, location, sampling season and pasture access also affect microbiota composition, but the relative effect of those factors is limited, explaining 2.3% to 6.4% of the observed variation in faecal microbiota composition in our study presented in Chapter 6 [40]. Extensive and detailed knowledge about the composition of the intestinal microbiome in healthy equids under normal housing and management conditions forms the foundation for future microbiome studies, including intervention studies as well as studies evaluating potential associations between microbiome composition and disease status.

Disturbances of the intestinal microbiota are associated with significant health problems in horses, such as colitis [42]. The administration of antimicrobial drugs profoundly affects the intestinal microbiota composition in adult horses, especially drugs administered orally, and can lead to dysbiosis and development of antimicrobial-associated diarrhoea in some horses [43-46]. In the study presented in Chapter 7, we have shown that successive transportation, hospitalisation and oral trimethoprim-sulfadiazine (TMS) treatment led to large and consistent changes in the equine faecal microbiota in healthy adult ponies [47]. These include a significant decrease in alpha-diversity and a decrease in relative abundance of several of the main phyla of the equine intestinal tract, such as Spirochaetes, Kiritimatiellaeota, Fibrobacteres and Verrucomicrobia as well as an increase in relative abundance of Firmicutes. A gradual recovery of the faecal microbiota composition was observed two weeks after cessation of treatment and discharge from the hospital. However, relative abundance of some of the larger phyla, such as Spirochaetes, Verrucomicrobia, Kiritimatiellaeota and Cyanobacteria, was still affected 6 months post hospitalization and oral treatment with TMS. In our study, we did not observe any clinical effects of these persistent changes in intestinal microbiota. In adult humans, antimicrobial drug use has been associated with increased odds for developing diabetes type 2 later (up to 15 years) in life [48]. Currently no studies have been performed in horses to evaluate the long-term health effects of antimicrobial-induced disturbances of the intestinal microbiota. In foals, the intestinal microbiota develops quickly after birth, with the greatest changes observed in the first 60 days of life [49]. In humans, it is known that exposure to antimicrobials during pregnancy or directly after birth affects development of microbiota composition in children and can have negative consequences on health later in life, such as an increased risk on allergy, atopy, asthma, and obesity [50]. One study in foals describes the effect of a combination of ampicillin and amikacin/gentamicin on the developing intestinal microbiota [51]. However, no control group was included in that study and no follow-up samples were available, limiting the authors ability to draw conclusions. Despite the relatively frequent use of antimicrobials in equine neonates to treat or prevent infections, there are currently no studies investigating the long-term health implications of administering antimicrobials to foals. Many clinicians will consider only short-term health effects when deciding whether or not to treat a foal with antimicrobials. As long as the true impact of antimicrobial treatment on the developing microbiota in foals and the potential long-term health effects later as adult horses are unknown, the results from studies in children from human medicine support careful consideration when administering antimicrobials to foals.

THE EFFECT OF ANTIMICROBIALS ON THE FAECAL RESISTOME

Similar to the effects on the microbiome, antimicrobial treatment also strongly affects the resistome. In the study described in Chapter 7, we observed that the relative abundance of antimicrobial resistance genes (ARGs) increased rapidly (at 24h) after the start of oral TMS treatment [47]. ARGs encoding for resistance to sulphonamides as well as to other classes of antimicrobials increased. These findings correspond to another study evaluating the effect of combined macrolide and rifampin treatment on the faecal resistome in foals treated for subclinical *Rhodococcus equi* infection in which the authors also observed an increase in ARGs encoding for resistance to other drugs than those used for treatment [52]. In our study presented in Chapter 7, we observed increases in relative abundance of ARGs that were already present before TMS treatment, as well as an increase in ARGs that were absent or below the limit of detection at the start of the study [47]. This might be explained by co-selection due to the presence of multiple ARGs on mobile genetic elements, such as plasmids. This is supported by the results of our study in Chapter 7, in which we observed several ARGs to be located on the same contigs. Furthermore, sulphonamide and tetracycline resistance genes were still detected in significantly higher numbers six months after hospitalisation and TMS treatment, despite the fact that microbiota composition largely returned to pre-treatment composition within two weeks after stopping the antimicrobial treatment [47]. Therefore, this is most likely the result from horizontal gene transfer between different species of bacteria or from a shift from susceptible to resistant strains within bacterial species that persist in the gut after antimicrobial treatment has been stopped. The rapid increase in ARGs after only one day of TMS treatment urges careful selection of horses for antimicrobial treatment by clinicians. Reducing the number of horses treated with antimicrobials might have a bigger impact on limiting the faecal spread of ARGs than, for example, shortening the duration of antimicrobial treatment in horses. The prolonged significant increase in ARGs in equine faeces after only five days of TMS treatment highlights the potential consequences of (injudicious) use of antimicrobials in horses. The equine hindgut might therefore be a potential reservoir of resistant bacteria and form a risk to animal and human health through transmission. However, not all ARGs pose an equally serious threat to human or animal health. ARGs present in bacteria that are known to cause infection in humans or animals comprise the highest threat, as well as ARGs that have a high gene mobility (risk for horizontal transfer) [53]. Regarding the faecal resistome of foals, little is known. One study evaluated the faecal resistome in healthy foals less than one month of age [54]. Several ARGs were detected, with genes encoding for tetracycline resistance being most abundant, followed by aminoglycoside resistance genes. The foals in that study had not been treated with

antimicrobials. However, information about antimicrobial treatment of their dams was not available. Foals develop their intestinal microbiota rapidly after birth and the dam is the main source of these micro-organisms [55, 56]. Therefore, in case of peri-partum treatment of the mare with antimicrobials, this might also affect the foal's intestinal microbiome and resistome. Subsequently, pathogenic resistant bacteria from their gut could lead to infections, or even sepsis, in these foals. The effects of antimicrobial treatment of mares in the peri-partum period and/or antimicrobial treatment of neonatal foals on the faecal resistome in foals and its potential clinical implications remain a topic of interest for further study.

ONE HEALTH: THE POTENTIAL ROLE OF THE HORSE IN SPREAD OF ANTIMICROBIAL RESISTANCE

Antimicrobial resistance is a One Health problem affecting people, domestic animals, wildlife, plants and the environment [57, 58]. Rapid emergence of resistant pathogens is an imminent threat to public health. Bacteria and antimicrobial resistance genes are not restricted to specific ecological compartments and can spread easily from one compartment to another [58-60]. Therefore, when studying antimicrobial resistance, the ecological context should be taken into consideration.

About 450,000 horses are kept in the Netherlands and equestrian sports are in the top ten of most popular sports, with approximately 500,000 people actively riding [61]. These people regularly come into close contact with horses. Antimicrobial drug use in horses in the Netherlands is low compared to other animals, such as dairy cows, poultry and even companion animals [62]. Nevertheless, large differences in antimicrobial drug use between practices were observed, presenting an opportunity for further improvement. As demonstrated in Chapter 7, faecal excretion of ARGs increases significantly in horses after oral treatment with antimicrobials and persists for a prolonged period of time (> 6 months). The equine hindgut, therefore, could be a potentially relevant reservoir of ARGs from a One Health perspective. However, not all ARGs pose an equally serious threat to human or animal health and not all ARGs have the same risk for spread from one ecological compartment to another [53]. ARGs located on mobile genetic elements are most important from a One Health perspective.

The horse has been described as a crucial part of One Health for many reasons, including antimicrobial resistance [63]. Historically, contact between horses and humans has been very close since their domestication about 5500 years ago [64, 65]. At first, horses were used as working animals and as means of transportation. Nowadays, horses are also often used for leisure purposes such as riding or racing. Equine manure has been,

and still is, used for agriculture, especially for growing mushrooms and in vegetable gardens. In a recent study, treatment of agricultural soil with equine manure, either fresh, composted or Bokashi (fermented), increased the presence of ARGs in the soil [66]. This demonstrates the potential of spread of ARGs from horse faeces to agricultural products and potentially also to humans consuming these products. Horse stable bedding, including that of equine hospitals, is sometimes repurposed as bedding for other animals, such as dairy cows. Under these circumstances, ARGs could spread to livestock. The results of the study presented in Chapter 7 indicate that equine manure should not be used for agricultural purposes for at least 6 months if horses have been treated with antimicrobials. Besides resistant bacteria and ARGs, faeces from horses treated with antimicrobials can also contain antimicrobial drug residues which could then further contribute to creating increased selection pressure for development of resistance by bacteria in the environment in which the manure is applied. Additional factors that potentially facilitate the spread of ARGs from horses to humans are the overlap in microbiome components (bacterial species and mobile genetic elements like plasmids) between horses and humans, the fact that many antimicrobials that are used in horses are also used in human medicine and the close contact between horses and humans. Horses produce a large volume of faeces that handlers come into contact with when cleaning the stables. Furthermore, many horses lie down in their own faeces thereby soiling their coat. Cleaning the coat by brushing off the faecal material creates an additional opportunity for exposure to resistant bacteria and ARGs originating from equine faeces either in the form of airborne dust particles or via direct contact.

The true relevance of the horse regarding the One Health topic of antimicrobial resistance is hard to assess and currently unknown. The number of horses in the Netherlands is lower than other companion animals (such as dogs and cats) and livestock and the antimicrobial drug use in horses is low compared to that in other species, at least in the Netherlands [61, 62]. However, several unique features regarding horses, as described above, do potentially pose a risk to animal, human and environmental health and should not be overlooked. These topics deserve further studying in order to assess their importance and to develop ways to limit their contribution to spread of antimicrobial resistance. The potential role of the horse in spread of antimicrobial resistance from a One Health perspective is presented in Figure 1.

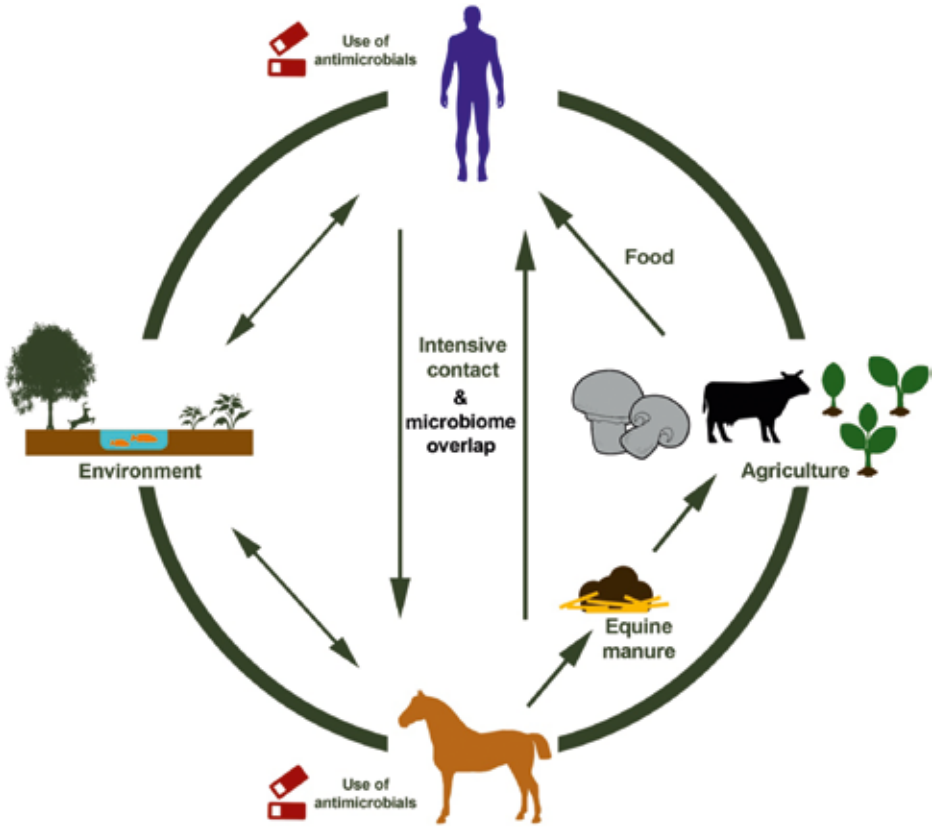

Figure 1: Potential spread of antimicrobial resistance genes from horses to humans, livestock, agriculture and the environment.

ANTIMICROBIAL STEWARDSHIP IN EQUINE (NEONATAL) MEDICINE

Antimicrobial stewardship involves the judicious use of antimicrobials balanced against the requirement to treat the presenting clinical condition [67]. The same principles apply to human medicine as well as veterinary medicine, including equine (neonatal) medicine. The studies included in this thesis provide information that underline the need for antimicrobial stewardship in equine practice and provide new insights that can be used to improve existing antimicrobial stewardship programmes. Implementation of an antimicrobial stewardship programme in companion animal medicine has demonstrated to be effective [68]. Furthermore, implementation of antimicrobial stewardship programmes in human medicine has also led to a significant healthcare cost reduction [69]. Antimicrobial use in equine practice varies significantly between practices, veterinarians and geographic location and in some regions,

restricted antimicrobials are still commonly used in equine practice [70-72]. Developing and incorporating an antimicrobial stewardship strategy are therefore important for all equine practices in order to reduce the use of antimicrobial drugs. Several papers have published guidelines for antimicrobial stewardship in equine medicine [67, 73, 74], however, papers evaluating the effectiveness of implementation of these programmes in equine practice are currently lacking. In Chapter 8, practical guidelines for antimicrobial stewardship in equine (neonatal) medicine are presented that can be applied by equine veterinarians.

FIGHTING ANTIMICROBIAL RESISTANCE IN FOAL SEPSIS: DOES THE GUT CONSPIRE AGAINST US?

Development of antimicrobial resistance in bacteria isolated from foals with sepsis has been observed over time [11]. This poses a direct threat to foal health as effective antimicrobial treatment increases the chances for survival [9]. Oral antimicrobial treatment in adult horses has long-term effects on the faecal microbiome and resistome and causes prolonged excretion of ARGs into the environment [47]. If these ARGs are present in pathogenic bacteria, these could subsequently lead to antimicrobial resistant bacterial infections, including sepsis in foals.

More research is needed to further evaluate the (potential long-term health) effects of antimicrobial drug administration on the intestinal microbiome and resistome in foals and adult horses, including pregnant mares. When we understand the true health effects of disturbances in intestinal microbiota composition, we can also start looking for ways to influence its composition. In human medicine, faecal microbiota transplantation has also been suggested as a potential way to reduce the antimicrobial resistance burden in the gut [75, 76]. In horses, studies into the curative or preventative use of (autologous) faecal microbiota transplant, or even specific micro-organisms, to affect intestinal microbiome composition are limited and provide an exciting field for future research [77-79]. By doing so, instead of conspiring against us, we might be able to get the gut to collaborate with us. Also, more insight into the development of, and risk factors for, infections with antimicrobial resistant bacteria in foals and horses, including healthcare-associated infections, is needed to develop effective intervention strategies. Furthermore, studies evaluating spread of antimicrobial resistance from horses to other animals, humans (owners, veterinarians, consumers of products for which equine manure has been used for production) and the environment can provide more information on the relevance of the horse in the One Health topic of antimicrobial resistance. Finally, studies evaluating the efficacy of the implementation of

antimicrobial stewardship programmes in equine medicine can assist in identifying which interventions are most effective and should be given priority.

For many years the focus has been mainly on reducing antimicrobial use in livestock to prevent antimicrobial resistance in human medicine. This has led to a significant reduction in the use of antimicrobial drugs in production animals worldwide [80]. Recently, the Quadripartite memorandum of understanding (MoU) was signed by four leading international organisations: the Food and Agriculture Organization of the United Nations (FAO), the World Organisation for Animal Health (WOAH/OIE), the UN Environment Programme (UNEP) and the World Health Organization (WHO) [81]. This MoU provides a legal and formal framework for these four organizations to tackle challenges, including antimicrobial resistance, at the human, animal, plant and ecosystem interface using a more integrated and coordinated approach. This framework will also contribute to reinforce national and regional health systems and services. This MoU is a promising next step in the One Health approach to address the global problem of antimicrobial resistance. It is time to focus more on antimicrobial stewardship in human and animal health, including production animals as well as companion animals, such as the horse. In order for the veterinary profession to take responsibility and contribute to the worldwide battle against antimicrobial resistance, all veterinary practices should develop and implement a practice-wide antimicrobial stewardship strategy.

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APPENDIX

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ENGLISH SUMMARY

Neonatal foals are susceptible to infectious diseases as they have immature innate and adaptive immune responses compared to adult horses. As a result, infectious diseases are common in neonatal foals. Sepsis is one of the most serious infectious conditions in neonatal foals and is associated with high mortality. Sepsis can present as primary disease, but is also observed frequently as comorbidity to other neonatal problems such as prematurity or neonatal maladjustment syndrome, and negatively affects prognosis. Sepsis results from the dysregulation of the systemic host response to cascading inflammatory and anti-inflammatory mediators induced by infecting organisms and is often defined as systemic inflammatory response syndrome (SIRS) caused by infection. Several risk factors for development of sepsis have been identified, with insufficient intake of good quality colostrum resulting in failure of passive transfer of immunity being the most important risk factor. Bacteria can enter the body via a variety of entry portals, such as the umbilicus, the respiratory tract, disrupted skin or mucous membranes or via translocation from the 'open gut' that allows for absorption of immunoglobins in the first 24 hours of life.

Many foals admitted to equine hospitals show clinical signs consistent with 'suspected sepsis', however, definite diagnosis of sepsis is complicated. Blood cultures are considered the gold standard, but these can also be false negative or false positive. Therefore, it is important that blood culture results are interpreted in the light of presence of clinical signs suggestive of sepsis. Progression of sepsis is often rapid, and sepsis can lead to multiple organ dysfunction syndrome (MODS), multiple organ failure syndrome (MOFS), septic shock and ultimately the death of the foal. Timely antimicrobial treatment can potentially avoid rapid clinical deterioration. In foals with sepsis immediate initiation of antimicrobial treatment is warranted, while awaiting culture and susceptibility testing results. Selection of antimicrobial drugs should be based on historic data of causative organisms and their susceptibility patterns. However, concerns are rising about development of antimicrobial resistance which complicates selection of antimicrobial drugs for treatment of sepsis in foals. *Escherichia coli* is the most common causative organism isolated from foals with sepsis, followed by *Enterococcus* spp., *Streptococcus* spp., *Actinobacillus* spp., *Enterobacter* spp., *Staphylococcus* spp. and *Klebsiella* spp.

Bacterial prevalence and antimicrobial susceptibility patterns vary between studies conducted in different geographic locations and in different time periods. Data on temporal trends in prevalence and antimicrobial susceptibility, as have been observed in human medicine, are scarce in equine medicine. In human medicine, critically ill

patients admitted to intensive care units are at risk of developing healthcare-associated infections, which often involve specific species or strains of bacteria that are resistant to many antimicrobial drugs and are also present in the hospital environment. Foals treated in an equine neonatal intensive care unit are likely at risk for healthcare-associated infections. However, no data on prevalence of healthcare-associated infections, the organisms involved and their antimicrobial susceptibility patterns have been published in equine neonatal medicine. In **Chapters 2 to 5,** antimicrobial susceptibility and emergence of resistance in bacteria isolated from foals with sepsis at the University of California, Davis, USA, were studied in order to provide guidance to clinicians in selecting antimicrobial drugs for treatment in foals with sepsis.

The equine gastrointestinal tract harbours a complex polymicrobial community (intestinal microbiota) of which bacteria form the largest part. A well-functioning gastrointestinal tract and a healthy intestinal microbiota community are essential for equine health and disturbances are associated with disease, such as diarrhoea. Until recently, the equine hindgut microbiota was relatively poorly characterized. By using next generation sequencing techniques, Firmicutes and Bacteroidetes have been identified as the most abundant bacterial phyla present in the equine intestinal tract of healthy horses. However, as a result of limited standardization of methods for microbiome analysis, combined with many other potential factors of influence, information on what is considered a 'healthy' or 'normal' equine intestinal microbiota composition and which factors shape it, is currently limited. In humans however, several distinct types of intestinal microbiota composition (enterotypes) can be distinguished in healthy individuals and several environmental- and host factors have been identified to affect intestinal microbiota composition.

Use of antimicrobials in horses carries the risk of development of antimicrobial-associated diarrhoea and the effects of antimicrobials on the intestinal microbiota in horses are poorly studied. Bacteria in the equine gastrointestinal tract carry genetic information encoding for metabolic pathways that are essential for digestion, but they also carry other genes, such as antimicrobial resistance genes. Antimicrobial resistance genes are naturally present in environmental bacteria and were identified in ancient environmental samples far predating the discovery of antimicrobials. All the antimicrobial resistance genes in a certain environment, of both pathogenic and non-pathogenic bacteria, are called the resistome. Use of antimicrobials places selection pressure on bacteria, including those in the intestines, which can lead to increases in relative abundance of antimicrobial resistance genes. The presence of antimicrobial resistant bacteria in the intestinal microbiota doesn't necessarily have

a negative impact on the host's health. However, if they cause an infection, it might be difficult to treat.

Resistant bacteria are not restricted to ecological compartments and can also spread to the environment by faecal excretion and subsequently cause infections in other animals or humans. This is especially important in a hospital setting in which contamination of the environment with antimicrobial resistant bacteria could be a source of healthcare-associated infections in other (already immunocompromised) patients. Currently, the effects of antimicrobial treatment on the equine faecal resistome are unknown. This is relevant not only for horses, but also from a One Health perspective as there is a close interaction between horses, their owners and the environment. In **Chapters 6 and 7,** we studied the intestinal microbiome and resistome in horses and evaluated their potential role as a reservoir of antimicrobial resistance. In **Chapter 8** practical guidelines for implementation of antimicrobial stewardship in equine practice are presented.

Chapter 1 covers a general introduction on sepsis in foals and the importance of selecting effective antimicrobials for treatment. Furthermore, an introduction is given on the intestinal microbiome and how it is essential for equine health and might also be a reservoir of antimicrobial resistance genes.

In **Chapters 2 and 3** we evaluated temporal trends in prevalence and antimicrobial susceptibility of 1091 isolates cultured from 588 foals with sepsis at the University of California, Davis, USA between 1979 and 2010. The percentage Gram-positive isolates increased significantly over the years. The percentage Enterobacteriaceae decreased over time. *Enterococcus* spp. isolates, often resistant to many antimicrobial drugs, were cultured more frequently in recent years. Emergence of antimicrobial resistance and increased minimum inhibitory concentration (MIC) values in important groups of bacteria, such as Enterobacteriaceae, *Actinobacillus* spp. and *Streptococcus* spp. to antimicrobial drugs that are frequently used in equine neonatal medicine such as gentamicin, amikacin and ceftiofur were observed. We also observed development of resistance to antimicrobial drugs that are not used in equine medicine, such as imipenem. In contrast, we also noticed trends in increased susceptibility of bacteria to antimicrobial drugs that are no longer used frequently in equine medicine, such as tetracyclines. Based on these results, we concluded the combination of ampicillin with amikacin appears to be an appropriate choice for initial treatment of foals suspected of sepsis at the University of California in Davis, USA. Continuous local monitoring of culture and susceptibility results is of utmost importance to ensure that empirical

selection of drugs for treatment is based on contemporaneous and locally applicable susceptibility results.

In **Chapter 4** the effect of initial antimicrobial treatment on outcome in 213 foals diagnosed with sepsis was studied. The likelihood of survival for foals from which all bacteria were susceptible to the initial antimicrobial treatment was 65.4%, versus 41.7%, if one or more isolates were resistant. These results indicate that empirical treatment of foals with antimicrobials to which the infecting bacteria are susceptible has a positive effect on outcome and supports the common practise of initiating antimicrobial treatment prior to culture and susceptibility results being available. Polymicrobial infections, in which more than one bacterial species is cultured from one foal, are common in foals with sepsis, ranging from 8% to 45%. Likelihood for survival was also affected by infection type in our study. Foals with single organism infections had a greater likelihood for survival (61.7%) compared to foals with a polymicrobial infection (40.6%).

Some foals fail to show clinical improvement after initial antimicrobial treatment has been started. In those cases, clinicians often adjust antimicrobial therapy based on culture and susceptibility testing results from samples collected at hospital admission. In **Chapter 5**, we compared bacterial culture and susceptibility testing results from samples collected from foals with sepsis at hospital admission to those collected after ≥48 hours of hospitalisation. Data from 231 foals were included. Samples collected after ≥48 hours of hospitalisation and after the start of initial antimicrobial treatment, were more often positive for *Acinetobacter* spp. (3.3% vs. 0,6%), *Enterococcus* spp. (19.6% vs. 4.8%), *Klebsiella* spp. (10.9% vs. 5.1%), *Pseudomonas* spp. (7.6% vs. 3.0%) and *Serratia* spp. (5.4% vs. 3.0%), which are all bacterial species that are associated with healthcare-associated infections in human as well as veterinary medicine. Furthermore, bacteria isolated after ≥48 hours of hospitalisation were less susceptible to all tested antimicrobial drugs, except for imipenem, and susceptibility profiles were unpredictable. Therefore, no general guidelines could be formulated regarding the choice of antimicrobials in cases that fail to respond to initial treatment. A large proportion (85%) of the positive samples collected after ≥48 hours hospitalisation met the criteria for potential healthcare-associated infections. These findings emphasize the importance of these infections in foals treated in neonatal intensive care units and underline the need for hygiene strategies to prevent and control healthcare-associated infections in equine hospitals. The decreased and unpredictable antimicrobial susceptibility of bacteria isolated after ≥48 hours of hospitalisation provides a rationale for routine repeated bacteriological culture and susceptibility testing at 48 hour intervals in hospitalised foals suspected of or at risk for sepsis.

The intestinal microbiota is considered essential for equine health. The single main predictor of microbiota composition is individual identity, and it is suggested that this explains about 50% of the variation. In **Chapter 6** we studied the faecal microbiota in 79 healthy horses and ponies kept under standard housing and management conditions. Bacteroidetes was the largest phylum found in the faecal microbiota (50.1%), followed by Firmicutes (28.4%). We also evaluated the effect of several host- and environmental factors on microbiota composition. Alpha diversity and richness decreased significantly with increasing age. Furthermore, location, age, season, horse type (horse vs. pony) and pasture access had a significant effect on beta-diversity, explaining 2.3% to 6.4% of the observed variation in faecal microbiota composition. Extensive and detailed knowledge about the composition of the intestinal microbiome in healthy equids under normal housing and management conditions forms the foundation for future microbiome studies, including intervention studies as well as studies evaluating potential associations between microbiome composition and disease status.

Every year large numbers of horses are hospitalised and even more are treated with antimicrobials in equine hospitals as well as in the field. In **Chapter 7,** in an experimental study in six ponies, the cumulative effects of transportation, hospitalisation and oral trimethoprim-sulfadiazine (TMS) treatment on the faecal microbiome as well as the effect on the faecal resistome, were studied. Immediately after the start of antimicrobial treatment, a significant decrease in alpha-diversity and a decrease in relative abundance of several of the main phyla of the equine intestinal tract, such as Spirochaetes, Kiritimatiellaeota, Fibrobacteres and Verrucomicrobia as well as an increase in relative abundance of Firmicutes were observed. A gradual recovery of the faecal microbiota composition was observed two weeks after cessation of treatment and discharge from the hospital. However, relative abundance of some of the larger phyla, such as Spirochaetes, Verrucomicrobia, Kiritimatiellaeota and Cyanobacteria, was still affected six months post hospitalisation and oral treatment with TMS. Similar to the effects on the microbiome, antimicrobial treatment also strongly affected the resistome. Relative abundance of antimicrobial resistance genes increased within 24h after the start of oral TMS treatment. Genes encoding for resistance to sulphonamides as well as to other classes of antimicrobials, such as tetracyclines and aminoglycosides, increased. This might be explained by co-selection due to the presence of multiple antimicrobial resistance genes on mobile genetic elements. Sulphonamide and tetracycline resistance genes were still increased six months after hospitalisation and TMS treatment, despite the fact that microbiota composition largely returned to pre-treatment composition. This is potentially the result from horizontal gene transfer between different species of bacteria or from a shift from susceptible to resistant

strains within bacterial species that persist in the gut after antimicrobial treatment has been stopped. The prolonged significant increase in antimicrobial resistance genes in equine faeces after only five days of TMS treatment highlights the potential consequences of (injudicious) use of antimicrobials in horses. The equine hindgut might, therefore, be a potential reservoir of resistant bacteria from a One Health perspective.

Antimicrobial stewardship involves the judicious use of antimicrobials balanced against the requirement to treat the presenting clinical condition. The same principles apply to human medicine as well as veterinary medicine, including equine (neonatal) medicine. In **Chapter 8,** practical guidelines for antimicrobial stewardship in equine (neonatal) medicine are presented that can be applied by equine veterinarians. Antimicrobial stewardship requires a multifaceted approach that combines several components, such as reduction of resistance reservoirs, improved clinical diagnosis of (bacterial) infections, improved infection control measures, improved use of preventative health measures, monitoring of culture and susceptibility testing results and monitoring of antimicrobial use, education/creating awareness of antimicrobial resistance and improved communication within the treatment team. Implementation of an antimicrobial stewardship programme in companion animal medicine as well as human medicine has demonstrated to be effective and to reduce healthcare costs. Strategies for antimicrobial stewardship are not one-size-fits-all and therefore may vary between practices/hospitals. Rather than an all-or-none approach, implementation of specific action points will help focus efforts to improve compliance and gradually bring about the needed institutional cultural change. Monitoring antimicrobial resistance and antimicrobial drug use within the veterinary practice can be used as tools to evaluate the effectiveness of the implementation of the antimicrobial stewardship programme. Developing and incorporating an antimicrobial stewardship strategy is important for all equine practices in order to take responsibility and contribute to the worldwide battle against antimicrobial resistance.

In **Chapter 9**, the main findings of this thesis are summarized and discussed in relation to previous research. Still, more research is needed to better understand the composition of the intestinal microbiome and the factors that shape it. Studies evaluating spread of antimicrobial resistance from horses to other animals, humans and the environment can provide more information on the relevance of the horse in the One Health topic of antimicrobial resistance. Furthermore, future studies into risk factors for healthcare-associated infections in equine (neonatal) patients could provide insights on which to base improvement of hospital hygiene protocols. Finally, studies evaluating the efficacy of the implementation of antimicrobial stewardship programmes in equine medicine can assist in identifying which interventions are most effective

and should be given priority. Several initiatives, such as the Quadripartite Memorandum of Understanding between the Food and Agriculture Organization (FAO), the World Organization for Animal Health (WOAH/OIE), the United Nations Environment Programme (UNEP) and the World Health Organization (WHO), have been launched recently in order to fight antimicrobial resistance on a global scale using an integrated and coordinated approach. These are promising steps in the One Health approach to address antimicrobial resistance. It is time to focus on antimicrobial stewardship in human and animal health. The latter should not only include production animals but also companion animals and horses. In order for the veterinary profession to take responsibility and contribute to the worldwide battle against antimicrobial resistance, veterinary practices should develop and implement a practice-wide antimicrobial stewardship strategy.

Nederlandse samenvatting

NEDERLANDSE SAMENVATTING

Pasgeboren veulens hebben een verhoogd risico op het oplopen van infecties ten opzichte van volwassen paarden omdat hun immuunsysteem nog niet volledig ontwikkeld is bij de geboorte. Sepsis (een ontstekingsreactie in het lichaam veroorzaakt door een infectie) is een potentieel levensbedreigende infectieuze aandoening die relatief vaak voorkomt bij neonatale veulens. Het kan voorkomen als primaire aandoening, maar het wordt ook vaak gezien als co-morbiditeit bij andere neonatale problemen zoals prematuriteit en neonatal maladjustment syndrome (NMS) en heeft in die gevallen een negatief effect op de prognose. Sepsis is het gevolg van een dysregulatie van de immunologische respons op een infectie en wordt vaak gedefinieerd als 'systemic inflammatory response syndrome' (SIRS), veroorzaakt door infectie. Er zijn een aantal risicofactoren die de kans op sepsis vergroten. De belangrijkste is onvoldoende opname van goede kwaliteit biest met als gevolg een te laag gehalte aan immuunglobulines in het bloed van het veulen. Bacteriën dringen het lichaam van het veulen binnen via verschillende routes, zoals de navel, de luchtwegen, huid- of slijmvlieslaesies of via translocatie vanuit de 'open darm' die normaal gesproken de opname van immuunglobulines faciliteert in neonatale veulens gedurende de eerste 24 uur. Het gevolg hiervan is dat er een bacteriemie kan optreden die kan leiden tot sepsis.

Een groot deel van de veulens dat intensieve zorg nodig heeft en waarvoor behandeling in een kliniek noodzakelijk is, vertoont tekenen van sepsis. Het stellen van de definitieve sepsis diagnose is echter niet eenvoudig. Een bloedkweek wordt gezien als de gouden standaard, maar deze kan ook vals negatief of vals positief zijn. Het is daarom belangrijk om klinische symptomen van sepsis mee te wegen bij de interpretatie van bloedkweekuitslagen. Sepsis kan zich erg snel ontwikkelen, het kan leiden tot orgaan dysfunctie (multiple organ dysfunction syndrome, MODS) gevolgd door orgaan falen (multiple organ failure syndrome, MOFS), septische shock en uiteindelijk tot de dood. Tijdige behandeling met antibiotica kan mogelijk deze complicaties voorkomen. Daarom wordt bij veulens met sepsis, in afwachting van de uitslagen van bacteriologisch onderzoek, vaak meteen gestart met een antibioticumbehandeling. Het selecteren van antibiotica voor deze initiële behandeling vindt dan meestal plaats op basis van resultaten van bloedkweken afgenomen bij eerdere patiënten. Echter, bij bacteriën die sepsis kunnen veroorzaken bij veulens komt antibioticumresistentie voor.

Escherichia coli is de bacterie die het vaakst gevonden wordt als veroorzaker van sepsis bij veulens, gevolgd door *Enterococcus* spp., *Streptococcus* spp., *Actinobacillus* spp., *Enterobacter* spp., *Staphylococcus* spp. en *Klebsiella* spp. De prevalentie van verschillende soorten bacteriën en hun gevoeligheidspatronen verschillen tussen studies die gedaan zijn in verschillende landen, klinieken en tijdperiodes. Er zijn weinig data gepubliceerd over trends in de tijd ten aanzien van prevalentie en antibioticumgevoeligheidspatronen van bacteriën die sepsis kunnen veroorzaken bij veulens. Vanuit de geneeskunde is bekend dat ernstig zieke patiënten die opgenomen worden op de intensive care het risico lopen een infectie met ziekenhuisbacteriën te ontwikkelen. Deze bacteriën zijn vaak resistent tegen verschillende soorten antibiotica. Veulens die behandeld worden in een veulen intensive care lopen mogelijk ook een risico op een infectie met ziekenhuisbacteriën. Op dit moment is hier echter nog geen informatie over gepubliceerd. In **Hoofdstuk 2 tot en met 5** worden de prevalentie en antibioticumgevoeligheidspatronen van bacteriën die gekweekt zijn uit veulens met sepsis in het Veterinary Medical Teaching Hospital van de University of California in Davis, USA, beschreven. Het doel van deze studies was om clinici te helpen bij het selecteren van antibiotica voor de behandeling van sepsis bij veulens.

In het maagdarmkanaal van paarden bevinden zich grote aantallen micro-organismen; samen het darmmicrobioom genoemd. Bacteriën maken hiervan het grootste deel uit. Een goed functionerend maagdarmkanaal en een gezond darmmicrobioom zijn essentieel voor de gezondheid van paarden. Verstoringen zijn geassocieerd met bepaalde ziektes, zoals diarree. Tot voor kort was er beperkte kennis over de samenstelling van het darmmicrobioom bij het paard, maar met behulp van next generation sequencing (NGS) technieken komt er steeds meer informatie beschikbaar. Firmicutes en Bacteroidetes zijn de meest voorkomende phyla in het maagdarmkanaal van gezonde paarden. Echter, een gebrek aan standaardisatie van studiemethodes en analyses, en vele andere factoren die verschillen tussen reeds gepubliceerde studies, maken het moeilijk om te bepalen wat een 'normaal' of 'gezond' darmmicrobioom is. Het is bekend dat bij mensen verschillende variaties in de samenstelling van het darmmicrobioom voorkomen bij gezonde individuen. Deze worden enterotypes genoemd. Verschillende omgevings- en gastheer-gerelateerde factoren hebben hier invloed op.

Het is bekend dat antibioticumgebruik bij paarden kan leiden tot een dysbacteriose en diarree. Op dit moment zijn er maar weinig studies gedaan naar het effect van antibiotica op de samenstelling van het darmmicrobioom en de ontwikkeling van antibioticaresistentie bij paarden. Darmbacteriën vervullen belangrijke functies bij de vertering van voedingsstoffen. Deze functies liggen vast in het genetische materiaal dat de bacteriën bij zich dragen en hierin bevindt zich ook informatie over de gevoeligheid van de bacteriën voor antibiotica (antibioticumresistentiegenen). Alle antibioticumresistentiegenen samen in een bepaalde omgeving, zoals in de darm, zowel in pathogene als niet-pathogene bacteriën, worden het resistoom genoemd.

Door het gebruik van antibiotica ontstaat een selectiedruk op bacteriën waardoor de hoeveelheid resistentiegenen in een bepaalde omgeving kan toenemen. Dit kan ook in het maagdarmkanaal plaatsvinden. De aanwezigheid van resistente bacteriën is op zichzelf niet schadelijk, maar als deze bacteriën een infectie veroorzaken kan het moeilijk zijn om deze te behandelen.

Mest, van paarden of van andere dieren, kan een bron kan zijn van resistentiegenen en resistente bacteriën. Deze kunnen verspreiden naar andere ecologische compartimenten zoals de mens, andere dieren en de omgeving. Deze bacteriën kunnen vervolgens een infectie veroorzaken in een ander dier of in een mens. Dit is vooral van belang in een ziekenhuisomgeving waar resistente bacteriën afkomstig uit de omgeving een bron kunnen zijn van infecties met (vaak resistente) ziekenhuisbacteriën bij andere patiënten met een reeds verzwakte afweer. Momenteel zijn de effecten van een antibioticumbehandeling op het resistoom bij paarden nog onbekend. Dit is dus niet alleen relevant voor de paarden zelf, maar mogelijk ook voor mensen en milieu, omdat er nauwe interactie bestaat tussen paarden, hun eigenaren en de omgeving. In de **Hoofdstukken 6 en 7** worden het darmmicrobioom en resistoom bij paarden en hun potentiële rol als reservoir van resistente bacteriën beschreven. Om ontwikkeling en spreiding van antibioticumresistentie zo veel mogelijk tegen te gaan worden in de geneeskunde en diergeneeskunde 'antimicrobial stewardship' programma's ontwikkeld. Antimicrobial stewardship betreft het zorgvuldig afwegen van de gevolgen van het gebruik van antibiotica ten opzichte van de noodzaak om een klinische infectie te behandelen. In **Hoofdstuk 8** worden praktische richtlijnen voor de implementatie van antimicrobial stewardship programma's in de paardengeneeskunde gepresenteerd.

Hoofdstuk 1 bestaat uit een algemene inleiding over sepsis bij veulens en het belang van het selecteren van effectieve antibiotica voor behandeling. Verder wordt een inleiding gegeven over het darmmicrobioom en er wordt ook ingegaan op de redenen waarom dit belangrijk is voor de gezondheid van paarden. Verder wordt geschetst hoe het darmmicrobioom mogelijk ook een rol zou kunnen spelen bij de verspreiding van resistente bacteriën.

In de **Hoofdstukken 2 en 3** worden trends in de tijd beschreven in prevalentie en antibioticumgevoeligheid van 1091 isolaten afkomstig uit 588 veulens met sepsis bij de University of California, Davis, USA tussen 1979 en 2010. Het percentage Gram-positieve bacteriën nam in de loop der jaren significant toe. Het percentage Enterobacteriaceae nam af. *Enterococcus* spp., vaak resistent tegen meerdere antibiotica, werd in de laatste jaren juist vaker aangetroffen. Er was een toename in antibioticumresistentie en verhoogde minimum inhibitory concentration (MIC) waarden zichtbaar in belangrijke groepen bacteriën, zoals Enterobacteriaceae, *Actinobacillus* spp. en *Streptococcus* spp. voor antibiotica die vaak worden gebruikt bij de behandeling van sepsis bij veulens, zoals gentamicine, amikacine en ceftiofur. Er bleek ook resistentie-ontwikkeling op te treden tegen antibiotica die niet worden gebruikt in de paardengeneeskunde, zoals imipenem. Er werden ook trends gezien waarbij juist een verhoogde gevoeligheid van bacteriën voor bepaalde antibiotica optrad voor middelen die niet meer vaak worden ingezet, zoals tetracyclines. Op basis van de resultaten van deze studies kan worden geconcludeerd dat de combinatie van ampicilline met amikacine een goede keuze is voor de initiële behandeling van veulens die verdacht worden van sepsis bij de University of California in Davis, USA. Selectie van antibiotica voor initiële behandeling moet idealiter gebaseerd worden op actuele en lokale informatie met betrekking tot veelvoorkomende bacteriesoorten en hun antibioticumgevoeligheidspatronen. Systematische monitoring van lokale kweek- en gevoeligheidsresultaten is hierbij essentieel.

In **Hoofdstuk 4** wordt het effect van de initiële antibioticumbehandeling op de overlevingskans bij 213 veulens met sepsis beschreven. De overlevingskans voor veulens waarvan alle gekweekte bacteriën gevoelig waren voor de initiële antibioticumbehandeling was 65,4%, versus 41,7% als één of meer bacteriën resistent waren. Deze resultaten tonen aan dat behandeling van veulens met antibiotica waarvoor de bacteriën die de infectie veroorzaken gevoelig zijn, een positief effect heeft op de overlevingskans. Dit ondersteunt de gangbare praktijk om behandeling met antibiotica te starten voordat de kweek- en gevoeligheidsresultaten beschikbaar zijn bij veulens verdacht van sepsis. Infecties waarbij meer dan één bacterie werd gekweekt uit één veulen (polymicrobiële infecties), komen regelmatig voor bij veulens met sepsis, variërend van 8% tot 45%. Veulens met een infectie veroorzaakt door één bacterie hadden een grotere overlevingskans (61,7%) in vergelijking met veulens met een polymicrobiële infectie (40,6%).

Sommige veulens knappen klinisch niet op nadat de initiële antibioticumbehandeling is gestart. In die gevallen wordt de therapie vaak aangepast op basis van de resultaten van kweek- en gevoeligheidsbepalingen van monsters die bij ziekenhuisopname zijn afgenomen. In **Hoofdstuk 5** worden de resultaten van een studie beschreven waarbij uitslagen van kweek en gevoeligheidsbepalingen van monsters die afgenomen zijn op het moment van ziekenhuisopname vergeleken worden met die van monsters die afgenomen zijn na ≥ 48 uur ziekenhuisopname. Data van 231 veulens zijn opgenomen in deze studie. Monsters die werden verzameld na ≥ 48 uur ziekenhuisopname en antibioticumbehandeling, waren vaker positief voor *Acinetobacter* spp. (3,3% vs. 0,6%), *Enterococcus* spp. (19,6% vs. 4,8%), *Klebsiella* spp. (10,9% vs. 5,1%), *Pseudomonas* spp.

(7,6% vs. 3,0%) en *Serratia* spp. (5,4% vs. 3,0%). Dit zijn allemaal bekende veroorzakers van ziekenhuisinfecties bij zowel mens als dier. Deze bacteriën waren minder gevoelig voor alle geteste antibiotica, behalve voor imipenem, en bovendien waren de gevoeligheidspatronen onvoorspelbaar. Hierdoor kon er geen algemene richtlijn worden opgesteld met betrekking tot de keuze voor antibiotica bij veulens met sepsis die niet reageren op de initiële behandeling. Een groot deel (85%) van de positieve monsters welke ≥ 48 uur na ziekenhuisopname werden afgenomen, voldeed aan de criteria voor ziekenhuisinfecties. Deze bevindingen benadrukken het belang van deze infecties bij veulens die worden behandeld in een veulen intensive care en onderstrepen de noodzaak voor strikte hygiëne. Daarnaast is het, op basis van bovenstaande bevindingen, raadzaam om bij veulens die behandeld worden in een veulen intensive care elke 48 uur een nieuw monster te verzamelen voor herhaalde kweek- en gevoeligheidsbepalingen. Hiermee kunnen infecties veroorzaakt door resistente bacteriën en ziekenhuisinfecties in een vroeg stadium worden opgespoord en kan een effectieve alternatieve antibioticumtherapie worden ingezet.

Het darmmicrobioom speelt een essentiële rol bij de gezondheid van paarden. Individuele identiteit is de belangrijkste bepalende factor voor de samenstelling en verklaart ongeveer 50% hiervan. In **Hoofdstuk 6** worden de resultaten van een studie beschreven waarbij de samenstelling van het darmmicrobioom is onderzocht bij 79 gezonde paarden en pony's die onder normale huisvesting- en managementomstandigheden werden gehouden. Bacteroidetes was het meest voorkomende phylum (50,1%) in deze studie, gevolgd door Firmicutes (28,4%). Verschillende gastheer- en omgevingsfactoren hadden effect op de samenstelling van het darmmicrobioom. Het aantal verschillende bacteriesoorten nam significant af bij toenemende leeftijd en ook veranderde hun onderlinge verhouding. Bovendien hadden locatie (verschillende stallen), leeftijd, seizoen (zomer/winter), type paard (paard/pony) en weidegang (wel/niet) een significant effect op de darmmicrobioomsamenstelling; 2,3% tot 6,4% van de variatie werd hiermee verklaard. Uitgebreide en gedetailleerde kennis over de samenstelling van het darmmicrobioom bij gezonde paarden, gehouden onder normale omstandigheden, vormt de basis voor toekomstige studies naar het darmmicrobioom bij paarden.

Elk jaar worden grote aantallen paarden in klinieken opgenomen en nóg meer worden er behandeld met antibiotica, zowel in paardenziekenhuizen als in praktijken. In **Hoofdstuk 7** worden de resultaten beschreven van een experimentele studie bij zes Welsh pony's waarbij de cumulatieve effecten van transport, ziekenhuisopname en orale behandeling met trimethoprim-sulfadiazine (TMPS) op het faecale microbioom en resistoom zijn onderzocht. Meteen na de start van de antibioticumbehandeling,

was er een significante afname van het aantal verschillende bacteriesoorten en hun onderlinge verhoudingen. Er was sprake van een verschuiving in het voorkomen van verschillende van de belangrijkste phyla. Na antibioticumbehandeling namen Spirochaetes, Kiritimatiellaeota, Fibrobacteres en Verrucomicrobia af, en bacteriesoorten in het phylum Firmicutes namen toe. Twee weken na het stoppen van de antibioticumbehandeling en ontslag uit het paardenziekenhuis was geleidelijk herstel van de samenstelling van het darmmicrobioom zichtbaar. Echter, sommige grotere phyla, zoals Spirochaetes, Verrucomicrobia, Kiritimatiellaeota en Cyanobacteriën, waren zes maanden later nog steeds niet op hetzelfde niveau als voor de antibioticumbehandeling en de opname in het paardenziekenhuis. Naast effecten op het darmmicrobioom, had de antibioticumbehandeling ook een grote invloed op het resistoom. De concentratie van antibioticumresistentiegenen in de mest nam binnen 24 uur na de start van de TMPS-behandeling sterk toe. Genen die coderen voor resistentie tegen TMPS, zoals sulfonamide-resistentiegenen, maar ook genen die coderen voor resistentie tegen andere typen antibiotica, zoals tetracycline- en aminoglycoside-resistentiegenen, namen toe. Dit kan mogelijk verklaard worden door co-selectie vanwege de aanwezigheid van meerdere antibioticumresistentiegenen op mobiele genetische elementen zoals plasmiden. Sulfonamide- en tetracycline resistentiegenen werden zes maanden na opname in het paardenziekenhuis en TMPS-behandeling nog steeds in significant verhoogde mate uitgescheiden via de faeces, ondanks het feit dat de samenstelling van het darmmicrobioom grotendeels teruggekeerd was naar de samenstelling van vóór de behandeling. Dit is mogelijk een gevolg van overdracht van antibioticumresistentiegenen tussen verschillende soorten bacteriën of van een verschuiving van gevoelige naar resistente stammen binnen bacteriesoorten die in de darm aanwezig blijven nadat de antibioticumbehandeling is gestopt. Vijf dagen TMPS-behandeling resulteerde dus in een zeer langdurige toename in uitscheiding van antibioticumresistentiegenen via de faeces in de pony's in onze studie. Paarden zijn daarom een potentieel reservoir van resistente bacteriën. Vanwege de eenvoudige verspreiding tussen ecologische compartimenten is dit niet alleen relevant voor paarden zelf, maar ook voor mensen, andere dieren en hun gedeelde omgeving (One Health perspectief).

Antimicrobial stewardship betreft het zorgvuldig afwegen van de gevolgen van het gebruik van antibiotica ten opzichte van de noodzaak om een klinische infectie te behandelen. Dezelfde principes zijn van toepassing in de geneeskunde en de diergeneeskunde (inclusief de paardengeneeskunde). In **Hoofdstuk 8** worden praktische richtlijnen voor antimicrobial stewardship in de paardengeneeskunde gepresenteerd. Antimicrobial stewardship vereist een aanpak die verschillende componenten combineert, zoals vermindering van resistentie-reservoirs, verbeterde klinische diagnostiek van (bacteriële) infecties, verbeterde maatregelen om infecties te beheersen,

preventieve gezondheidsmaatregelen, monitoring van resultaten van kweek- en gevoeligheidsbepalingen en monitoring van antibioticumgebruik, voorlichting/ bewustwording van antibioticumresistentie en verbeterde communicatie binnen het behandelteam van een patiënt. De implementatie van antimicrobial stewardship programma's in gezelschapsdierenpraktijken en ziekenhuizen zijn effectief gebleken en dragen bij aan kostenreductie van de gezondheidszorg. Er bestaat geen 'one-size-fits-all' antimicrobial stewardship programma. De implementatie van enkele specifieke actiepunten binnen de lijst aan mogelijkheden kan helpen om voor dierenartsen (en artsen) de drempel om deel te nemen te verlagen en om de naleving van antimicrobial stewardship beleid te verbeteren. Op die manier kan geleidelijk de culturele verandering tot stand gebracht worden die noodzakelijk is voor een effectieve implementatie en verdere uitbreiding van antimicrobial stewardship. Systematische monitoring van antibioticumresistentie en het antibioticumgebruik binnen een veterinaire praktijk kunnen worden gebruikt als instrumenten om de effectiviteit van een antimicrobial stewardship programma te evalueren. Het ontwikkelen en invoeren van een antimicrobial stewardship strategie is belangrijk voor alle paardenpraktijken zodat dierenartsen bijdragen aan het tegengaan van antibioticumresistentie.

In **Hoofdstuk 9** worden de belangrijkste bevindingen van dit proefschrift samengevat en besproken in relatie tot eerder onderzoek. Er is meer onderzoek nodig naar het darmmicrobioom en de factoren die invloed hebben op de samenstelling ervan. Studies waarin de verspreiding van antibioticumresistentie van paarden naar andere dieren, mensen en het milieu worden onderzocht, kunnen meer informatie geven over de relevantie van het paard ten aanzien van het One Health probleem van antibioticumresistentie. Toekomstige studies naar risicofactoren voor ziekenhuisinfecties bij (neonatale) patiënten in de paardengeneeskunde zouden meer inzicht kunnen geven waarmee hygiëneprotocollen in paardenziekenhuizen verbeterd kunnen worden. Tenslotte kunnen onderzoeken die de effectiviteit van antimicrobial stewardship programma's in de paardengeneeskunde evalueren, helpen in kaart te brengen welke interventies het meest effectief zijn en prioriteit zouden moeten krijgen. Verschillende initiatieven, zoals het Quadripartite Memorandum of Understanding tussen de Food and Agriculture Organization (FAO), de World Organisation for Animal Health (WOAH/OIE), de United Nations Environment Programme (UNEP) en de World Health Organization (WHO), zijn recent gestart om antibioticumresistentie op wereldschaal te inventariseren en te bestrijden door middel van een geïntegreerde en gecoördineerde aanpak. Dit zijn veelbelovende stappen in de One Health-aanpak om antibioticumresistentie te bestrijden. Het is tijd om te focussen op antimicrobial stewardship in de geneeskunde en de diergeneeskunde. Dit laatste heeft tot nu toe veelal betrekking gehad op productiedieren, maar moet verder uitgebreid worden naar gezelschapsdieren en paarden. Om ervoor te zorgen dat de veterinaire beroepsgroep verantwoordelijkheid neemt en bijdraagt aan de wereldwijde strijd tegen antibioticumresistentie, zouden alle dierenartspraktijken een strategie voor antimicrobial stewardship moeten ontwikkelen en implementeren.

List of publications

LIST OF PUBLICATIONS

Related to this thesis

Theelen MJP, Luiken REC, Wagenaar JA, Sloet van Oldruitenborgh-Oosterbaan MM, Rossen JWA, Schaafstra FJWC, Van Doorn DA & Zomer AL. *Longitudinal study of the short- and long-term effects of hospitalization and oral trimethoprim-sulfadiazine administration on the equine facial microbiome and resistome.* Submitted.

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Dankwoord

DANKWOORD

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Mathijs

About the author

ABOUT THE AUTHOR

Mathijs Theelen was born on December 2nd 1981 in Venray, the Netherlands. He finished his secondary school at Valuas College in Venlo in 2000. Subsequently he started studying Veterinary Medicine at Utrecht University. Mathijs was actively involved in several extra-curricular activities, including one year as board member of the veterinary student association (D.S.K.). As part of his studies, he performed a research externship at the Department of Medicine and Epidemiology, School of Veterinary Medicine at the University of California in Davis, USA. Under supervision of Prof. David Wilson, he studied bacteria causing foal sepsis. In 2007, Mathijs received his degree in veterinary medicine (with distinction). After his graduation, he returned to UC Davis for several months to continue to work on the collaborative research project as Junior Research Specialist. After his return to the Netherlands, he started working as an equine veterinarian at Lingehoeve Diergeneeskunde in Lienden. To pursue his dream of becoming a Specialist in Equine Internal Medicine, Mathijs returned to Utrecht University in 2009 for an internship, followed by a residency. In 2014 he passed his exams and became a board-certified member of the European College of Equine Internal Medicine (Dipl.ECEIM). Mathijs continued to work in the equine hospital at Utrecht University as an equine internist focusing on neonatology, hepatology and gastro-intestinal diseases and he also obtained his basic university teaching qualification (BKO). In 2016 he became coordinator of the equine neonatal intensive care unit (NICU). Besides his clinical work, he also started a part-time PhD in 2014 building on his previous work regarding foal sepsis. His PhD project was a collaboration between the Department of Clinical Sciences (Equine Internal Medicine) and the Department of Infectious Diseases and Immunology (Clinical Infectiology). His supervisors were Prof. dr. Marianne Sloet van Oldruitenborgh-Oosterbaan, Prof. dr. Jaap Wagenaar, Dr. Aldert Zomer and Dr. Roosmarijn Luiken. The two main objectives of his PhD project were, first, to study antimicrobial susceptibility and emergence of resistance in bacteria isolated from foals with sepsis in order to provide guidance to clinicians in selecting antimicrobial drugs for treatment and second, to evaluate the role of the intestinal microbiome and resistome as a reservoir of antimicrobial resistance. In 2016 Mathijs received an Utrecht University Short Stay PhD Grant, which enabled him to visit UC Davis again as part of his PhD. The results of all studies included in his PhD are described in this thesis.

Mathijs regularly speaks at national and international conferences. He is currently also Member of the Equine Health Advisory Committee of the Morris Animal Foundation, Chair of the Advanced Training Advisory Committee of the European College of Equine Internal Medicine (ECEIM), Member of the sounding board for equine veterinarians of the Royal Dutch Veterinary Association (KNMvD) and Member of the Equality, Diversity and Inclusion Committee of the Faculty of Veterinary Medicine.