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Choice of antimicrobial treatment for anaerobic infections with *Bacteroides* spp. in horses

Val av antibiotika vid anaeroba infektioner med *Bacteroides* spp. hos häst

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SUMMARY

The purpose of this study was to species-identify clinical isolates of genus *Bacteroides* previously submitted to the National Veterinary Institute (SVA) and to determine their antimicrobial susceptibility to penicillin and other antimicrobials commonly used in horses in Sweden. The 29 isolates included in the study were identified as *Bacteroides fragilis* (n=10), *Bacteroides heparinolyticus* (n=7), *Bacteroides ovatus* (n=2), *Bacteroides thetaiotaomicron* (n=2), *Bacteroides pyogenes* (n=1), *Parabacteroides distasonis* (n=2), *Prevotella* sp. (n=2), *Prevotella dentasini* (n=1), *Porphyromonas* sp. (n=1) and *Fusobacterium* sp. (n=1). Identification of *Bacteroides* spp. using matrix-assisted-laser-desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) has proved to be a time and cost effective method for species-identification of bacteria. *Bacteroides fragilis, B. ovatus, B. thetaiotaomicron and P. distasonis* all showed resistance against penicillin. The only *Bacteroides* spp. showing susceptibility to penicillin was *B. heparinolyticus* and *B. pyogenes. Bacteroides fragilis* showed resistance to many other antimicrobials used in horses such as ampicillin, gentamicin, ceftiofur erythromycin and trimethoprim/sulphamethoxazole. Few antimicrobials available for treatment of horses in Sweden proved effective against *B. fragilis.* Unfortunately it was not possible to test the isolates for susceptibility towards metronidazole; however, internationally, metronidazole resistance is extremely rare even in human isolates, and it would not be expected to find resistant isolates from horses in Sweden.

SAMMANFATTNING

Syftet med denna studie var att artidentifiera kliniska isolat av genus *Bacteroides* som tidigare har skickats till Statens Veterinärmedicinska Anstalt (SVA) och testa deras känslighet för antibiotika som används till häst i Sverige som till exempel penicillin. De 29 isolaten som inkluderades i denna studie identifierades som *Bacteroides fragilis* (n=10), *Bacteroides heparinolyticus* (n=7), *Bacteroides ovatus* (n=2), *Bacteroides thetaiotaomicron* (n=2), *Bacteroides pyogenes* (n=1), *Parabacteroides distasonis* (n=2), *Prevotella* sp. (n=2), *Prevotella dentasini* (n=1), *Porphyromonas* sp. (n=1) och *Fusobacterium* sp. (n=1). Identifiering med matrix-assisted-laser-desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) visade sig vara en snabb och säker metod för artidentifiering av bakterier. *Bacteroides fragilis, B. ovatus, B.thetaiotaomicron* och *P. distasonis* var alla resistenta mot penicillin. Endast *B. heparinolyticus* och *B. pyogenes* var känsliga för penicillin av alla *Bacteroides*-arter. *Bacteroides fragilis* uppvisade resistens mot många andra antibiotika som ingick i studien. *Bacteroides fragilis* var resistant mot ampicillin, gentamicin, ceftiofur, erythromycin och trimetoprim/sulfametoxazol. Få antibiotika tillgängliga på den svenska marknaden var effektiva mot *B. fragilis.* Tyvärr var det ej möjligt att testa isolaten för känslighet mot metronidazol. Internationell litteratur rapporterar dock en väldigt låg frekvens av metronidazol-resistens för isolat från djur och människor, och det är osannolikt att isolat av *Bacteroides* spp. från häst i Sverige är resistenta.

CONTENT

INTRODUCTION

Bacteria of the genus *Bacteroides* are important pathogens involved in many different infections in horses. It is critical that these horses are treated with appropriate antimicrobial therapy. The current guidelines for the use of antimicrobials in horses in Sweden recommend the use of penicillin as a primary antimicrobial in most infectious processes. However, it is not clear to which extent penicillin-resistant *Bacteroides* spp. exist in Sweden. In the literature it is possible to identify *Bacteroides fragilis* as highly resistant to penicillin, whilst other members of the genus would appear to be more susceptible to penicillin. In human literature it is possible to identify an increasing trend in resistance to antimicrobials within the genus *Bacteroides*. Therefore it is important to know which species of *Bacteroides* that is involved in the infection to determine the most appropriate antimicrobial agent.

Unfortunately antimicrobial susceptibility results for clinical samples of *Bacteroides* spp. sent to the National Veterinary Institute are not available, as the testing is not performed. Because of this, it is possible that horses are treated with antimicrobials with little or no effect against these pathogens. Currently the laboratory only identifies the clinical isolates of *Bacteroides* spp. to genus level, and species level identification is unavailable for these samples. However, with new emerging techniques for identification of isolated organisms it would be possible to species- identify these pathogens. The purpose of this study was to determine the antimicrobial susceptibility of clinical isolates of previously identified *Bacteroides* spp. and to species identify these isolates using matrix-assisted-laser-desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) or 16S rRNA gene sequencing. This is important as it needs to be determined to which extent penicillin-resistant *Bacteroides* spp. exist in clinical isolates from horses. This was done in order to gain more understanding of the *Bacteroides* spp. involved in equine infections and to suggest treatment regimens for these infections.

LITTERATURE REVIEW

Genus Bacteroides

Bacteria of the genus *Bacteroides* are anaerobic, Gram-negative and non-spore forming rods. The *Bacteroides* genus has undergone several recent taxonomic changes and organisms formerly known as *Bacteroides* have now been moved into several other genera including *Parabacteroides, Prevotella, Porphyromonas* and *Tannerella*. (Jousimies-Somer *et al.*,2003; Sakamoto & Benno, 2006; Sakamoto *et al.*, 2002, Shah & Collins, 1988; 1989; 1990; Wexler, 2007). This might cause confusion but is important to both clinicians and microbiologists as the taxonomic placement can be an indicator of potential virulence and antimicrobial resistance (Wexler, 2007).

Much research has been done on *Bacteroides* spp. in humans, but by comparison little has been done in animals and clinical cases involving *Bacteroides* spp. They are however an important part of infections in animals and according to a recent study make up about 8% of anaerobic infections in horses, 11% in dogs and 24% in cats (Lawhon *et al.*, 2013). Earlier reports state that *Bacteroides* spp. make up 24% of anaerobic infections in dogs and 22% in

cats (Jang *et al.,* 1997). An older study establishes that *Bacteroides* spp. make up about 44% of all anaerobic isolates (Hirsh *et al.,* 1985, Jang *et al.,* 1991) which reflects the results of an even older study that found *Bacteroides* spp. in 46% of clinical anaerobic isolates from animals (Hirsh *et al.,* 1979). However, it is important to note that since then the species included in the *Bacteroides* genus has changed dramatically which might explain why the more recent data suggest a lower percentage involvement of *Bacteroides* spp. It is also important to note that when looking at more specific categories of disease involving *Bacteroides* spp. the percentage of infections involving the genus is higher. For example, in lower respiratory tract infections in horses research shows that 26% of all samples cultured are positive for anaerobic growth (Hirsh *et al.,* 1985).

Bacteroides spp. are part of the normal flora of the gastrointestinal tract in both humans and animals and they are also part of the normal flora of the mouth, urogenital tract and the upper respiratory tract in humans (Jousimies-Somer *et al.,* 2003). Fecal isolates from the gut flora in humans is comprised of about 30% of bacteria belonging to the genus *Bacteroides* (Salyers, 1984) and the genus is the most commonly isolated of the anaerobic bacteria in clinical samples with *Bacteroides fragilis* being the most commonly isolated pathogen (Jousimies-Somer *et al.,*2003; Nagy *et al.,* 2010; Polk & Kasper, 1977). One study looking at the bovine intestinal flora found that 17% was *Bacteroides* spp. (Wagner *et al.,* 2011). *Bacteroides fragilis* is considered the most virulent of the *Bacteroides* spp. as the number of isolates of *B. fragilis* is 10 to 100-fold less than other *Bacteroides* spp. isolates in the gut, yet in humans it is the most frequently isolated from clinical specimens (Wexler, 2007). In animals *B. fragilis* used to be isolated very infrequently (Kimsey & Hirsh 1978, Hirsh *et al.,* 1979). However, with better methods for culturing of anaerobic bacteria there has been an increase in the isolation of *B. fragilis* from animals (Hirsh *et al.,* 1985).

Virulence

The genus *Bacteroides* is important because the bacteria have several mechanisms which make them well suited to turn from a commensal to a pathogen. *Bacteroides fragilis* in particular have fimbriae and agglutinins that function as adhesins, which allow them to become established in host tissues where you will normally not find *Bacteroides* (Wexler, 2007). They possess various enzymes that protect the bacteria from the host's immune system and immune response and it has been shown that after interaction with *B. fragilis* macrophages will show decreased nitric oxide (NO) production. Macrophages produce NO as an important early immune response which normally has a microbiocidal effect on bacteria (Vieira *et al.,* 2005). This allows *B. fragilis* to effectively evade the host's immune system. *Bacteroides fragilis* also possess a capsule with structural polysaccharides which makes it highly capable of inducing abscess formation as a lone infecting organism (Tzianabos *et al.,* 1993). *Bacteroides fragilis* also produce enzymes which can cause tissue destruction. These are histolytic enzymes, some of which attack the host's extracellular matrix (Rudek & Haque 1976). Some of the proteases produced by *B. fragilis* have been implicated in destroying the brush border enzymes required for digestion of food and required for selective absorption of nutrients. It has also been shown that *B. fragilis* produce an enterotoxin which may cause

diarrhea by destroying tight junctions in intestinal epithelium (Wu *et al.,* 1998, Almeida *et al.,* 2007). These potent virulence factors contribute to *B. fragilis* being the most commonly isolated of the anaerobic pathogens (Polk & Kasper, 1977).

To illustrate the virulence of *B. fragilis* it has been noted in humans that the mortality of infections involving *B. fragilis* is more than 19%, and if an infection with *B. fragilis* is left untreated the mortality rate is about 60% (Goldstein, 1996).

Antimicrobial susceptibility

Internationally there are few recent reports on antimicrobial susceptibility in *Bacteroides* spp. in animals and most treatment decisions are based on older studies. The few recent reports would suggest however, that *Bacteroides* show resistance to many antimicrobials used in horses, cattle, dogs and cats (Lawhon *et al.,* 2013, Wagner *et al.,* 2011, Almeida *et al.,* 2007**)** and studies conducted in humans echo the same results (Nagy *et al.,* 2010; Ulger-Toprak *et al.,* 2004**).** Resistance to penicillin, ampicillin, amoxicillin, ceftiofur and cefoxitin is not uncommon.

Frequently, susceptibility patterns of anaerobes isolated from humans are used in veterinary practice since limited data is available for animals. However, this might not be optimal as susceptibility patterns of anaerobes in animals might be different to those in humans as the antimicrobial use differs between the two groups. Both the frequency of which an antimicrobial is used and which antimicrobials are used differs between countries and species. Care needs to be taken interpreting susceptibility data for *Bacteroides* spp., as the species are usually grouped into their genus when results are published. However, the antimicrobial susceptibility varies within the group and data displayed in this fashion can be misleading to clinicians and can cause confusion when making decisions of antimicrobial therapy. Also, general statements are commonly made that the anaerobes are susceptible to penicillin. This is true for many other anaerobes which generally are susceptible, but is not suitable when discussing *Bacteroides* spp.

Studies on isolates from human patients demonstrate a high resistance to different antimicrobials in *Bacteroides* spp. and treatment needs to be selected based on the outcome of susceptibility tests. The medical community is seeing a rapid rise in resistance to many antimicrobials to which *Bacteroides* spp. were previously sensitive such as ampicillin, amoxicillin/clavulanic acid, cefoxitin and clindamycin (Nagy *et al.,* 2010). Resistance to penicillin is almost universal in *Bacteroides* spp. isolated from humans (Franklin *et al.,* 2006; Aldridge *et al.,* 2001; Nagy *et al.,* 2010; Ulger-Toprak *et al.,* 2004). Isolates of *Bacteroides* spp. from different animal species show similar resistance to antimicrobials as those from humans, but the frequency at which resistant strains are encountered is lower (Franklin *et al.,* 2006). It is of importance to note that many strains of *Bacteroides* spp. produce β-lactamases and would therefore make them highly unsuitable candidates for treatment with penicillin, ampicillin and most of the cephalosporins (Ulger-Toprak *et al.,* 2004; Jousimies-Somer *et al.,* 2003). High resistance has been noted to these substances in both human and animal

literature. One study found β-lactamase production in up to 99% of tested isolates from humans (Ulger-Toprak *et al.,* 2004). It has been noted by Hirsh *et al.* even as early as 1985 that members of the *Bacteroides* genus isolated from animals show high resistance to penicillin, ampicillin and cephalothin. *Bacteroides fragilis* made up 68% of the resistant isolates in that study. The same study also noted that one third of penicillin-resistant *Bacteroides* also showed resistance to tetracycline. It is also known that members of the genus *Bacteroides* can be resistant to β-lactam antimicrobials even without β-lactamase production. These strains have alternate means of resistance such as alteration of their penicillin binding proteins (PBPs) or by having an altered permeability to β-lactams (Fang *et al.,* 2002; Hedberg *et al,*. 1997). One study looking at bovine intestinal bacteria has shown that the antimicrobials ceftiofur and ceftriaxone are degraded in the intestine by several different bacteria, and that up to 96% of intestinal organisms inactivate ceftiofur to some extent (Wagner *et al.,* 2011). The same study showed that *Bacteroides* spp. as a group was the second most efficient at ceftiofur degradation.

Bacteroides spp. are still showing very low frequency of resistance to antimicrobials such as imipenem, metronidazole, chloramphenicol and tigecycline (Nagy *et al.,* 2010; Almeida *et al.,* 2007; Aldridge *et al.,* 1994). Resistance to piperacillin/tazobactam is still low, but would appear to be increasing (Aldridge *et al.,* 2001; Nagy *et al.,* 2010).

Susceptibility testing of *Bacteroides* spp. is not routinely performed at the National Veterinary Institute (SVA), which leaves out a vital piece of information for the clinician. Isolates recognized as *Bacteroides* spp. are not identified further to species-level since there have not been methods readily available to perform this. Knowledge about antimicrobial susceptibility is important in the decision-making in determining the most appropriate antimicrobial treatment in clinical cases. This is true for any bacterial infection, but very important in infections with bacteria that are known to be resistant to many antimicrobials. In the clinical setting, choice of antimicrobial treatment is decided before results of culture and susceptibility is known. Knowledge of the susceptibilities of the most common microbes is therefore central to a correct choice of antimicrobial before the final result of the susceptibility test. For example it has been noted that *Bacteroides thetaiotaomicron* displays resistance to more antimicrobials than *B. fragilis* (Wexler 2007; Ulger-Toprak *et al.,* 2004).

When considering which antimicrobial to use for a patient, the veterinarian not only needs to consider which would be the most appropriate for the isolated organism, but also if it is possible to reach the levels required for the antimicrobial to have its effect. The veterinarian also needs to consider if the antimicrobial to be used is time-dependent or concentration-dependent to ensure correct dosage regimens. For antimicrobials that are concentration-dependent the aim would be to achieve a high plasma concentration relative to the minimum inhibitory concentration (MIC) whereas if the antimicrobial is time-dependent the objective is to keep the concentration above MIC for the duration of the dosage intervals (Giguère 2013). There are a few drugs which are both time and concentration-dependent, but they are used in extremely rare cases of horses in Sweden.

When deciding which antimicrobial to use it is also important to consider the clinical MIC breakpoint of the antimicrobial against a specific organism in the target location in the horse. Many clinical breakpoints are derived from human literature and for other organs or tissues in the body. These breakpoints might not be transferrable to horses or to the target site of infection. Clinical breakpoints are relevant only for the specific drug in a specific organ system against a specific bacterium (Giguère & Afonso, 2013). However, not all of this information is available in the horse as less research has been done in horses, and often veterinary clinicians have to use clinical breakpoints set in humans when evaluating which antimicrobial to use. Often used clinical breakpoints are those from the Clinical and Laboratory Standards Institute (CLSI) and EUCAST (European Committee on Antimicrobial Susceptibility Testing).

In Sweden frequently used antimicrobials in horses include penicillin, gentamicin, and trimethoprim-sulfonamide combination. Other antimicrobials used in horses include tetracycline, ceftiofur and metronidazole. Rifampicin is used in foals with *Rhodococcus equi* infections. Penicillin, tetracycline, trimethoprim-sulfonamide, and ceftiofur are all time-dependent and frequent administrations are required to keep the concentration above MIC (Giguère, 2013). Gentamicin and metronidazole are concentration-dependent, and it is not necessary to keep the concentration of the drug above MIC between doses (Giguère, 2013). Many other antimicrobials are not readily available for use in horses and many are restricted by law for use in animals as they are reserved for severe cases of disease in humans (Statens Jordbruksverks Författningssamling, SJVFS 2012:32). Some antimicrobials that exist in an appropriate solution abroad do not exist in Sweden such as chloramphenicol which is only available in ophthalmic solutions.

It has been suggested that a combination of penicillin against Gram-positive and anaerobic bacteria together with gentamicin against Gram-negative bacteria give good coverage in severe bacterial infections in horses until results of culture and susceptibility testing is received (Giguère & Afonso, 2013).

Bacteroides spp. in horses

To the author's knowledge, there are no studies on antimicrobial susceptibility of *Bacteroides* spp. isolated from horses in Sweden. The current consensus in Sweden is to treat horses with infections involving *Bacteroides* spp. with penicillin as a first choice antimicrobial (Sveriges Veterinärmedicinska Sällskap, 2013) as it is not clear to which extent penicillin resistant *B. fragilis* occur in Sweden.

Since *Bacteroides* spp. are part of the normal anaerobic flora in the horse and because of its easy transition from commensal to pathogen it is not surprising that it is isolated from clinical cases in horses. However, the true frequency of infections involving *Bacteroides* spp. might be higher as frequently no anaerobic culture will be requested as it means added costs for the client. It also demands that the clinician knows the proper technique for anaerobic sampling of the affected area. There are few international studies done on *Bacteroides* spp. and

antimicrobial susceptibility exclusively from horses, and case reports are few and far between. Examples of infections involving *Bacteroides* spp. in horses are paraoral and lower respiratory tract infections (Bailey & Love, 1991; Sweeney *et al.,* 1985; 1991a; Mair & Lane, 1989), endometritis (Hariharan, 1994), abscesses (Spiers *et al.*,1986; Jang & Hirsh, 1991)**,** peritonitis (Sweeney *et al.,* 1991b; Mair *et al.,* 1990) diarrhea in foals (Myers *et al.,* 1987) and wounds (Lawhon *et al.,* 2013; Jang & Hirsh, 1991)**.** But there are also examples of more uncommon occurrences of infections with *Bacteroides* spp. such as keratitis (Johns, 2009).

Infections involving the respiratory tract

It has been shown in horses that bacteria involved in lower respiratory tract (LRT) infections and paraoral infections most likely come from the bacterial flora from the oral cavity (Bailey & Love, 1991). The same species were isolated from the normal pharyngeal tonsillar surfaces and from LRT and paraoral infections. It was shown that *Bacteroides* spp. comprised about 50% of the isolates from normal pharyngeal tonsillar surface samples and about 21% of anaerobic isolates from LRT and paraoral infections, with LRT infections having a slightly higher percentage at 30%.

These results are similar to what Sweeney *et al.* (1991a) reported which is that out of all the anaerobic bacteria isolated, *Bacteroides* spp. made up 20.2% of anaerobes found in clinical cases of pneumonia and pleuropneumonia. The same study also reported that strictly anaerobic infections made up about 2.0% of pleuropneumonia or pneumonia cases. But anaerobes in mixed infections with aerobes represent about 24.8% of cases. In a review of 45 cases of pneumonia and lung abscesses, 6 of 11 cases of primary pneumonia and lung abscess cases were tested for anaerobes. Three of the six (50%) samples resulted in anaerobic isolates and they all contained at least one *Bacteroides* sp. (Mair & Lane 1989)

There was also a poorer prognosis for horses with anaerobic or mixed infections with survival rates of 38.3% versus horses with only aerobic isolates with a survival rate of 81.4% (Sweeney *et al.,* 1991a). In an earlier study from Sweeney *et al.* (1985) it was found that in 46% of horses with pleuropneumonia anaerobic bacteria were isolated, and *Bacteroides oralis* and *Bacteroides melaninogenicus* (now classified as *Prevotella oralis* and *Prevotella melaninogenica*) were the anaerobes most frequently isolated. The two studies show a similar survival rate for horses with an involvement of anaerobic bacteria in pleuropneumonia to be 33.3% (Sweeney *et al.,* 1985) and 38.3% (Sweeney *et al.,* 1991a) which indicates that infection with anaerobic bacteria is a serious condition and rapid correct therapy is vital.

Bacteroides *spp. in wounds and abscesses*

The anaerobic microbiology of equine wounds is poorly researched, and few reports describing the types of bacteria inhabiting equine wounds exist. However, using information from other species it is clear that anaerobes are involved and is a relevant component of these infectious processes. A recent study on resistance in obligate anaerobes from animals reports the presence of *Bacteroides* spp. in subcutaneous tissue and wounds in horses (Lawhon *et al.,* 2013). A study by Jang & Hirsh (1991) reported frequent isolation of *Bacteroides* spp. from

abscesses and wounds in horses. However, a recent study looking solely at the microbiology of equine wounds found no involvement of *Bacteroides* spp. (Westgate *et al.*, 2011). This is interesting as in humans it has been recorded that 7% of anaerobes in soft-tissue infections belong to the genus *Bacteroides* (Wexler *et al.*,, 1998), and another study concludes that *Bacteroides* was the most common organism isolated from mixed aerobic and anaerobic infections (Elliot *et al*., 2000). It is also interesting to note that *Bacteroides* spp. is very frequently involved in abscesses from fight-wounds in cats. One study found involvement of *Bacteroides* spp. in 44.5% of subcutaneous abscesses (Love *et al.*, 1989). Love *et al*. (1989) draws the conclusion that the species involved in subcutaneous fight-wound abscesses in cats originate from the flora of the mouth. Interestingly, they noted a virtual absence of *B. fragilis* in these abscesses. The most commonly isolated species in this study was *B. tectum, B. salivosus, B. gingivalis* and *B. heparinolyticus.*

Anaerobic infections should always be considered when aerobic cultures are negative. This is especially true for cases of osteomyelitis, drainage tracts and abscess formation. But both anaerobic and aerobic cultures should always be submitted (Spurlock & Hanie, 1989)

Bacteroides *spp. in cases of peritonitis*

Obligate anaerobic bacteria are isolated frequently from peritoneal cavities. *Bacteroides* spp. is the anaerobic genus isolated most often and it has been noted that *B. fragilis* can be involved in approximately 10-20% of positive cases (Hirsh & Jang 1987). In dogs and cats it has been noted that anaerobes are involved in 31% and 40% of cases of peritonitis respectively (Jang *et al.,* 1997). The most commonly isolated species in this study included *Bacteroides* spp.

Because many different species are involved in cases of peritonitis in the horse, it is crucial that correct culturing and susceptibility testing is performed to ensure correct antimicrobial therapy. Treatment in cases with peritonitis poses as problem as the infection is in the transcellular space and it can be difficult to get the concentration of antimicrobials high enough. However, Sweeney *et al. (*1986) showed that it is possible to achieve even higher levels of metronidazole in the peritoneal fluid than in serum, making it an ideal antimicrobial to treat horses with peritonitis involving anaerobic organism such as *Bacteroides* spp.

In humans, the most common infection caused by *Bacteroides* spp. is intra-abdominal sepsis. A disruption of the intestinal wall caused by surgery or other malignancies such as traumatic injury or appendicitis, cause the flora of the gut to invade the sterile environment of the peritoneal cavity. *Bacteroides* spp. predominates in the second more chronic stage of infection, when sufficient oxygen has been removed (Wexler, 2007). Another study which looks at the microbiology of the peritoneal cavity in children after a perforated appendix found that *Bacteroides* spp. was recovered in 93% of cases (Brook, 2003).

MALDI TOF MS

Identifying *Bacteroides* spp. from a clinical sample takes time as it needs to grow at least 48 hours before any kind of identification can be made. Most likely, another 48 hours is required

for identification of samples that are not in pure culture. After that, more work is needed for phenotypic identification. This is time consuming and high in cost. A new and quickly emerging way of identification of bacteria is by using matrix-assisted-laser-desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). With this method minimum sample preparation is necessary. Material from a single colony is taken and spotted onto a ground-steel MALDI target plate which is then introduced into the MALDI-TOF MS. Results take less than 1 minute to process per spot on the plate. This can be done from the primary culture and several colonies with different characteristics can be run simultaneously (Wieser *et al.,* 2012). In human medicine, it is now possible to get results from blood-cultures within hours after the blood culture bottle is positive for growth through acquiring material by centrifuging liquid from the bottle, extract and purify the bacterial proteins and using this sample to spot onto the MALDI plate (Reviewed by Wieser *et al.,* 2012).

MALDI-TOF MS works by exposing each fixed sample to multiple pulsing laser beams that ionizes part of the sample. The ions travel to a detector through a flight tube. At the detector, differences in mass-to-ionic charge ratios cause the ions to separate. The results are collected electronically in spectral channels and converted into a mass value that is displayed as a spectrum. This spectrum is then compared to a library of spectrums in a software program (Reviewed by Wieser *et al.,* 2012).

MATERIALS AND METHODS

Bacterial isolates

A total of 31 bacterial isolates from clinical submissions between the years 2006-2013 were used in this study. The isolates were determined to be of genus *Bacteroides* by different phenotypical methods and frozen at the National Veterinary Institute (SVA) in Uppsala, Sweden at the time of submission. However the isolates were not species-identified as no method for this was available at the laboratory. All isolates were from equine patients from different clinics and hospitals around Sweden. The sources of the samples were wounds (n=7), peritoneal fluid (n=3), abscesses (n=7), aspirates from lungs and sinuses (n=7), fecal samples (n=2), one uterine sample (n=1), blood (n=1), synovial fluid (n=2) and unknown sample sites (n=1). The type strain *B. fragilis* ATCC 25285 was included as a control due to its known susceptibility pattern.

Culturing and identification

The isolates were cultured on fastidious anaerobe agar (FAA) supplemented with 10% horse blood (SVA, Uppsala, Sweden**)**. The samples were incubated for 48 h in 37°C in a 2.5 liter anaerobic jar. The anaerobic environment was created using Oxoid AnaeroGen sachets (Oxoid Ltd, Basingstoke, UK). The samples were then recultured twice on FAA and incubated in 37°C for 48 h to ensure vitality of the bacteria.

All isolates were analyzed by MALDI –TOF MS (Bruker Daltonik GmbH, Bremen, Germany) to identify the species. Mass spectra were compared against the 4613 spectra in the

MALDI Biotyper database using the MALDI Biotyper 3.0 Realtime Classification (RTC) software (Bruker Daltonik GmbH, Bremen, Germany).

Material from a single colony from FAA was spotted on a MALDI-plate without pretreatment. The spots were then covered with 1µl matrix solution consisting of α-cyano-4-hydroxycinnamic acid (HCCA) and left to air-dry in room temperature. The plate was then introduced into the MALDI-TOF mass spectrometer for analysis. The spectra of all isolates were compared to the spectra in the database and identification was provided with a score of reliability. A score <1.7 is considered an unreliable identification. A score ≥1.7 and <2.0 is considered a genus identification, however it is also considered an unreliable identification. Scores ≥2.0 were considered species-level identification. In those samples where MALDI-TOF MS could not provide reliable results, or could not provide any results because the spectra did not exist in the database, the identification of the samples was carried out using 16S rRNA gene sequencing described below.

PCR

All isolates to be 16S rRNA gene sequenced were prepared as boiled lysates. A 1µl plastic loop of colony material was washed in 200µl of phosphate buffered saline (PBS). The solution was vortexed until homogenous. It was then centrifuged for 5 min at 13,000 rpm. The liquid phase was discarded and the pellet dissolved in 200µl of new PBS-buffer. The solution was again centrifuged for 5 min at 13,000 rpm. After the liquid phase was discarded, 50 µl of DNase and RNase free water (Sigma Aldrich Company Ltd, Irvine, UK**)** was added to the pellet and vortexed. The solution was boiled in a heating block (98°C) for 10 min and then immediately cooled on ice for 10 min. The solution was then centrifuged for 10 minutes at 13,000 rpm. The supernatant was transferred to a clean tube and the pellet discarded. As template for the PCR a 1/10 dilution of the supernatant was made.

The PCR was performed using primers Bac27F (5'-AGAGTTTGGATCMTGGCTCAG-3') and Univ1492R (5'-CGGTTACCTTGTTACGACTT-3') (Jiang *et al.,* 2006). A mastermix consisting of 21µl of DNase and RNase free water, 25 µl of Qiagen HotStarTaq® Master Mix (Qiagen, Hilden, Germany), 1 µl Bac27F primer and 1 µl Univ1492R primer was added to 2 µl of template for a total volume of 50 µl. 16S rRNA gene amplification was then carried out as follows: initial heat-activation of the HotStar Taq® Master mix for 15 min at 95°C, 30 cycles of denaturing (30 s at 95°C), annealing (30 s at 55°C), and extension (90 s at 72°C); and a final extension at 72°C for 10 min. To verify that the PCR was successful, gel-electrophoresis in a 1.5% agarose gel was performed.

16S r RNA gene sequencing

Before sequencing the PCR product was treated enzymatically using FastAP™ Thermosensitive Alkaline Phosphatase (Thermo Scientific, Waltham, USA**)** and Exonuclease I *E.coli* (Thermo Scientific, Waltham, USA**)** according to the manufacturer's description. The phosphatase inactivates unincorporated nucleotides. The nuclease digests single-stranded DNA. In our samples that means A-overhang on the PCR products and left-over primers. The

samples were loaded into the 2720 Thermal cycler (Applied Biosystems, Foster City, USA), heated to 37°C for optimal enzymatic activity for 15 minutes and then increased to 80°C for 15 minutes to denature and thereby inactivate the enzymes.

For the sequencing reaction the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, USA) was used. Two master-mixes was prepared for each template by taking 2 µl of Terminator ready reaction mix (v3.1) and adding 1 µl of BigDye Sequencing buffer and 1 µl of either the Bac27F primer or the Univ1492R primer, each at a concentration of 10µM. Between 1-4 µl of each template was then added to their Master-mix depending on the concentration from the first PCR, interpreted by reading the intensity of the bands on (the) gel-electrophoresis. The templates with the Master-mix were then run through a new PCR according to the manufacturer's description.

The PCR products from the BigDye Terminator reaction was cleaned by solidifying the DNA by sodium acetate and ethanol and centrifugation. The resulting pellet was washed once with ethanol and thereafter dried at 50°C for 3 minutes. The DNA was dissolved in formamide and sequenced using the 3100 Genetic Analyzer (Applied Biosystems, Foster City, USA) according to the manufacturer's protocol.

Antimicrobial susceptibility tests

All isolates were tested for antimicrobial susceptibility using VetMIC GP-mo (ver.2) microdilution plates (SVA, Uppsala, Sweden). The plates contain the following antimicrobials in the following ranges of concentration: penicillin (0.015-2 µg/ml), cephalothin (0.03-4 µg/ml), oxacillin +2% NaCl (0.06-8 µg/ml), erythromycin (0.125-16 µg/ml), chloramphenicol (0.25-32 µg/ml), clindamycin (0.125-16 µg/ml), tetracycline (0.25-32 µg/ml), fusidic acid (0.03-4 µg/ml), gentamicin (0.25-32 µg/ml), kanamycin (0.125-16 µg/ml), ciprofloxacin (0.03-2 µg/ml), trimethoprim (0.25-16 µg/ml). The plate also contains a control well with a citrate buffer. No growth in this well will imply a sensitivity of the strain to the citric acid which is included in the buffer used in the wells with penicillin. In these cases reading the penicillin sensitivity is not possible. For the control of growth one well with only the bacterial suspension was included. The panel is not exclusively designed for isolates from horses and contains antimicrobials not intended for use in horses such as clindamycin. It also contains antimicrobials not readily available in suitable formulations in Sweden for use in horses such as chloramphenicol. However this panel was the best choice for this study because of the long penicillin range included.

All but one (that was obtained late in the study) of the isolates identified as *Bacteroides fragilis* were because of resistance to many of the substances in the GP-mo panel tested on two additional panels, VetMIC Stordjur (SVA, Uppsala, Sweden) and VetMIC CLIN GN (SVA, Uppsala, Sweden). The VetMIC Stordjur panel is designed for bacteria isolated from horses but also from other animals such as cattle and pigs and therefore contains antimicrobials not used in horses in Sweden. The VetMIC CLIN GN is designed for use with Gram-negative bacteria and also contains antimicrobials not used in horses in Sweden. The

VetMIC Stordjur panel contains the following antimicrobials in the following ranges: penicillin (0.5-0.06 µg/ml), ampicillin (0.5-4 µg/ml), ceftiofur (0.125-1 µg/ml), spiramycin (2-16 µg/ml), neomycin (2-16 µg/ml), gentamicin (1-8 µg/ml), streptomycin (2-16 µg/ml), trimethoprim/sulphamethoxazol (0.25/4.75-2/38 µg/ml), enrofloxacin (0.06-0.5 µg/ml), oxitetracycline (0.5-4 µg/ml), florfenicol (1-8 µg/ml), oxacillin (0.25-0.5 µg/ml). The VetMIC CLIN GN panel contain the following antimicrobials in the following ranges: ampicillin (1-8 µg/ml), cefotaxime (0.125-1 µg/ml), amoxicillin/clavulanic acid (2/1-16/8 µg/ml), colistin (0,5-4 µg/ml), nitrofurantoin (4-32 µg/ml), trimethoprim/sulphamethoxazole (0.25/4.75-2/38 µg/ml), gentamicin (1-8 µg/ml), streptomycin (4-32 µg/ml), neomycin (2-16 µg/ml), tetracycline (1-8 µg/ml), enrofloxacin (0.06-2 µg/ml). Both panels include a citrate buffer control and a growth control**.**

Isolates were tested according to the Clinical and Laboratory Standards Institute's guidelines for antimicrobial susceptibility testing for anaerobic bacteria (CLSI M11-A8). The following procedure was used for all three different microtitre panels. For each sample, colony material was suspended in 2 ml of Brucella broth with hemin (5µg/ml) and vitamin K_1 (1µg/ml) (SVA, Uppsala, Sweden). The turbidity of the suspensions was adjusted to be equivalent to a 0.5 McFarland solution which corresponds to a concentration of approximately $1\text{-}5x10^8$ CFU/ml. For inoculum density control viable counts of the type strain were made by taking 10 µl of the suspension and adding it to 10 ml of sterile saline and then taking 100 µl of this dilution and spreading it on an FAA plate. These plates were incubated in an anaerobic environment at 37°C for 48 h.

To get a final concentration of $1\text{-}5x10^5$ CFU per well 150 µl of the suspended colony material was added to 15 ml of Brucella broth with hemin (5µg/ml) and vitamin K_1 (1µg/ml) supplemented with 750 µl of lysed horse blood. Each well of the microtitre plate was inoculated with 100 µl of this dilution. All plates were incubated in an anaerobic environment at 37°C for 48 h before final reading. All isolates, except the one obtained late in the study, that were identified as *B. fragilis* with MALDI-TOF MS were tested as described above and additionally tested using Brucella broth stored at -20°C for 11 months. This was done to evaluate if broth could be stored frozen and then be used for antimicrobial susceptibility testing of *Bacteroides* spp.

Final reading of Minimum Inhibitory Concentration (MIC) was performed using both a BIOMIC V3 96-well microdilution plate reader (Giles Scientific, Santa Barbara, CA, USA) and by manual visual reading. The MIC was determined to be the lowest concentration of an antimicrobial agent completely preventing visible growth. To make sure the suspension used in the microdilution plates was not contaminated, a culture was made on FAA agar from the very same suspension used in the microtiter plate, and incubated in an anaerobic environment at 37°C for 48 h.

β-lactamase production

All samples were tested for β-lactamase production using Cefinase™ (CEF-F) paper discs (bioMérieux® sa, Lyon, France). These discs are impregnated with nitrocefin, a chromogenic β-lactam, which turns red when the β-lactam ring is broken by a β-lactamase. Colony material was rubbed onto a disc moistened with sterile water and a color change within 30 min was considered as positive. *Bacteroides fragilis* ATCC 25285 was used as a positive control.

Accepted breakpoints for resistance

The MICs were interpreted by using clinical breakpoints set by the CLSI (M11-A8, M100-S23), EUCAST and SVA and are outlined in table 1. For some of the antimicrobials there is no breakpoint in either CLSI or EUCAST and in these cases the breakpoints have been set using the interpretive criteria used for the VetMIC panels designed by SVA (VetMIC Stordjur, VetMIC CLIN GN and VetMIC Smådjur). VetMIC Smådjur is a microdilution plate for isolates from dogs and cats. The accepted clinical breakpoints used are adapted for *Bacteroides* spp. isolated from humans since no complete guideline for anaerobic bacteria isolated from horses exists. Not all of the tested substances have accepted breakpoints. Table 1 shows the clinical breakpoints available for the tested antimicrobials. Not all antimicrobials tested are included in this table as they are not relevant to this study.

Table 1. *Clinical MIC breakpoints for resistance for selected antimicrobials*

	CLSI	**EUCAST**	**VetMIC**
Antimicrobial	**S|I|R**	**S|R**	**S|I|R**
Penicillin	≤0.5|1|>1	≤0.25|>0.5	
Ampicillin	≤0.5|1|>1	≤0.5|>2	
Amoxicillin-Clavulanic Acid			≤8/4| - |>8/4
Erythromycin			>4
Chloramphenicol	≤8|16|>16		
Clindamycin	≤2|4|>4	< 4|>4	
Tetracycline	≤4|8|>8		
Fusidic Acid			≤4| - |>4
Gentamicin			≤4|8|>8 (NR)
Kanamycin	NR	NR	NR
Ciprofloxacin	NR	NR	NR
Trimethoprim	NV	NV	NV
Cefotaxime			≤0.25| - |>0.25
Nitrofurantoin			≤64| - |>64
Trimethoprim/sulfamethoxazole			≤0.5/9.5|1/19-4/76|>4/76
Ceftiofur			≤2| - |>2

S: Sensitive, I: Intermediate, R: Resistant,

NR: not relevant, because of limited or uncertain effect for treatment of anaerobic infections in general (see discussion)

NV: No value available for trimethoprim only

Carbapenemase-production

Strains of *B. fragilis* harboring a gene for carbapenemase production (*cfiA*) can be separated from other *B. fragilis* by their MALDI-TOF spectra (Wybo *et al.*, 2011). In addition to the antimicrobial testing, the spectra generated by the MALDI-TOF MS from isolates identified as *Bacteroides fragilis* were sent to Åsa Johansson, Centrallasarettet in Växjö, for analysis to evaluate if they belonged to the *cfiA* positive cluster.

Medical Records

All available medical records have been reviewed for diagnosis, treatment and outcome to determine if the horses were treated with antimicrobials to which the isolate was susceptible.

RESULTS

Bacterial species identification

Of the 31 isolates frozen and previously identified as *Bacteroides* spp., it was not possible to get a viable culture from 2 isolates. A total of 29 isolates were identified by MALDI-TOF MS and 16S rRNA gene sequencing. Isolates were identified as *Bacteroides* spp. (n=22) *Parabacteroides distasonis* (n=2), *Prevotella* spp. (n=3), *Fusobacterium* sp. (n=1), and *Porphyromonas* sp. (n=1). Table 2 shows the outcome of the MALDI-TOF and DNA sequencing for all isolates. A dendrogram from all spectra except one (*Fusobacterium* sp.) was created with the MALDI Biotyper software (Fig 1). It shows the relationship between the isolates. The isolates clustered together are assumed to be of the same species and not all isolates were therefore 16S rRNA sequenced. Interestingly the two *Bacteroides thetaiotaomicron* do not cluster together, and it was only possible to identify one using MALDI-TOF MS, the other was identified by 16S rRNA sequencing. Table 2 outlines the results of MALDI-TOF MS and 16S rRNA gene sequencing. Table 3 illustrates the number of each species identified.

Table 2. *Results of identification with MALDI-TOF MS and 16S rRNA gene sequencing*

Isolate	MALDI-TOF MS	Score	16S rRNA	Identification derived from dendrogram*
4528	*Bacteroides fragilis*	2.305	-	
4533	*Bacteroides fragilis*	2.283	-	
4666	*Parabacteroides distasonis*	2.383	-	
4693	No reliable identification	1.328	*Fusobacterium* sp.	
4704	Not viable	-	-	
4713	No reliable identification	1.331	*Prevotella dentasini*	
4764	No reliable identification	1.375	*Porphyromonas* spp.	
4829	*Bacteroides ovatus*	2.172	-	
4860	*Bacteroides fragilis*	2.279	-	
4928	*Bacteroides fragilis*	2.392	*Bacteroides fragilis*	
4930	*Bacteroides fragilis*	2.344	-	
4963	No reliable identification	1.317	*Prevotella* sp.	
4977	*Bacteroides fragilis*	2.293	-	
4987	*Bacteroides thetaiotaomicron*	2.263	-	
4999	No reliable identification	1.586		*Bacteroides heparinolyticus*
5039	No reliable identification	1.565	*Bacteroides heparinolyticus*	
5135	No reliable identification	1.441	*Prevotella* sp.	
5233	*Parabacteroides distasonis*	2.358	-	
5393	Not viable	-	-	
5421	No reliable identification	1.554		*Bacteroides heparinolyticus*
5677	No reliable identification	1.418	*Bacteroides heparinolyticus*	
5712	No reliable identification	1.409	*Bacteroides heparinolyticus*	
5742	*Bacteroides fragilis*	2.361	-	
5743	*Bacteroides pyogenes*	2.225	-	
5745	No reliable identification	1.359	*Bacteroides thetaiotaomicron*	
5749	No reliable identification	1.443		*Bacteroides heparinolyticus*
5794	*Bacteroides fragilis*	2.4	-	
5817	*Bacteroides fragilis*	2.358	-	
5827	*Bacteroides ovatus*	2.221	-	
5992	No reliable identification	1.47	*Bacteroides heparinolyticus*	
88957	*Bacteroides fragilis*	2.301	-	
ATCC 25285	*Bacteroides fragilis*	2.376	-	

*These isolates have been identified with the help of the dendrogram (Fig. 1). Isolates clustering together in the dendrogram are assumed to be of the same species as their spectra produced by the MALDI-TOF MS are very similar.

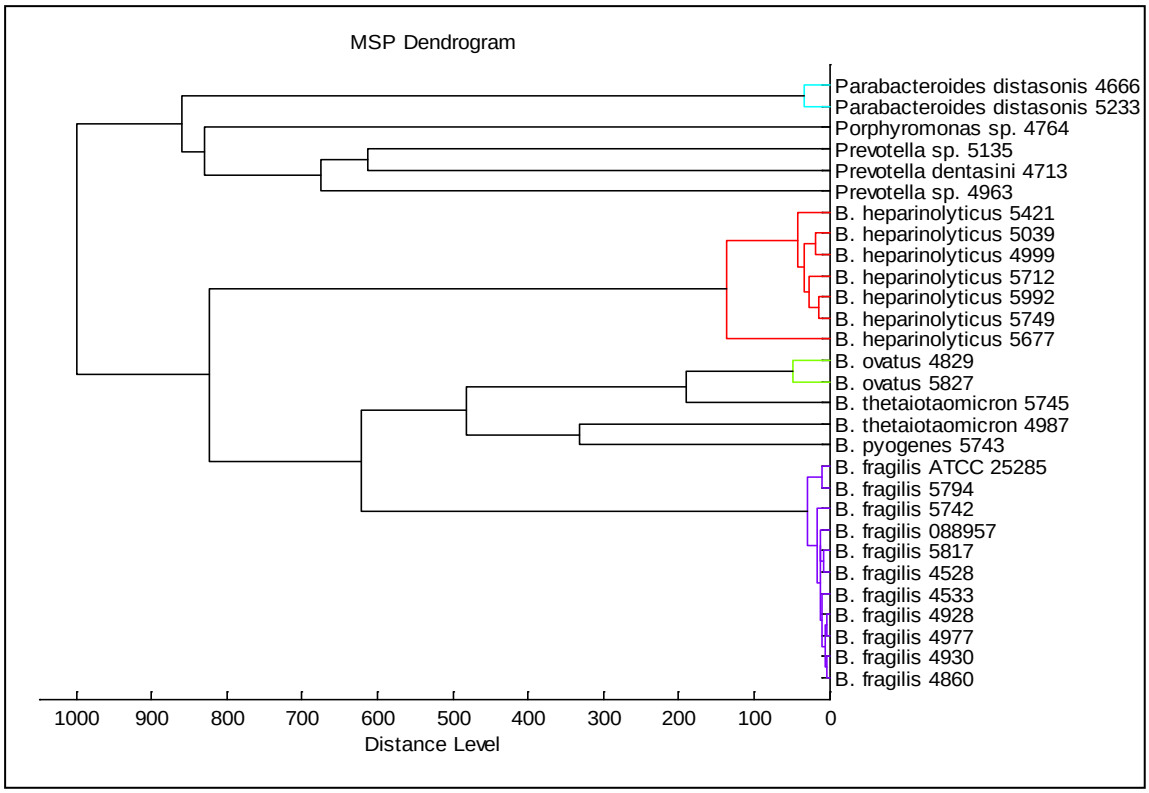

Fig 1. *Dendrogram of spectra generated using MALDI-TOF MS.*

Table 3. *Identified species*

Species	**N**
Bacteroides fragilis	10
Bacteroides heparinolyticus	7
Bacteroides ovatus	2
Bacteroides thetaiotaomicron	2
Bacteroides pyogenes	1
Parabacteroides distasonis	2
Prevotella sp.	2
Prevotella dentasini	1
Porphyromonas sp.	1
Fusobacterium sp.	1
Total	**29**

All the isolates identified as *B. heparinolyticus* were prepared as ethanol extracts and sent to Bruker Daltonics to be included in the next version of their database. This will allow for future identification of this species with MALDI-TOF MS.

Source of isolation

The samples came from different sources. Table 4 summarizes the source of the samples from which they were isolated. The *B. fragilis* isolates came from a variety of sources whereas 5 of the 7 (71%) isolates of *Bacteroides heparinolyticus* came from infections involving the lower respiratory tract or paraoral infections.

Table 4. *Source of isolation*

Species	**Source of isolation**	**N**
Bacteroides fragilis	Abscess	5
	Wound	2
	Synovial fluid	1
	Peritoneal fluid	1
	Tracheal aspirate	1
Bacteroides heparinolyticus	Sinus	3
	Tracheal aspirate	1
	Bronchoalveolar lavage	1
	Wound	1
	Peritoneal fluid	1
Bacteroides ovatus	Abscess	1
	Uterus	1
Bacteroides thetaiotaomicron	Abscess	1
	Faecal sample	1
Bacteroides pyogenes	Wound	1
Parabacteroides distasonis	Blood	1
	Synovial fluid	1
Prevotella sp.	Wound	1
	Tracheal aspirate	1
Prevotella dentasini	Peritoneal fluid	1
Porphyromonas sp.	Wound	1
Fusobacterium sp.	Fecal sample	1
Total		**29**

Antimicrobial susceptibility

Table 5 illustrates the results of the antimicrobial susceptibility testing for a selection of the antimicrobials in the VetMIC GP-mo panel.

Table 5. *MIC of selected antimicrobials for the isolates*

		CLSI S|I|R		≤0.5|1|>1	≤2|4|>4		≤8|16|>16	≤2|4|>4	≤4|8|>8		N/R	N/R	N/R	N/K
		EUCAST S|R		≤0.25|>0.5				>4			N/R	N/R	N/R	N/K
		VetMIC S|I|R				>4					N/R	N/R	N/R	N/K
		Range µg/ml		0.015-2	0.03-4	0.125-16	0.25-32	0.125-16	0.25-32	0.03-4	0.25-32	0.125-16	0.03-2	0.25-16
Isolate	**Source**	**Species**	**Cefinase**	**Pc**	**Ct**	**Em**	**Cm**	**Cl[a]**	**Tc**	**Fu**	**Gm**	**Km**	**Ci**	**Trim**
4528	Hoof-abscess	*B. fragilis*	pos	>2	>4	16	8	1	0.25	>4	>32	>16	>2	>16
4533	Hoof-abscess	*B. fragilis*	pos	>2	>4	>16	8	1	1	>4	>32	>16	>2	>16
4977	Synovia	*B. fragilis*	pos	>2	>4	>16	8	0.5	32	>4	>32	>16	>2	>16
5817	Abscess	*B. fragilis*	pos	>2	>4	>16	8	0.5	1	>4	>32	>16	>2	>16
4860	Peritoneal fluid	*B. fragilis*	Pos	>2	>4	16	4	0.125	0.25	>4	>32	>16	>2	>16
4928	Wound	*B. fragilis*	Pos	>2	>4	16	4	1	0.25	>4	>32	>16	>2	>16
4930	Wound	*B. fragilis*	Pos	>2	>4	>16	8	0.5	0.5	>4	>32	>16	>2	>16
5742	Hoof-abscess	*B. fragilis*	Pos	>2	>4	>16	4	0.5	0.25	>4	>32	>16	>2	>16
5794	Tracheal-aspirate	*B. fragilis*	Pos	>2	>4	>16	8	0.5	0.5	>4	>32	>16	>2	>16
B88957	Hoof-abscess	*B. fragilis*	Pos	>2	>4	>16	8	2	0.5		>32	>16	2	>16
4999	Tracheal-aspirate	*B. heparinolyticus*	Neg	0.12	1	1	4	0.125	0.25	0.5	>32	>16	>2	>16
5421	Peritoneal fluid	*B. heparinolyticus*	Neg	0.06	1	1	2	0.125	0.25	0.5	>32	>16	>2	>16
5677	Sinus	*B. heparinolyticus*	Neg	0.25	2	1	4	0.125	0.25	1	>32	>16	>2	>16
5712	Sinus	*B. heparinolyticus*	Neg	N/D	0.5	1	2	0.125	4	0.5	>32	>16	2	>16
5749	Wound	*B. heparinolyticus*	Neg	0.03	0.5	0.5	2	0.125	0.25	0.5	>32	>16	2	>16
5992	Sinus	*B. heparinolyticus*	Neg	0.25	0.5	1	4	0.125	0.25	1	>32	>16	>2	>16
5039	BAL*	*B. heparinolyticus*	Neg	N/D	0.5	0.5	4	0.125	0.25	1	>32	>16	>2	>16
4829	Hoof-abscess	*B. ovatus*	Pos	>2	>4	16	8	2	0.25	>4	>32	>16	>2	>16
5827	Uterus	*B. ovatus*	Pos	>2	>4	16	8	1	1	>4	>32	>16	>2	>16
5743	Wound	*B. pyogenes*	Neg	0.03	0.25	0.5	1	0.25	0.25	0.5	>32	>16	0.5	>16
4987	Fecal sample	*B. thetaiotaomicron*	Pos	>2	>4	>16	8	4	0.5	>4	>32	>16	>2	>16
5745	Abscess	*B. thetaiotaomicron*	Pos	>2	>4	>16	8	16	0.25	>4	>32	>16	>2	>16
ATCC 25285		*B. fragilis*	Pos	>2	>4	>16	8	0.5-2	0.25-0.5	>4	>32	>16	>2	>16

S: Sensitive, I: Intermediate, R: Resistant,
N/D: Not determined
N/R: not relevant, because of limited or uncertain effect for treatment of anaerobic infections in general
N/K: No known clinical breakpoint available
a. Lethal in horses – therefore not relevant
Pc: penicillin, Ct: Cephalotin, Em: Erythromycin, Cm: Chloramphenicol, Cl: Clindamycin,
Tc: Tetracycline, Fu: Fusidic Acid, Gm: Gentamicin, Km: Kanamycin, Ci: Ciprofloxacin, Trim: Trimethoprim
*Bronchoalveolar lavage

Table 6. *MIC of selected antimicrobials against isolates not identified as* Bacteroides *spp.*

		CLSI S|I|R		≤0.5|1|>1	≤2|4|>4		≤8|16|>16	≤2|4|>4	≤4|8|>8				N/R	N/K
		EUCAST S|R		≤0.25|>0.5				>4					N/R	N/K
		VetMIC S|I|R				>4					≤2|4|>4		N/R	N/K
		Range µg/ml		0.015-2	0.03-4	0.125-16	0.25-32	0.125-16	0.25-32	0.03-4	0.25-32	0.125-16	0.03-2	0.25-16
Isolate	**Source**	**Species**	**Cefinase**	**Pc**	**Ct**	**Em**	**Cm**	**Cl[a]**	**Tc**	**Fu**	**Gm**	**Km**	**Ci**	**Trim**
4693	Fecal sample	*Fusobacterium* sp.	Neg	0.25	0.5	>16	1	0.125	0.25	2	>32	>16	1	>16
4666	Synovia	*Parabacteroides distasonis*	Neg	>2	>4	>16	8	8	16	4	>32	>16	>2	>16
5233	Blood	*Parabacteroides distasonis*	Neg	>2	>4	>16	8	8	16	>4	>32	>16	>2	>16
4764	Wound	*Porphyromonas* sp.	Neg	0.015	0.5	0.125	1	0.125	0.25	0.12	1	>16	1	>16
4963	Wound	*Prevotella* sp.	Neg	0.015	0.03	0.125	1	0.125	0.25	0.12	4	>16	0.5	>16
4713	Peritoneal fluid	*Prevotella dentasini*	Neg	0.015	0.06	0.25	2	0.125	0.25	1	>32	>16	2	8
5135	Tracheal-aspirate	*Prevotella* sp.	Neg	0.015	0.5	4	2	0.25	0.25	0.25	>32	>16	2	>16

S: Sensitive, I: Intermediate, R: Resistant
N/R: not relevant, because of limited or uncertain effect for treatment of anaerobic infections in general
N/K: No known clinical breakpoint available
a. Lethal in horses – therefore not relevant
Pc: penicillin, Ct: Cephalotin, Em: Erythromycin, Cm: Chloramphenicol, Cl: Clindamycin,
Tc: Tetracycline, Fu: Fusidic Acid, Gm: Gentamicin, Km: Kanamycin, Ci: Ciprofloxacin, Trim: Trimethoprim

Table 7 illustrates the results for all *B. fragilis* for selected antimicrobials from the CLIN GN and VetMIC Stordjur panels.

Table 7. *MIC of* Bacteroides fragilis *tested on CLIN GN and VetMIC Stordjur panels.*

	CLSI S|I|R	≤0.5|1|>1	≤4/2|8/4|>8/4			≤0.25|-|-	
	EUCAST S|R	≤0.5|>2					
	VetMIC S|I|R			≤64|-|>64	≤0.5/9.5|-|>0.5/9.5		
	Range µg/ml	1-8	2/1-16/8	4-32	2.25/4.75-2/38	0.125-1	1-8
Isolate	**Source**	**Am**	**A/C**	**Ni**	**T/S**	**Ce**	**Ff**
4528	Abscess	>8	4/2	8	>2/38	>1	2
4533	Abscess	>8	4/2	8	>2/38	>1	4
4977	Synovia	>8	4/2	8	>2/38	>1	>8
5817	Abscess	>8	4/2	8	2/38	>1	4
4860	Peritoneal fluid	>8	4/2	8	>2/38	>1	2
4928	Wound	>8	8/4	8	>2/38	>1	2
4930	Wound	>8	4/2	16	>2/38	>1	4
5742	Hoof-abscess	>8	4/2	8	>2/38	>1	4
5794	Tracheal-aspirate	>8	4/2	8	2/38	>1	4

S: Sensitive, I: Intermediate, R: Resistant
Am: Ampicillin, A/C: Amoxicillin/Clavulanic Acid, Ni: Nitrofurantoin,
T/Trimethoprim/sulfamethoxazole, Ce: Ceftiofur, Ff: Florfenikol

It is important to note that the antimicrobial testing for *B. heparinolyticus* contains an error for two isolates. Isolate 5712 and 5039 were sensitive to the tri-cit buffer used in the penicillin wells. Therefore, it is not possible to draw conclusions about the MIC of penicillin for these two isolates. The remaining *B. heparinolyticus* showed growth in the tri-cit wells and the MIC of penicillin was assumed to be correct.

Some isolates appear to be more resistant than others such as 4677 (*B. fragilis*) which demonstrates a much higher MIC of tetracycline than the other *B. fragilis*. Also, both *P. distasonis* isolates were more resistant than other isolates showing resistance to both clindamycin and tetracycline.

Not all of the antimicrobials tested are displayed. One of the reasons for this being that some of the antimicrobials are only included in the VetMIC panels to identify specific bacteria. For example, oxacillin is included in the panels to identify methicillin resistant *Staphylococcus aureus* (MRSA). Therefore those results are not relevant to this study. Other antimicrobials have been included, even though they are not used in horses. This is because of potential value for other species of animals with *Bacteroides* spp. infections. Clindamycin is lethal in horses but is used in for example dogs. The results reflect that *Bacteroides* sp. are sensitive to clindamycin and so the results for this antimicrobial have value in being included. As are results for nitrofurantoin, it is an antimicrobial used off-label in dogs. Kanamycin is an aminoglycoside just like gentamicin. It have been included to demonstrate the general lack of susceptibility of *Bacteroides* spp. to aminoglycosides, even though kanamycin is not used in

horses in Sweden. Also, resistance to gentamicin does not automatically mean isolates will be resistant to kanamycin. Ciprofloxacin is included in the results even though no approved pharmaceutical exists for use in horses in Sweden. It is approved for use in humans and is used off-label in small animals.

β-lactamase production

All of the *B. fragilis* isolates were positive for β-lactamase production on the Cefinase™ nitrocefin discs whereas all of the *Bacteroides heparinolyticus* isolates were negative for β-lactamase production. Also a much lower MIC for penicillin (≤0,015-0,25 µg/ml) was recorded for the *B. heparinolyticus* isolates compared to *B. fragilis* (>2 µg/ml) and would be considered susceptible. All other *Bacteroides* spp. isolates identified, apart from one isolate of *B. pyogenes*, were positive for β-lactamase production and the MICs of penicillin were high (MIC >2 µg/ml). Interestingly, the two isolates identified as *P. distasonis* do not display β-lactamase production, but still the MIC of penicillin were high (MIC >2 µg/ml).

Frozen Brucella broth

The isolates of *B. fragilis* that were run on the VetMIC GP-mo panels using frozen Brucella broth gave corresponding results to results using fresh Brucella broth.

Carbapenemase-production

All MALDI-TOF MS spectra sent to Åsa Johansson at Centrallasarettet in Växjö belonged to the *cfiA* negative group of *B. fragilis*.

Medical Records

No general conclusions can be drawn from the medical records as the quality of the information varies. It was not possible to obtain the medical records for two horses. It is clear that horses have been treated with antimicrobials to which the isolate is resistant. Table 8 summarizes the medical records. For some isolates, the same antimicrobial will appear more than once in the treatment column. This means the horse has been treated more than once with the antimicrobial and has either been reexamined, readmitted or has changed treatment protocol. It is also possible that the route of administration has been altered, in which case this is indicated with intravenous (i.v.) or intramuscular (i.m.).

Table 8. *Summary of antimicrobial treatment and duration of treatment obtained from the medical records*

Isolate	Species identified	Diagnosis	Treatment	Days treated	Outcome
4528	*Bacteroides fragilis*	Hoof abscess	Penicillin (i.v)	Unknown	Recovered
4533	*Bacteroides fragilis*	Hoof abscess	unknown	Unknown	Unknown
4666	*Parabacteroides distasonis*	Synovitis	Penicillin (i.v)	11	Unknown
			Gentamicin (i.v)	15	
			Penicillin (i.m)	12	
4693	*Fusobacterium* sp.	Colic	unknown	Unknown	Recovered
4977	*Bacteroides fragilis*	Synovitis and osteomyelitis	Penicillin (i.v)	Unknown	Euthanized
			Gentamicin (i.v)	Unknown	
5817	*Bacteroides fragilis*	Perianal abscess	Penicillin (i.m)	6	Recovered
			Penicillin (i.v)	4	
4713	*Prevotella dentasini*	Peritonitis	Penicillin (i.v)	2	Euthanized
			Gentamicin (i.v)	2	
4764	*Porphyromonas* sp.	Hoof-abscess with involvement of distal phalanx	Penicillin (i.v)	27	Recovered
			Gentamicin (i.v)	21	
			Compress with Fucidic Acid	Unknown	
			Trimethoprim/Sulfadiazine (p.o)	8	
4829	*Bacteroides ovatus*	Hoof abscess	Unknown	Unknown	Unknown
4860	*Bacteroides fragilis*	Peritonitis	Penicillin (i.v)	30	Recovered
			Gentamicin (i.v)	6	
4928	*Bacteroides fragilis*	Fistula formation after apical tooth root abscess	Unknown	Unknown	Recovered
4930	*Bacteroides fragilis*	Sutureline infection after surgery	Penicillin (i.v)	26	Recovered
			Gentamicin (i.v)	10	
4963	*Prevotella* sp.	Funiculitis	Penicillin (i.m)	15	Recovered
			Trimethoprim/Sulfadiazine (p.o)	14	
			Penicillin (i.v)	13	

4987	*Bacteroides thetaiotaomicron*	Right dorsal colitis caused by NSAID treatment	No antimicrobial treatment		Euthanized
4999	*Bacteroides heparinolyticus*	Pleuropneumonia (esophageal obstruction)	Gentamicin (i.v)	Unknown	Euthanized
			Penicillin (i.v)	4	
			Ceftiofur	1	
			Metronidazole	Unknown	
5039	*Bacteroides heparinolyticus*	Unknown	Unknown	Unknown	Unknown
5135	*Prevotella* sp.	Pleuropneumonia (esoophageal obstruction)	Penicillin (i.m)	8	Unknown
5233	*Parabacteroides distasonis*	Purulent artrit och sepsis	Penicillin (i.v)	10	Euthanized
			Trimethoprim/Sulfadiazine (i.v)	7	
			Amikacin (i.a)	1	
			Gentamicin (i.v)	4	
5421	*Bacteroides heparinolyticus*	Peritonit	Penicillin (i.v)	15	Recovered
			Gentamicin (i.v)	12	
5677	*Bacteroides heparinolyticus*	Sinusitis (apical tooth root abscess)	Penicillin (i.v)	9	Recovered
			Penicillin (i.v)	7	
			Trimethoprim/Sulfadiazine (p.o)	12	
5712	*Bacteroides heparinolyticus*	Sinusitis	Penicillin (i.v)	16	Recovered
			Gentamicin (i.v)	15	
5742	*Bacteroides fragilis*	Hoof-abscess	Penicillin (i.v)	15	Recovered
			Trimethoprim/Sulfadiazine (p.o)	11	
5743	*Bacteroides pyogenes*	Wound infection	Penicillin (i.m)	6	Recovered
			Trimethoprim/Sulfadiazine (p.o)	6	
			Medicinal honey	Unknown	
			Penicillin (i.v)	7	
			Penicillin (i.m)	5	
			Penicillin (i.v)	8	
5745	*Bacteroides thetaiotaomicron*	Abscess	Unknown	Unknown	Unknown
5749	*Bacteroides heparinolyticus*	Septic arthritis	Trimethoprim/Sulfadiazine (p.o)	8	Recovered

			Penicillin (i.m)	3	
			Penicillin (i.v)	11	
			Gentamicin (i.v)	8	
5794	*Bacteroides fragilis*	Pleuritis following esophageal rupture	Penicillin (i.v)	3	Euthanized
			Gentamicin (i.v)	3	
5827	*Bacteroides ovatus*	Chronic endometritis	Penicillin (i.m)	5	Unknown
5992	*Bacteroides heparinolyticus*	Sinusitis	Penicillin (i.v)	4	Euthanized
			Penicillin (i.m)	11	
B88957	*Bacteroides fragilis*	Hoof-abscess	Penicillin (i.v)	5	Euthanized

DISCUSSION

Even though infection with *Bacteroides* spp. can be severe, the treatment is seldom guided by results of susceptibility testing as it is not routinely performed. This could be potentially life-threatening as we in this study have seen that isolates of *B. fragilis* are resistant to penicillin, a frequently used antimicrobial in Sweden. It is important to note when reading antimicrobial susceptibility reports and studies that the anaerobes are frequently grouped into one category, and general statements are made about their susceptibility. This has consequences as it might lead clinicians to believe that all anaerobes are sensitive to for example penicillin, when in fact several members of *Bacteroides* spp. are resistant. When the anaerobes are separated, it is usually into genus. This is also misleading, with the results of this study indicating that different species within the same genus have different susceptibilities.

As described in the literature review, *Bacteroides* spp. are important in infections in horses and it is important to know their antimicrobial susceptibility to start correct treatment as soon as possible. This again places emphasis on the importance that the clinician knows the correct technique for sampling and on the importance of requesting anaerobic cultures and antimicrobial susceptibility tests for the anaerobes. If all horses with infections, in which anaerobes could be suspected, were tested for anaerobic isolates then perhaps more horses would receive correct treatment. This is well demonstrated by Mair & Lane (1989) in a review of horses with lung abscesses and primary pneumonia. Only samples from 6 of 11 horses were cultured anaerobically and 3 of these 6 cultures (50 %) were positive for anaerobic growth. All of these returned at least one *Bacteroides* spp. This might well mean that several of the horses not tested for anaerobic isolates had anaerobic infections and might therefore have received incorrect treatment.

Identification

The use of MALDI-TOF MS in species-identification of bacteria will notably speed up the identification-process and also reduce costs incurred by the laboratory. The strains identified as *B. heparinolyticus* has been prepared as ethanol extracts and sent to Bruker Daltonics for incorporation into their new database. Because of this study it will now be possible to identify *B. heparinolyticus* using MALDI TOF MS. The percentage of isolates successfully identified using MALDI-TOF MS in this study was 55% (16/29). With the addition of *Bacteroides heparinolyticus* to the software it would then be possible to identify 79% (23/29) of isolates in this study. With the upgraded database, and when excluding the isolates incorrectly identified as *Bacteroides* spp., the software will be able to identify 95% (21/22) of the *Bacteroides* spp. isolates in this study. With the exception of one isolate of *B. thetaiotaomicron* all isolates ultimately identified as *Bacteroides* spp. in this study could be identified using the MALDI-TOF MS.

Of interest to note is the identification of isolates as *Bacteroides* spp. when they are in fact *Prevotella* spp., *Parabacteroides* spp., and *Porphyromonas* spp. However, they were up until recently part of the *Bacteroides* genus and are most likely very similar in their phenotypic profile, and so it might be impossible to identify these correctly using more traditional

methods. One isolate was found to be an unnamed *Fusobacterium* sp. which is also a Gram-negative genus and which include species that may phenotypically resemble *Bacteroides* spp. By using MALDI-TOF MS to identify isolates it will be possible to reduce the risk of incorrect identification in the future.

Source of isolation

It is interesting to note that 5/7 (71%) of the isolates identified as *Bacteroides heparinolyticus* came from paraoral or lower respiratory tract infections. A search for *B. heparinolyticus* in animals in databases reveals that they are commonly found in the oral cavity which would explain their involvement in these types of infections (Bailey & Love, 1991). Careful speculation can be made that paraoral and respiratory tract infections will have a higher incidence of *B. heparinolyticus* involved whereas abscesses and wounds will have a higher incidence of *B. fragilis* because of contamination from the fecal flora. Of the 5 isolates which came from hoof abscesses, 4 of these (80%) were identified as *B. fragilis.* One isolate which came from an abscess could not be closer source-identified as the medical record was never obtained. This isolate is nevertheless identified as *B. fragilis* which makes involvement of *B. fragilis* in abscesses in this study 5/6 (83%). This can be important information to clinicians as early conclusions about the susceptibility can be made when culture results are reported before susceptibility results arrive. The results in this study indicate that *B. heparinolyticus* are susceptible to penicillin and *B. fragilis* are resistant. And perhaps tentative suggestions can be made that when *Bacteroides* spp. are involved in lower respiratory tract infections they are likely to be susceptible to penicillin whereas when they are involved in abscesses or wounds they are more likely to be resistant against penicillin.

Antimicrobial susceptibility

It is important to remember that the results found in this study are *in vitro* results obtained under controlled laboratory conditions. It is not guaranteed that an antimicrobial will behave and have the same effect *in vivo* as the condition at a site of infection is different. Clinical results are dependent on many different host factors such as anatomy, physiological and pathological barriers, different bacterial properties and pharmacokinetic and pharmacodynamic properties such as volumes of distribution, protein-binding and attainable tissue concentrations. If it is not possible to achieve the concentration required at the site of infection the treatment with antimicrobials will not be successful. For example, the ability of *Bacteroides* spp. to harvest thymidine from its surroundings in a necrotic process will render trimethoprim-sulfonamides useless in an *in vivo* situation, even if the *in vitro* results are promising (Indiveri & Hirsh, 1992). This difference between *in vitro* and *in vivo* results is demonstrated in two early case reports using metronidazole in horses with pleuropneumonia, that even though the organisms were sensitive to the previous antimicrobials used, the horses did not improve until an additional antimicrobial was used (Mair & Yeo 1987). It is also pertinent to remember that not all infections involve one single agent, but infections are usually mixed. This would mean that whilst one bacterial species shows resistance, the other involved organisms might be susceptible. It is important that the clinician bears this in mind.

Also, it is important to remember that currently no susceptibility testing is done for *Bacteroides* spp. which would give a false indication of susceptibility in a mixed sample where the other infecting organisms are susceptible to certain antimicrobials. This might lull the clinician into a false sense of security which might lead to the withdrawal of certain antimicrobials, possibly effective against *Bacteroides* spp., from the treatment regime.

To determine if an antimicrobial will be effective against an organism *in vivo* the clinician needs to take into consideration more than just the results of susceptibility testing. Pharmacokinetic and pharmacodynamic properties needs to be considered for each pharmaceutical and the doses recommended. Unfortunately, not all antimicrobials will have detailed information on their behavior in tissues and distributions to sites of infection in horses. Table 9 outline pharmacokinetic data for some of the most commonly used antimicrobials in horses in Sweden. These are serum levels of the antimicrobial. With this information, visualization of the problems facing the clinician becomes easier to understand. It is not possible to maintain concentrations required for certain antimicrobials to be effective over extended periods of time. And although the concentrations required for inhibition of bacterial growth might seem achievable, the quick half-lives of most antimicrobials bring plasma levels down to concentrations too low to be considered effective. However, it is important to remember; that for some antimicrobials, whilst the concentration in plasma is low, the distribution of the antimicrobial into tissues and transcellular spaces such as the peritoneal fluid might be higher, as demonstrated by Sweeney *et al.* (1986) in regards to metronidazole. The study shows that metronidazole reaches even higher levels in peritoneal fluid than in serum when given intravenously or orally. Because it is a lipophilic weak base it penetrates cell membranes very well, allowing for good distribution in tissues (Dowling, 2013). So there are many factors influencing the effectiveness of the antimicrobials.

Table 9. *Pharmacokinetics data in horses for relevant antimicrobials from selected references*

Antimicrobial	Dose (unit)	Route of administration	C_{max} (µg/ml) (hours after administration)	Half-life (h)	Conc(µg/ml) (hours after administration)	Reference
Benzylpenicillin sodium	20,000(IU/kg)	iv	>40 (0.25)	0.8	0.5 (2.92)	Love *et al.*, 1983
	20.000 (IU/kg)	im	>10 (0.25-0.5)	1.5	0.5 (6.2)	Love *et al.*, 1983
Procaine-benzylpenicillin	20,000 (IU/kg)	im	~1.5 (1)	19.68	0.5 (18.75)	Love *et al.*, 1983,
	22,000 (IU/kg)	im	2.06 (4)		0.17(24)	Sullins *et al.*, 1984
Gentamicin	6.6 (mg/kg)	iv	71.9±15.7	3.0±2.8	22.0±4.9 (1.31), 1 (~11)	Magdesian *et al.*, 1998
Trimethoprim/Sulfadiazine	2.5(mg/kg)/12.5(mg/kg)	iv	2.42/52.7	2.8/4.6		Gustafsson *et al.*, 1999
	5 (mg/kg)/25(mg/kg)	po	1.06/22.4	5.1/8.2		Gustafsson *et al.*, 1999
Ceftiofur						
Oxitetracycline	10(mg/kg)	Iv	135.1±21.0	12.95	>1 µg/ml (36)	Horspool & McKellar, 1990
Metronidazole	20(mg/kg),	po	22±8 (~1)	3.5±0.5	~1 (13.3)	Steinman *et al.*, 2000
	25(mg/kg)	po	12.6±2.4 (1-2)	2.5	~2 (10)	Sweeney *et al.*, 1986

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Studies have shown that not all *Bacteroides* strains produce β-lactamases but are still resistant to penicillin showing that these bacteria have some alternative mechanism for penicillin resistance (Fang *et al.,* 2002)**.** This means that testing for β-lactamase production only is not enough to determine if the patient can be treated using penicillin in the case of *Bacteroides* infections. However it is a quick test to determine the β-lactamase production from bacteria and penicillin can quickly be ruled out as a treatment option in a positive test. It cannot however be assumed that the infecting organism will be susceptible in a negative test result. In this study, only the *P. distasonis* displayed a negative result for β-lactamase production but were still resistant against penicillin. The results indicated that all of the *Bacteroides* spp. isolates in this study with β-lactamase production are highly resistant to penicillin. A promising result of the study is that the nitrocefin disc test can be performed early in the identification of the isolates as minimal colony material is required for this test. This would quickly give indications about the susceptibility of the isolate to penicillin.

Penicillin would appear to be a poor choice of antimicrobial in infections with *B. fragilis, B. thetaiotaomicron* and *B. ovatus* as they in this study show resistance to penicillin because of β-lactamase production. However, it might be efficient in infections involving *B. heparinolyticus* or *B. pyogenes* as they do not demonstrate β-lactamase production and have a lower MIC for penicillin in this study. Penicillin is the first-choice antimicrobial in Sweden and most horses are initially treated with this. Therefore, using the β-lactamase test in conjunction with species identification by MALDI-TOF MS and antimicrobial susceptibility testing can quickly rule it in or out for treatment of infections. When combining pharmacokinetic studies and the MIC values obtained in this study, it is clear that it is not possible to achieve effective concentrations of penicillin in the horse against *B. fragilis, B. thetaiotaomicron* and *B. ovatus.* However, for *B. heparinolyticus* and *B. pyogenes,* which have considerably lower MIC for penicillin, it is a good choice of antimicrobial. As seen in this study the MIC for *B. heparinolyticus* and *B. pyogenes* is below or equal to the 0.25 µg/ml clinical breakpoint set by EUCAST and well below the 0.5 µg/ml clinical breakpoint set by the CLSI. At the recommended dose of 22.000 IU/kg given once or twice intramuscularly the concentration of penicillin in the horse stays above the clinical breakpoints for extended periods of time and well above the MIC for *B. heparinolyticus* and *B. pyogenes* which makes it a very suitable antimicrobial for treatment of these infections in horse (Sullins *et al.,* 1984; Love *et al.* 1983).

Gentamicin is approved for use in horses in Sweden; however, it was shown early that aminoglycosides such as gentamicin and kanamycin are relatively ineffective against anaerobes, requiring over ≥128 µg/ml to inhibit 50% of isolates (Kimsey & Hirsh 1978). None of the aminoglycosides tested (gentamicin, kanamycin, streptomycin) were effective against the 29 isolates of *Bacteroides* spp. This would be expected given that aminoglycosides cannot function in an anaerobic environment since uptake of the antimicrobial is oxygen-dependent. However, as gentamicin is the antimicrobial used most frequently in conjunction with penicillin in horses it was considered relevant to present data that supports it has no effect on *Bacteroides* spp.

Tetracycline showed promising effect against all but two of the isolates. However, given the high risk of side-effects such as enterocolitis it would not be wise to recommend this antimicrobial to be used in horses for extended periods of time. Also, horses treated with tetracycline need to be carefully monitored for signs of developing colitis. Currently there is no authorized tetracycline antimicrobial in Sweden and use is off-label.

Cephalotin showed promising *in vitro* results against *B. heparinolyticus* and the one isolate of *B. pyogenes*. Unfortunately *B. fragilis* and the remaining *Bacteroides* spp. in this study showed resistance against this substance. Since *B. heparinolyticus* and *B. pyogenes* are both susceptible to penicillin it means there is no need to use cephalosporins in the treatment of *Bacteroides* spp. infections in the horse.

Ceftiofur was only tested on *B. fragilis* in this study, and all isolates showed resistance. Hence, ceftiofur is a poor choice of antimicrobial for isolates of *Bacteroides* spp. with β-lactamase production. Interestingly, one study comparing anaerobic equine isolates to bovine isolates and their susceptibility to ceftiofur found a higher rate of resistance in equine isolates (Samitz *et al.*, 1996).

All isolates were tested against trimethoprim, but only *B. fragilis* was tested against a combination of trimethoprim and sulfonamides. Both of these compounds have been determined to be more efficient against anaerobes when combined in animals (Indiveri & Hirsh 1986). However in necrotic tissues and exudates the thymidine content may inhibit the effect (Indiveri & Hirsh, 1992) and the outcome of the treatment is uncertain. Approved substances for intravenous and oral administration are available for horses in Sweden.

Unfortunately we were not able to test the isolates against metronidazole, but other studies show that there is currently very little resistance among *Bacteroides* spp. to metronidazole even globally and in humans (Ulger-Toprak *et al.*, 2004; Aldridge *et al.*, 1994; 2001; Nagy *et al.*, 2010; Jousimies-Somer *et al.*, 2003; Hirsh *et al.*, 1985) and it would be an effective alternative for treatment of these infections in horses. However, metronidazole does not exist on the market specifically for horses or other animals in Sweden, and the off-label use of drugs intended for humans quickly gets expensive because of the large dosages required. Metronidazole has a high bioavailability when given orally, up to 85%, and penetrates bone, abscesses and the central nervous system well (Dowling, 2013). Metronidazole shows a very good distribution to peritoneal fluid in horses which would make it an ideal pharmaceutical to treat horses with peritonitis involving anaerobic organisms resistant to other antimicrobials available. The clinical breakpoint for resistance in *Bacteroides* spp. is frequently reported as >16μg/ml (Lawhon *et al.*, 2013; Nagy *et al.*, 2010; Aldridge *et al.*, 1994; Hirsh *et al.*, 1985), although EUCAST uses ≤4 μg/ml (sensitive) and >4 μg/ml (resistant). The MIC of *Bacteroides* spp. isolated from animals is reported by Lawhon *et al.* (2013) to be <1.5 μg/ml, and over 85 % have a MIC of ≤0.75 μg/ml. The pharmacokinetic data shows that the level of metronidazole stay well above this for at least 13 hours (Steinman *et al.*, 2000; Sweeney *et al.*, 1986). A large study spanning 20 years of research reported the MIC_{90} for *Bacteroides* spp. to be 1-2 μg/ml (Nagy *et al.*, 2010).

One of the earliest reports of metronidazole used in two horses with pleuropneumonia demonstrates improvement when given metronidazole intramuscularly after being treated for prolonged periods with both penicillin and trimethoprim-sulphadoxine. Susceptibility test revealed however that all the isolates, including four *Bacteroides* spp. isolates, were sensitive to both penicillin and trimethoprim-sulphadoxine (Mair & Yeo 1987). This demonstrates that although an organism might be sensitive in vitro to a pharmaceutical it might not be effective *in vivo*. And although it is not possible to attribute the successful outcome in these two cases only to the use of metronidazole, it suggests that it played a role in the recovery of the horses since they had both been treated for prolonged periods with other antimicrobials without improvement.

The isolates identified as *Parabacteroides distasonis* showed significant resistance to many of the antimicrobials tested. The only antibiotic to which they appear to be susceptible is chloramphenicol. This is not readily available in Sweden other than in ophthalmic solutions. It would pose quite a problem when deciding which antimicrobial to use. One of these isolates came from the synovial fluid from a foal which suffered from sepsis and septic arthritis. The foal was euthanized after failure to respond to treatment. See case 2 under medical records below.

Developing new methods for susceptibility testing

Using Brucella broth frozen 8 months previous to this study was done in an attempt to determine whether it is possible to do antimicrobial testing for *Bacteroides* spp. using frozen broth. The fresh broth used in these trials is not readily available and must be ordered specifically for every occasion. This is time consuming and delays the process of antimicrobial testing. It might also be one explanation as to why susceptibility testing is not currently performed for *Bacteroides* spp. The results reflect that using frozen broth works very well and the results for *B. fragilis* antimicrobial susceptibility test are the same for both fresh and frozen broth. This means the laboratory could use frozen broth and speed up the process of antimicrobial susceptibility testing. Using frozen broth would most likely prove both time and cost effective.

Medical Records

As previously stated, it is not possible to draw general conclusions from the medical records as the quality varies amongst them and because of the insufficient number of samples included in this study. Also it was not possible to obtain all records. It is however worth noting that several of these cases were treated with antimicrobials to which the isolated organism was resistant.

Case 1 – 4930

A horse developed a subcutaneous infection in the suture line after an exploratory laparotomy. It was found that the horse had nephrosplenic entrapment of the colon with a complete retroflexion of the pelvic flexure. An enterotomi was performed to empty the large colon of its contents. The horse was treated with penicillin, gentamicin and flunixin-meglumine. The

horse recovered well from the surgery and the incision and suture line looked good with no signs of infection. However, the horse started to develop pain upon inspection of the suture line and eventually developed a mild ventral edema along the suture line. Ultrasound revealed a small collection of fluid in the caudal end of the suture line. A few staples were removed to obtain a culture. However, no visual signs of infection besides the ventral edema and pain were present. The first visual signs were seen six days after the surgery when a small collection of pus was seen in the suture line. The horse was still undergoing medication with penicillin and gentamicin.

The Laboratory reported growth of *Bacteroides* spp. and the horse was sent home with treatment with intramuscular procaine-penicillin and daily cleaning of the wound. However, the horse continued experiencing discomfort and pus started to drip from the suture line. The horse was returned to the clinic for assessment and continuing i.v. medication with penicillin. The horse recovered after being treated with penicillin for 26 days, including 10 days with gentamicin, and daily cleaning of the wound. Our results show that this horse had an infection with *B. fragilis* not susceptible to penicillin or gentamicin. If susceptibility tests had been performed perhaps a more suitable treatment could have been initiated. One can speculate that the recovery of the horse might more be attributed to the constant cleaning of the wound than the antimicrobial treatment received.

Case 2 - 5233

A 3 week old foal was treated for sepsis and purulent arthritis in the right tarsal joint. The foal was treated with an intravenous dose of plasma, penicillin and trimethoprim-sulfonamides, along with flunixin-meglumine, omeprazole, vitamin-K and intravenous fluids. Synovia was collected for analysis and amikacin deposited locally in the joint. The joint was also flushed. Laboratory results showed infection with *Bacteroides* spp. At first the foal started to recover and showed signs of improvement. The foal was assessed to be well enough to be sent home and about to be discharged when it suddenly started to swell up in the other tarsal joint. The foal underwent flushing of both tarsal joints under general anesthesia and the medication was supplemented with gentamicin. The general condition of the foal was good, but the joints continued to swell and an increasing lameness was seen. Eventually both the carpal joints also showed signs of involvement and the decision was made to euthanize the foal.

The isolate was identified in this study as *Parabacteroides distasonis.* This is an example of where antimicrobial susceptibility testing could have increased the chances for this foal. According to our results, this isolate was resistant to penicillin, trimethoprim and gentamicin. Treatment with metronidazole could have been effective, however since the isolate has not been tested against metronidazole it is only possible to speculate.

CONCLUSION

This study demonstrates the importance of antimicrobial susceptibility testing. The recommendation would be to initially test isolates of genus *Bacteroides* for β-lactamase production using the nitrocefin discs. This is a quick test which immediately lets you know if

the *Bacteroides* spp. involved in the infection will be resistant to penicillin and an alteration in treatment could be quickly implemented whilst waiting for results of broader antimicrobial susceptibility testing. If the isolate is found to be β-lactamase producing then the recommendation is to use antimicrobials other than penicillin.

Unfortunately it would appear that there are very few treatment options available for the most resistant of strains of *B. fragilis* as not all antimicrobials are readily available in suitable formulations in Sweden. Also, the antibiotics that are available such as tetracycline are used only in rare cases of disease, being currently only recommended for treatment of anaplasmosis and *Lawsonia intracellularis* infections. Tetracycline has a high risk of side-effects such as enterocolitis which would make it an unsuitable antimicrobial for use over extended periods of time. Chloramphenicol does not exist in the formulation required for treatment of infections from which these samples have come. In Sweden, chloramphenicol is available for ophthalmic infections only. Clindamycin appears to be effective; however this antimicrobial is lethal in horses and cannot be used. It is however useful in other species such as dogs, and the results in this study might help clinicians when dealing with patients other than horses.

If it is a serious case with a rapidly deteriorating patient, metronidazole should be considered to be included in the treatment regimen because of its effect on anaerobes. Unfortunately we were not able to test the susceptibility of our isolates against metronidazole, but other studies show that metronidazole is efficient against *Bacteroides* spp. and this should be considered as a treatment option in horses with penicillin-resistant *Bacteroides*-infections. The benefits of this antimicrobial outweigh the added costs of treatment as it has a high bioavailability and only acts on anaerobes. And since it has a high displacement into transcellular spaces such as the peritoneal fluid, it makes it an excellent antimicrobial to be used in cases of peritonitis and pleuropneumonia.

It is extremely important to remember in infections with *B. fragilis*, with the ability for abscess formation, that treatment of wounds and abscesses should never solely be attempted with antimicrobials. Proper drainage of the abscess and debridement of wounds are the cornerstones of good medical practice and cannot be replaced by antimicrobial therapy only. Instead antimicrobial therapy should be used in cases where it is absolutely necessary to complement the other strategies for the treatment of abscesses and wounds.

As a final recommendation, as a limited number of isolates were included in this study, it would be appropriate to perform future extended studies on *Bacteroides* spp. from horses to further define and establish new treatment regimens for these infections.

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