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Studies on Equine *Actinobacillus* spp

Susanna Sternberg

SWEDISH UNIVERSITY OF AGRICULTURAL SCIENCES

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Abstract

Studies on equine *Actinobacillus* spp. found in the normal flora of the oral cavity of the horse, as well as isolates from clinical cases, are summarised in this thesis.

The major disease caused by equine *Actinobacillus* spp. is septicaemia in foals. In adult horses, respiratory infections can be seen, but various other opportunistic infections are also associated with these bacteria. The taxonomic status of equine actinobacilli is still uncertain, and future revisions can be expected.

The prevalence of *Actinobacillus* spp. in the normal flora of the oral cavity of healthy horses was found to be high but varied substantially over time, and most horses were carriers. The bacterial population was complex with a large number of different strains present at the same time. No overall phylogenetic differences could be detected by ribotyping or biochemical fingerprinting of isolates from clinical cases and isolates obtained from healthy horses. So far, there is no evidence of any difference in pathogenic potential between various subtypes of *Actinobacillus* spp. or between different strains. However, studies on bacterial culture supernatants from some clinical isolates of equine *Actinobacillus* spp., indicated the presence of leukotoxic metabolites causing a decrease in total number of granulocytes in neutrophil function studies. Immunodiffusion studies demonstrated that *Actinobacillus* spp. carried in the oropharynx of healthy horses provoke a humoral immune response that can be transferred from mares to their foals via colostrum. This finding indicates that prompt and successive passive transfer of colostral antibodies is of specific importance in protecting the neonatal foal from *Actinobacillus* infection. Antimicrobial susceptibility testing showed that resistance against commonly used antimicrobial substances, such as penicillin and trimethoprim-sulfa, is present among Swedish strains of equine *Actinobacillus* spp. However, most of the bacterial population is susceptible. The possible presence of plasmid-mediated β-lactamase may change this situation by causing a rapid spread of penicillin resistance.

The pathogenesis of equine *Actinobacillus* spp. infections is still largely unknown. This thesis demonstrates that the bacteria are present in most horse populations and that interactions between the infectious agent and the host animal is important for the transition from commensal to pathogen.

Keywords: equine *Actinobacillus* spp., *Actinobacillus equuli*, infectious disease, bacterial infection, horse, foal

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For where is the man
that has incontestable evidence
of the truth of all that he holds,
or of the falsehood of all that he condemns...?

John Locke

Abstract

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The prevalence of *Actinobacillus* spp. in the normal flora of the oral cavity of healthy horses was found to be high but varied substantially over time, and most horses were carriers. The bacterial population was complex with a large number of different strains present at the same time. No overall phylogenetic differences could be detected by ribotyping or biochemical fingerprinting of isolates from clinical cases and isolates obtained from healthy horses. So far, there is no evidence of any difference in pathogenic potential between various subtypes of *Actinobacillus* spp. or between different strains. However, studies on bacterial culture supernatants from some clinical isolates of equine *Actinobacillus* spp., indicated the presence of leukotoxic metabolites causing a decrease in total number of granulocytes in neutrophil function studies.
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The pathogenesis of equine *Actinobacillus* spp. infections is still largely unknown. This thesis demonstrates that the bacteria are present in most horse populations and that interactions between the infectious agent and the host animal is important for the transition from commensal to pathogen.

Keywords: equine *Actinobacillus* spp., *Actinobacillus equuli*, infectious disease, bacterial infection, horse, foal

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Studier av *Actinobacillus* spp. från häst

Sammanfattning

Fem studier av *Actinobacillus* spp. från normalfloran i munhålan hos friska hästar, och från kliniska prover från sjuka hästar, sammanfattas i denna avhandling.
Actinobacillus spp. orsakar framför allt generaliserade infektioner, blodförgiftning, hos föl. Fölen insjuknar strax efter födelsen och blir snabbt mycket svaga, får feber och vägrar äta. Ibland ses tecken på ledinflammation eller lunginflammation. Hos vuxna hästar ger *Actinobacillus* spp. ofta sekundära luftvägsinfektioner, men även andra opportunistiska infektioner kan härledas till dessa bakterier.
Tidigare har ofta alla *Actinobacillus* spp. isolerade från häst benämnts *Actinobacillus equuli*. Dock pågår förändringar inom klassificeringen av *Actinobacillus* spp. från häst och eventuellt kan taxonomiska omgrupperingar förväntas i framtiden. Taxonomiska studier har ej varit avsikten med avhandlingen, utan denna belyser främst problem som rör patogenes.
Förekomsten av *Actinobacillus* spp. i normalfloran hos friska hästar, både vuxna och föl, befanns vara hög och flertalet, kanske t.o.m. alla, hästar tycks bära på bakterierna. Vid upprepade provtagningar av samma hästar under en vecka sågs stor variation i förekomsten av *Actinobacillus* spp. mellan olika provtagningstillfällen. Bakteriepopulationen i normalfloran var komplex och ett antal olika stammar befanns kolonisera samma häst. Ribotypning och biokemisk typning av stammar från kliniska prover från sjuka hästar och från munhålan hos friska hästar visade inga övergripande skillnader mellan kliniska isolat och normalfloreisolat. Hittills har inga skillnader i patogenitet kunnat påvisas mellan olika subtyper av *Actinobacillus* spp. från häst, men undersökning av effekten av *Actinobacillus* spp. på neutrofiler från häst indikerade en möjlig förekomst av leukotoxiska metaboliter. De leukotoxiska effekterna sågs främst som en minskning i totalantalet granulocyter, medan funktionen hos överlevande celler ej påverkades nämnvärt.
Immunodiffusion visade att *Actinobacillus* spp. i oropharynx hos friska hästar stimulerar till ett immunsvar, som kan överföras från sto till föl via råmjölk. Detta antyder att ett snabbt och effektivt upptag av antikroppar från råmjölk är särskilt viktigt för att skydda det nyfödda fölet mot infektion med *Actinobacillus* spp.
Undersökning avseende känslighet för antimikrobiella substanser visade att resistens mot vissa substanser som ofta används för behandling av häst, såsom penicillin och trimetoprim-sulfa, förekommer i den svenska populationen av *Actinobacillus* spp. Flertalet stammar är dock ännu känsliga för de flesta substanser. Resultaten tyder på en möjlig förekomst av plasmidburen β-laktamresistens, vars spridning skulle kunna förändra denna gynnsamma situation. En ökad frekvens penicillinresistens hos denna viktiga fölpatogen skulle försvåra och begränsa valet av förstahandspreparat vid behandling av akut sjuka föl.
Patogenesen för *Actinobacillus* spp.-infektioner hos häst är fortfarande otillräckligt känd, varför vidare studier är nödvändiga för att effektivisera behandling och förebyggande åtgärder. Denna avhandling visar att bakterierna är allmänt förekommande i hästpopulationen och att samspelet mellan infektionsämnet och värddjurets immunsystem är av stor betydelse för övergången från normalflorebakterie till sjukdomasframkallande patogen.

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Appendix

Papers I-V

This thesis is based on the following papers, which will be referred to in the text by their corresponding Roman numerals:

I. Sternberg, S., 1998.
Isolation of *Actinobacillus equuli* from the oral cavity of healthy horses and comparison of isolates by restriction enzyme digestion and pulsed-field gel electrophoresis. Veterinary Microbiology 59:147-156.

II. Sternberg, S., and B. Brändström, 1999.
Biochemical fingerprinting and ribotyping of isolates of *Actinobacillus equuli* from healthy and diseased horses. Veterinary Microbiology 66:53-65.

III. Sternberg, S., A. Johannisson, U. Magnusson, and M. Jensen-Waern, 1999
Effects of *Actinobacillus equuli* culture supernatants on equine neutrophil functions and survival. Journal of Veterinary Medicine B 46:595-602.

IV. Sternberg, S.
Specific immune response of mares and their newborn foals to *Actinobacillus* spp. present in the oral cavity. Submitted for publication.

V. Sternberg, S., C. Greko, and B. Olsson-Liljequist, 1999.
Antimicrobial susceptibility of equine isolates of *Actinobacillus* spp. and identification of β-lactamases in some strains. Microbial Drug Resistance, vol. 5, issue 4. In press.

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Abbreviations

AGID	Agar gel immunodiffusion
ASLO	*Actinobacillus suis*-like organisms
AUC	area under the curve
BCIP	5-bromo-4-chloro-3-indonyl-phosphate
cDNA	complementary DNA (reversely transcribed from RNA)
CFU	colony forming units
CL	chemiluminescence
EDTA	ethylenediaminetetraacetic acid
FACS	fluorescent-activated cell sorting (flow cytometry)
FPT	failure of passive transfer (of colostral immunoglobulins)
IEF	isoelectric focusing
Ig	immunoglobulin
LPS	lipopolysaccharide
MIC	minimum inhibitory concentration
NBT	nitro blue tetrazolium
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PFGE	pulsed-field gel electrophoresis
PhP	pheneplate
rDNA	ribosomal RNA gene
RFLP	restriction fragment length polymorphism
RLU	relative light units
rRNA	ribosomal RNA
RTX	repeats in toxins
TSB	trypticase soy broth
UPGMA	unweighted pair group method with arithmetic mean (for clustering)

Introduction

Actinobacillus equuli (*A. equuli*), the best known equine *Actinobacillus* sp., has, since the beginning of this century been recognised as an important cause of neonatal death in foals in most countries of the world. Generalised *A. equuli* infections typically occur very shortly after birth. The disease has been named sleepy foal disease or joint ill, shigellosis, actinobacillosis, equulosis, neonatal pyosepticaemia and, in French, maladie du poulain triste. A review of the history of the first discoveries of this bacterial species was given by Kim (1976). It was first described by Meyer in 1910, who isolated a pleomorphic Gram-negative organism from a case of purulent nephritis in a horse in South Africa. The organism was named *Bacillus nephritidis equi*, and was reported to have been observed already in 1902 by Theiler. McFadyean and Edwards mentioned in 1919 that the organism described by Meyer had been isolated from a case of pyaemic nephritis in a horse in London, stating that a striking characteristic was the viscosity of the culture and that it died out before the organism could be further studied. In 1917, Magnusson found the organism in Swedish foals and, not recognising it as being the same as that described by Meyer, named it *Bacterium viscosum equi*. Other names used for this organism include *Bacillus equuli*, *Bacillus pyosepticus equi*, *Bacterium pyosepticum viscosum*, *Bacterium pyosepticum viscosum equi*, *Bacterium pyosepticum*, *Bacterium equi*, *Bacillus pyosepticus*, *Eberthella viscosa*, *Shigella viscosa*, *Shigella equirulis*, *Shigella equuli*, *Shigella viscosum equi*, and *Achromobacter equuli*. Since the 1957 version of Bergey's manual of Determinative Bacteriology, it has been referred to as *Actinobacillus equuli*.

Taxonomy

Genus Actinobacillus

Genus *Actinobacillus* belongs to the family *Pasteurellaceae*, which also includes the genera *Haemophilus*, *Pasteurella* and *Mannheimia* (Angen *et al*, 1999). *Actinobacillus* species are facultatively anaerobic, nonmotile, Gram-negative, medium-sized, pleomorphic rods. The term pleomorphic indicates that the micromorphology is variable, individual cells can be either spherical, oval or rod-shaped. There is a significant variation in the micromorphology depending on which culture medium is used (Kim, 1976). Colonies, especially those of *A. equuli*, are typically sticky and difficult to remove from the agar surface, a characteristic that has been attributed to the presence of extracellular slime (Quinn *et al.*, 1994). The actinobacilli ferment carbohydrates, with acid production but no gas, within 24 h (Mayer, 1981, Quinn *et al.*, 1994). Most species, including *A. equuli*, produce urease and grow on MacConkey agar. However, the ability to grow on MacConkey agar has been reported to be a variable feature, dependent on type and batch of medium used (Mraz, 1983,

Sneath and Stevens, 1985), and in some studies equine actinobacilli did not grow on MacConkey agar (Blackall *et al.*, 1997). In the latest edition of Bergey's Manual of Systematic Bacteriology, which reflects the information available up to about 1981 (Philips, 1984), five species were listed under the genus *Actinobacillus*. In the 9th edition of Bergey's Manual of Determinative Bacteriology (Holt *et al.*, 1994), another six species had been added or transferred to this genus, and the taxonomy has not yet been comprehensively established. This is not surprising, since many bacterial species were defined as a result of subjective consideration at a time when more refined tests were missing. Diseases were known clinical entities before their causative agents were discovered, a fact that may have influenced the initial classification. Historically, classification of the *Pasteurellaceae* family has been based on growth factor requirements, a limited number of phenotypic charactersistics, and the ability to cause disease in vertebrates. Genetically based typing methods have led to a revised taxonomy, but phenotypic characters are still the basis for isolation and identification of clinical isolates. For phylogenetic studies, i.e. attempts to classify organisms based on evolutionary relationships rather than general resemblance, the comparison of 16S rRNA sequences is particularly useful (Olsen and Woese, 1993). Such sequences are available from different databases, for a vast number of bacteria. The universal regions of the 16S rRNA molecule are highly conserved between different species, while the so-called oligonucleotide signature sequences, that are situated in the evolutionary variable regions, are characteristic for a particular taxon. However, in some cases, DNA-DNA hybridisation may be more useful for the categorising of individual species (Stackenbrandt and Goebel, 1994).
After sequencing the 16S rRNA of a number of strains of different species within the family *Pasteurellaceae*, Dewhirst and co-workers (1993) concluded that the division of this bacterial family into phylogenetically and phenotypically coherent genera is very difficult. There are no obvious characteristics that clearly separate genus *Actinobacillus* from the other genera within the family.

Equine Actinobacillus *spp.*

Actinobacilli associated with clinical disease in horses have historically been reported as *A. equuli*. However, further identification of equine isolates has resulted in various reports on other *Actinobacillus* spp. (Chladeck and Ruth, 1976, Harbourne *et al.*, 1978, Zaharija *et al.*, 1979, Carman and Hodges, 1982, Baum *et al.*, 1984) as well as subtypes of, or new species related to, *A. equuli* (Bisgaard *et al.*, 1984, Jang *et al.*, 1985, Jang *et al.*, 1987, Samitz and Biberstein, 1991). It is exceedingly difficult to determine, from the reports on clinical cases, whether the cultured organism was *A. equuli sensu stricto* or some other, related, organism. Biochemical and cultural characteristics are sometimes, but not often, presented, and, for some biochemical tests, reactions vary between strains within the same species and the interpretation of some tests appears to vary between authors. For example, the reaction of *A. equuli* in the oxidase test is reported to be variable (Mayer, 1981, Philips, 1984, Quinn *et al.*, 1994) and its use for the

separation between the *Pasteurellaceae* and the nonmotile *Enterobacteriaceae* has been questioned (Bisgaard, 1982). The ability to ferment L-arabinose with acid production is another debatable feature as regards *A. equuli*. It has sometimes been reported as variable (Mayer, 1981, Philips, 1984), while others have stated that *A. equuli sensu stricto* does not ferment L-arabinose, although this is a constant feature of *Actinobacillus suis* (Frederiksen, 1973, Bisgaard *et al.*, 1984, Mutters, 1984). Carman and Hodges (1982), who isolated what they called *A. suis* from horses in New Zealand, stated that the ability of their isolates to ferment L-arabinose was difficult to interpret. L-arabinose positive strains of *A. equuli* have frequently been isolated from equine samples in the diagnostic laboratories of the Faculty of Veterinary Medicine and the National Veterinary Institute in Uppsala (Sternberg, 1998) and also in other parts of the world (Blackall *et al.*, 1997).

There are numerous reports on the isolation of *A. equuli* from swine (Zimmermann, 1964, Jones and Simmons, 1971, Bell, 1973, Werdin *et al.*, 1976, Jones, 1980, Okolo, 1987, Szazados, 1991) and *A. suis* from horses (Harbourne *et al.*, 1978, Carman and Hodges, 1982, Vaissaire *et al.*, 1988, Nelson *et al.*, 1996). The similarities and differences between *A. equuli* and *A. suis* have frequently been discussed (Kim *et al.*, 1976, Pedersen, 1977, Bercovier *et al.*, 1984, Escande *et al.*, 1984, Sneath and Stevens, 1985, Dewhirst *et al.*, 1992, Bada *et al.*, 1996). Antigenic cross-reactivity between *A. suis* and *A. equuli* has been demonstrated (Kim *et al.*, 1976, Bada *et al.*, 1996) and similarities in the outer membrane proteins of these two species have also been detected (Frey *et al.*, 1996, Hartmann *et al.*, 1996). Sequencing of the 16S rRNA gene of type strains of *A. equuli* and *A. suis* has indicated a very close relationship (Dewhirst *et al.*, 1992, Dewhirst *et al.*, 1993) and DNA-DNA hybridisation showed a 51-75% relatedness between *A. equuli* and *A. suis* strains (Escande *et al.*, 1984). Bercovier and co-workers (1984) characterised *Actinobacillus* strains by their morphology, physiology and biochemical reactions and found that some *A. equuli* strains clustered together with *A. suis* strains, while others formed a separate, but related, cluster. The authors stated that undue weight had been given to the animal hosts in species determination, and that reorganisation of the *Pasteurellaceae* should be based on DNA studies. Other authors have noted that host specificity is often observed in the *Pasteurellaceae* (Nicolet, 1990, Bisgaard, 1995), possibly related to the composition of the lipopolysaccharide in the outer membrane (Fenwick, 1995). It has also been stated that reports on isolations from species other than the known hosts should be questioned unless a thorough identification can be demonstrated. (Bisgaard *et al.*, 1984). The varying results of taxonomic studies are not entirely surprising. Calculations of diversity can be based on various similarity coefficients and using different clustering programs. All of these have their own merits but give different results. The reliability of phylogenetic trees has also been debated, especially as the choice of "outgroups" or the use of a different algorithm can significantly change the topology of the tree (Dewhirst *et al.*, 1992). However, further studies on equine actinobacilli have resulted in the re-classification of some strains previously identified as

A. equuli or equine *A. suis* (Blackall *et al.*, 1997). The characteristics of equine *Actinobacillus* strains designated Bisgaard's taxon 11, subtype I-IV (Bisgaard *et al.*, 1984) and *Actinobacillus suis*-like organisms (ASLO), subtype I-IV (Jang *et al.*, 1987, Samitz and Biberstein, 1991), have been published and subsequent clinical reports on infections with these organisms can also be found (Wilson and Madigan, 1989, Elad *et al.*, 1991, Mohan *et al.*, 1997). It would appear that subtype I of taxon 11 and subtype I of ASLO may actually be identical, but that the other subtypes differ. In the diagnostic laboratories of the Faculty of Veterinary Medicine and the National Veterinary Institute in Uppsala, the classification as presented by Bisgaard has been used for the past decade. The biochemical characteristics on which this typing system has been based are presented in Table 1. Considering the heterogeneity of this group of bacteria, a typing system based on only a few biochemical tests is, of course, far from perfect. However, it would be impractical to use too large a number of tests in routine diagnostics.

Table 1. Biochemical characteristics used for the identification of equine actinobacilli in the veterinary diagnostic laboratories in Uppsala

	A. equuli	Arabinose+ *A. equuli*	Taxon 11 biovar 1	Taxon 11 biovar 2	Taxon 11 biovar 3	Taxon 11 biovar 4
Ornithine	-	-	-	-	-	-
L-arabinose	-	+	-	-	-	-
Cellobiose	-	-	+	(+)	-	-
Mannitol	+	+	-	+	-	-
Salicin	-	-	+	(+)	-	(+)
Trehalose	+	+	+	+	+	+
Urease	+	+	+	+	+	+
Indole	-	-	-	-	-	-
Esculin	-	-	+	+	-	-

(+) sometimes positive reaction delayed

A common feature of the majority of the taxon 11 and ASLO strains is their ability to haemolyse sheep erythrocytes. A review of clinical reports on the isolation of *A. equuli* from diseased horses reveals that many of the described isolates were indeed haemolytic (Cottew and Francis, 1954, Wetmore *et al.*, 1963, Carter *et al.*, 1971, Kim, 1976), although this trait could be lost on storage or subculture (Hughes, 1972). Some older textbooks describe *A. equuli* as being usually non-haemolytic, but state that some strains are haemolytic (Philips, 1984, Timoney *et al.*, 1988, Quinn *et al.*, 1994). Some authors even describe *A. equuli* as being typically haemolytic (Whitford, 1976). Based on more recent taxonomic studies it can be concluded that *A. equuli sensu stricto* does not haemolyse sheep or horse erythrocytes, however, some other equine *Actinobacillus* spp. do. Many of the organisms in reports on *A. suis* from horses seem to match the criteria of either taxon 11 or ASLO, although some authors seem to think that all reports of *A. suis* in horses describe *A. suis sensu stricto* (Harbourne *et al.*, 1978, Nelson *et al.*, 1996). This is a questionable conclusion given the current state of taxonomy. The pathogenic potential of taxon 11 and ASLO has been debated, some authors

think that they are at best opportunistic pathogens that may occasionally cause disease (Kim, 1976, Blackall *et al.*, 1997). Other reports indicate that they may be more frequently associated with respiratory disease in adult horses than *A. equuli sensu stricto* (Sternberg, 1998, Wood *et al.*, 1998). A further study of clinical reports on *A. equuli* from swine gives a similar result as that on horses, with many of the described strains matching the criteria for taxon 11 or ASLO better than *A. equuli* (Bell, 1973, Schwartz, 1986, Fodor *et al.*, 1990). There are also a few reports on the isolation of *Actinobacillus lignieresii* from diseased horses (Chladeck and Ruth, 1976, Zaharija *et al.*, 1979, Baum *et al.*, 1984, Vaissaire *et al.*, 1988). *A. lignieresii* typically causes disease in ruminants. However, it is not clear whether the reported isolates were indeed *A. lignieresii* or another variant of equine *Actinobacillus* spp.
In conclusion, the taxonomic status of equine actinobacilli is still unclear, and future revisions, including the naming of new species besides *A. equuli*, are expected. It has been recommended that once genotypic groups have been established, distinguishing phenotypic tests should be re-evaluated for practical use (MacInnes and Borr, 1990). Despite several efforts in this field, the *Pasterurellaceae* family is still poorly defined, and have been described as "interesting but at times frustrating" (Macinnes and Smart, 1993). A definitive set of discriminating tests for the identification of equine actinobacilli is not yet available.

Equine disease caused by *Actinobacillus* spp.

Foal septicaemia

The most important disease complex associated with equine actinobacilli is that of neonatal foal septicaemia (Farrelly and Cronin, 1949, Kim, 1976, Mayer, 1981, Philips, 1984, Timoney *et al.*, 1988, Phillips, 1992). Usually, the infectious agent matches the criteria for *A. equuli sensu stricto* but, as discussed above, this identification cannot always be ascertained. Therefore, in the following text the infectious agents will be referred to as *Actinobacillus* spp., whether they are reported as *A. equuli*, *A. suis*, *A. suis*-like or simply actinobacilli.
Cases of foal septicaemia due to *Actinobacillus* spp. have been reported in all parts of the world, including North America (Shideler and Kelly, 1976, King, 1991, Yong and Griffin, 1992, Darien and Williams, 1993, Golenz *et al.*, 1994, Nelson *et al.*, 1996), South America (Lutzelschwab *et al.*, 1987), Australia (Cottew and Francis, 1954, Carter *et al.*, 1971), Europe (Radnai, 1976, Molenda *et al.*, 1992, Costecalde, 1997, Rycroft *et al.*, 1998), Africa (Mohan *et al.*, 1997), and Asia (Kamada *et al.*, 1985, Kanemaru *et al.*, 1985, Elad *et al.*, 1988, Al Darraji *et al.*, 1989, Varshney and Uppal, 1993). Infection with *Actinobacillus* spp. is mentioned as one of the most common causes of foal septicaemia in various reviews of foal disease (Gunning, 1947, Bain, 1963, Rossdale, 1972, Bredin, 1973, Platt, 1973, Rossdale and Leadon, 1975, Hosli, 1977, Knight, 1978, Liu, 1980, Dennis, 1981, Fiolka, 1982, Deem Morris, 1984, Carter and

Martens, 1986, Koterba, 1987, Madigan, 1987, Clabough, 1988). The frequency of isolation of *Actinobacillus* spp. from septicaemic foals ranges between 5% and 50%, depending on the report (Dimock *et al.*, 1947, Miller, 1950, Platt, 1973, Whitwell, 1980, Koterba *et al.*, 1984, Brewer and Koterba, 1986, Vaissaire *et al.*, 1987, Vaissaire *et al.*, 1988, Blood *et al.*, 1989, Wilson and Madigan, 1989, Brewer and Koterba, 1990, Raisis *et al.*, 1996), with the lowest frequencies being found in the more recent US studies. It is not clear whether the differences in isolation frequency between various studies reflects a true temporal and/or geographical difference or if the differences in isolation procedures, the interpretation of bacterial cultures or the presentation of results could fully explain the variations in prevalence. In some publications, cases with pathological lesions typical of *A. equuli* infection are included even if *A. equuli* could not be isolated by culture (Platt, 1977, Al Darraji *et al.*, 1989) and sometimes it is not clear as to whether the diagnosis was based on culture results or on post mortem examination only (Varshney and Uppal, 1993). Older publications generally report higher frequencies of *Actinobacillus* infection. A general improvement of hygienic conditions, horse feed, and the disease status of horses may have contributed to this apparent decrease in the prevalence of *Actinobacillus* septicaemia, as well as the prophylactic administration of penicillin to newborn foals that has become common in some parts of the world. In Sweden, based on samples taken in the horse clinic at the Faculty of Veterinary Medicine in Uppsala during the last decade, it can be estimated that approximately 20% of all positive blood cultures from foals yield growth of *Actinobacillus* spp.

The diagnosis of foal septicaemia has been extensively discussed (Rossdale and Leadon, 1975, Brewer and Koterba, 1986, Koterba, 1987, Madigan, 1987, Brewer and Koterba, 1988). It is obvious that a bacteriological culture from blood and/or local sites of infection is crucial in order to determine the cause of infection and choice of treatment, although this is not always clearly stated (Rossdale, 1972). A scoring method for the identification of foal septicaemia has been developed and is widely used (Brewer and Koterba, 1988). However, this is intended for quick diagnosis pending culture results, not as a substitute for bacteriological examinations. A negative blood culture does not necessarily rule out septicaemia (Madigan, 1987), and sometimes post mortem cultures reveal generalised infections that could not be identified *in vivo* (Koterba *et al.*, 1984). However, great care should be taken in interpreting positive post mortem cultures as evidence of a false negative or misleading blood culture, although this is frequently done (Wilson and Madigan, 1989, Brewer and Koterba, 1990). Despite claims to the contrary (Eisenfeld *et al.*, 1983, Brewer and Koterba, 1990), the post mortem invasion of intestinal bacteria can result in false positive post mortem organ cultures. Even truly positive post mortem cultures cannot rule out the possibility that some other infectious agent, or none at all, was present *in vivo* and was replaced by the presently detected organism at, or just before, death. For example, antimicrobial treatment may suppress a susceptible primary infectious agent, leaving room for a more resistant microbe to invade an already

critically ill foal. This may contribute to the finding of a high prevalence of infections with *Escherichia coli* and *Klebsiella* spp., that are resistant to several antimicrobial substances, in neonatal intensive care units (Brewer and Koterba, 1990). Primary infections with bacterial agents such as streptococci and actinobacilli, that are generally more susceptible to antimicrobial substances, may go undetected in some cases where no immediate blood culture is performed.

Foals with septicaemia due to infection with *Actinobacillus* spp. are characteristically depressed and feverish, they refuse to suckle and quickly become prostrate. The clinical symptoms usually appear shortly after birth, and almost always within the first week of life. Some foals may show symptoms of polyarthritis, enteritis, or pneumonia. On post mortem examination, lesions may be absent in cases of peracute death, or they may appear in the form of peteachiae, microabscesses, or necrotic foci in various parenchymatous organs such as the kidneys, liver, lungs and spleen. The renal lesions are usually the most prominent. A purulent glomerulonephritis, with microabscesses sometimes being visible on macroscopic examination of the renal cortex, has been recognised as being almost pathognomonic for *A. equuli* infection (Doll, 1963) and it has been suggested that this bacterial species has a predilection for the kidneys (Rossdale, 1972). However, adhesion studies using Swedish strains of *A. equuli* on equine renal cells have not been able to confirm this hypothesis (Sternberg, unpublished results). The presence of Gram-negative coccobacilli in various organs is common (Papparella *et al.*, 1987). The pathological lesions may be explained by the presence of a bacteriaemia that causes great exposure of vascular tissue, particularly in organs with a slow blood flow in fine capillaries (Farrelly and Cronin, 1949), and by an activation of the complement cascade and inflammatory mediators (Darien and Williams, 1993). The extent of the lesions usually reflects the course of the disease, with peracute cases lacking visible lesions while microabscesses, septic thrombi and necrosis are more evident in protracted cases. Cases are usually sporadic (Derksen, 1983, Phillips, 1992), but occasional outbreaks involving several foals in the same season or foals from the same dam in consecutive years have been reported (Baker, 1972, Kim, 1976, Philips, 1984, Yong and Griffin, 1992). It is not known whether some common trigger factor was involved in reported outbreaks.

Other diseases

Although a frequent cause of disease in the newborn foal, the role of actinobacilli in the aetiology of abortion is debatable. Cases of abortion associated with the isolation of *Actinobacillus* spp. have been reported (Prickett, 1970, Webb *et al.*, 1976, Vaissaire *et al.*, 1986, Vaissaire *et al.*, 1988), but mostly as a rare event (Dimock *et al.*, 1947, Bain, 1963, Whitford, 1976, Whitwell, 1980) and it has been questioned whether bacterial infection is ever the primary cause of abortion (Platt, 1975, Tizard, 1996).

Arthritis is a common feature of *Actinobacillus* spp. infection in foals (Thein, 1981, Poyade-Alvarado and Marcoux, 1993, Dolvik and Gaustad, 1995,

Waterhouse and Marr, 1997, Franzen, 1998), usually as a sequel to septicaemia. In adult horses, *Actinobacillus* spp. have occasionally been isolated from synovia, wounds, surgical sites and abscesses (Snyder *et al.*, 1987, Kanoe *et al.*, 1988, Vaissaire *et al.*, 1988, Peremans *et al.*, 1991). More generalised infections such as pericarditis (Dill *et al.*, 1982) and endocarditis (Roussel and Kasari, 1989, Hillyer *et al.*, 1990, Maxson and Reef, 1997) in adult horses have also been associated with *Actinobacillus* spp. Actinobacilli appear to be a fairly common cause of peritonitis, with migrating larvae of *Strongylus vulgaris* being mentioned as a probable predisposing factor (Gay and Lording, 1980, Golland *et al.*, 1994), and have occasionally been found in cases of enteritis (Al Mashat and Taylor, 1986) or periorchitis (Belknap *et al.*, 1988). The majority of *Actinobacillus* spp. infections in adult horses, however, are opportunistic respiratory tract infections. These range from retropharyngeal infections (Todhunter *et al.*, 1985), subepiglottis cysts (Stick and Boles, 1980), sinusitis (Harbourne *et al.*, 1978, Elad *et al.*, 1991) and tracheitis (Ward *et al.*, 1998) to bronchopneumonia (Bayly *et al.*, 1982, Hoffman *et al.*, 1998, Wood *et al.*, 1998), pleuropneumonia (Collins *et al.*, 1994) and necrotising pneumonia (Carr *et al.*, 1997). Usually, the case history in such reports involve predisposing factors such as viral infections or trauma.

In summary, the major diseases caused by equine actinobacilli are septicaemia in foals and respiratory infections in adult horses, but various other opportunistic infections are also associated with these bacteria. This situation is reflected in reports from diagnostic laboratories (Whitford, 1976, Lavoie *et al.*, 1991). Similar disease patterns have been noted in wild equidae, such as zebras (Bath, 1979, Penzhorn, 1984).

Some of the different types of *Actinobacillus* spp. isolated from various types of infectious disease, in horses of different ages, are shown in Tables 2a-b. The figures are based on case records from the diagnostic laboratories of the Faculty of Veterinary Medicine, and the National Veterinary Institute in Uppsala from August 1991 to May 1997.

Table 2a. No. of isolates of different subtypes of *Actinobacillus* spp. isolated from horses of various age.

Subtype	Age		
	<1 mo	1mo-1yr	>1 yr
A. equuli	25	6	18
Arabinose+ *A. equuli*	9	4	10
Taxon 11 biovar 1	7	9	48
Taxon 11 biovar 2	3	3	29
Taxon 11 biovar 3	0	1	1
Taxon 11 biovar 4	0	0	1
No. of horses	44	23	107

Table 2b. No. of isolates of different subtypes of *Actinobacillus* spp. isolated from horses with various types of disease.

Subtype	Septicaemia	Respiratory disease	Arthritis	Wound or abscess	Other
A. equuli	25	14	3	4	2
Arabinose+ *A. equuli*	8	12	2	1	0
Taxon 11 biovar 1	4	38	11	6	5
Taxon 11 biovar 2	3	26	0	4	2
Taxon 11 biovar 3	0	2	0	0	0
Taxon 11 biovar 4	0	0	1	0	0
No. of horses	40	92	17	15	9

A. equuli infection in non-equidae

As previously mentioned, bacteria described as *A. equuli* have been isolated from pigs, mainly in association with neonatal septicaemia in piglets (Zimmermann, 1964, Bell, 1973, Schwartz, 1986, Okolo, 1987), endocarditis in both piglets and adult swine (Jones and Simmons, 1971, Jones, 1980, Roussel and Kasari, 1989, Szazados, 1991), and abortion (Werdin *et al.*, 1976). However, it would appear from the biochemical reactions presented in some of these reports that not all isolates were *A. equuli sensu stricto*. The same might be suspected of the reports on the isolation of *A. equuli* from laboratory rodents (Lentsch and Wagner, 1980, Simpson and Simmons, 1980), lagomorphs (Kim, 1976, Philips, 1984), goats (Ameh *et al.*, 1993), calves (DuPlessis *et al.*, 1967, Osbaldiston and Walker, 1972) and a dog (Philips, 1984). Isolation of *A. equuli* from primates (Kim, 1976, Philips, 1984), including humans (Sakazaki *et al.*, 1984) has also been described, with some cases of human disease being linked to horse contact (Peel *et al.*, 1991, Richard *et al.*, 1991). Again, these isolates do probably not all represent *A. equuli sensu stricto*.

Pathogenesis of equine *Actinobacillus* infection

Source of infection

Actinobacillus infections in horses are typically opportunistic. Actinobacilli are commensals of the mucous membranes of healthy animals (Biberstein, 1990, Phillips, 1992, DeBey *et al.*, 1996, Costecalde, 1997) and are readily isolated from the oropharynx (Platt, 1973, Whitwell, 1980, Bisgaard *et al.*, 1984, Philips, 1984, Elad *et al.*, 1988, Timoney *et al.*, 1988, Bailey and Love, 1991, Samitz and Biberstein, 1991). The organisms have, albeit rarely, been isolated from the cervix of mares (Cottew and Francis, 1954, Fluge *et al.*, 1972, Platt, 1973, Varshney *et al.*, 1994) both in the absence and presence of concurrent reproductive problems. Several authors state that *Actinobacillus* spp. are commonly found in the intestinal tract of horses (Gay and Lording, 1980, Philips, 1984, Timoney *et al.*, 1988). However, the actual reports on the isolation of actinobacilli from faecal or intestinal material are rare (Laudien, 1923, Al Mashat and Taylor, 1986, Elad *et al.*, 1988). The study by Laudien appears to form the

basis for many statements regarding the presence of *Actinobacillus* spp. in horse faeces. However, the isolation procedure used by Laudien has not, so far, yielded any *Actinobacillus* isolates from faecal samples from Swedish horses, despite repeated efforts (Sternberg, unpublished results). An attempt to culture a number of equine *Actinobacillus* strains on the selective medium described by Laudien revealed no growth of these strains. In comparison, *Escherichia coli* was easily cultured (Fig. 1).

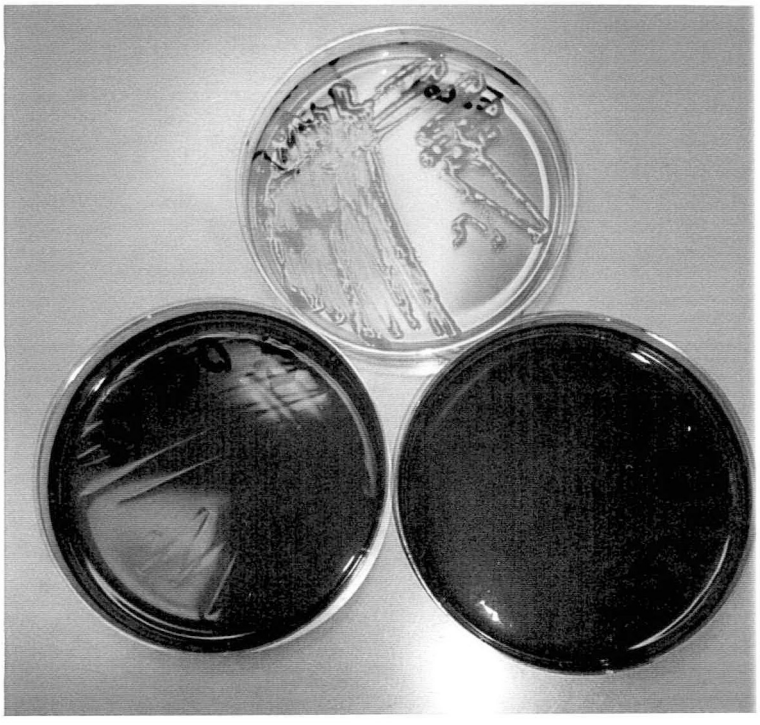

Fig. 1 Culture of *E. coli* (yellow colour change, profuse growth) and *A. equuli* (blue, no colour change, no or little growth) on the selective culture medium used by Laudien. The size of inoculum was identical for all plates.

In the report by Elad, colonies similar to those of *A. equuli* isolated from the sick foal were seen on primary culture of pharynx and faecal swabs from the mare, on both blood agar and MacConkey agar. It is not clear if the isolates from the mare were further identified. Al Mashat isolated *Actinobacillus* spp. from the small and large intestine of 11 out of 25 necropsied horses, from both normal-looking and congested mucosa with excess mucus. However, Cottew and Francis (1954) failed to culture actinobacilli from rectal swabs of horses in which *Actinobacillus* spp. had been found in the mouth and cervix. Based on this evidence, it may be concluded that *Actinobacillus* spp. are commonly present in the oropharynx and perhaps in the gastrointestinal tract, but that they are not abundant in faeces or genitalia. Thus, the pharynx of the mare would appear to be the most probable

source of a postnatal infection in the foal, most likely conveyed by the mare licking the foal. With regard to respiratory tract infections in adult horses, the oropharynx would seem to be the most likely source of *Actinobacillus* spp. The number of actinobacilli present in the nasal and oral cavities have been noted to increase in horses that were experimentally infected with respiratory viruses (Sutton *et al.*, 1998) or exposed to transport stress (Foreman *et al.*, 1991, Raidal *et al.*, 1995).

Time of infection

Because of the characteristically early age of onset of clinical disease, the possibility of prenatal infection has been proposed. The presence of *Actinobacillus* spp. in aborted foetuses would suggest a prenatal infection (Dimock *et al.*, 1947) and it has been suggested that such infections are ascending and secondary (Platt, 1975). However, it is generally believed that most neonatal infections are contracted at, or shortly after, birth and that the acute onset and generally short and fatal course of the disease does not dispute this assumption (Dimock *et al.*, 1947, Gunning, 1947, Doll, 1963, Platt, 1973, Kim, 1976, Webb *et al.*, 1976, Blood *et al.*, 1989).

Route of infection

Actinobacillus bacteria gain entrance to the host via the oral, nasal or, in the case of newborn foals, the umbilical route. Another possible port of entry is through a wound or abrasion. In foal septicaemia, the foal is generally believed to ingest or inhale the infectious organism, or become infected via the umbilicus (Doll, 1963, Kim, 1976, Mayer, 1981, Derksen, 1983, Blood *et al.*, 1989). Once the bacteria enter the systemic circulation, they proliferate, disseminate, and cause damage to various organs, via both direct and indirect mechanisms. In respiratory tract infections the obvious route of infection would be via inhalation or aspiration of the bacteria from the upper respiratory tract, something that would be facilitated by impairment of the mucociliary transport mechanism, as caused by a viral infection, transport stress, or some other predisposing factor.

Predisposing factors

Actinobacillus infections are typically opportunistic and in general, some predisposing factor has to be present in order for disease to occur (Biberstein, 1990). This could be in the form of a concurrent infection (Kim, 1976), an environmental problem such as poor feed quality (Radnai, 1976), or a weakened or immature immune system of the host animal. Predisposing factors as regards foal septicaemia, such as an abnormal gestation length, dystocia, a poor environment or management, have been extensively discussed. However, the failure of passive transfer, i.e. the lack of uptake of colostral immunoglobulins, has been mentioned as probably the most important factor (Deem Morris, 1984, Koterba *et al.*, 1984, Brewer and Koterba, 1986, Carter and Martens, 1986, Clabough, 1988). The failure of passive transfer (FPT) may be due to a number

of factors: I) A low immunoglobulin (Ig) content of the colostrum caused by premature lactation, premature foaling or a failure to concentrate Ig in the mammary gland. II) A lack of intake of colostrum due to a weak foal failing to suckle, or poor mothering. III) A failure to absorb Ig in the intestine due to neonatal stress. The theory that stress lies behind the failure to absorb Ig is supported by the fact that the experimental administration of corticosteroids impairs Ig uptake (Jeffcott, 1974a). Some estimates suggest that as many as 10-25% of all neonatal foals fail to absorb sufficient Ig (McGuire *et al.*, 1977, Tizard, 1996). It has been demonstrated that foaling early in the season, poor general health or the birth of an immature foal is associated with an increased risk of FPT (Clabough *et al.*, 1991). In addition, foals born to older mares (>15 years old) and those born in a colder climate with little sunshine (LeBlanc *et al.*, 1992), are also at increased risk. It is generally believed that the foal needs to acquire a serum concentration of IgG above 800 mg/dl in order to get adequate protection from infection. Levels below 200 mg/dl are regarded as total FPT and concentrations between 200 and 400 mg/dl indicate partial FPT. In septicaemic foals, however, a low serum level of IgG is not necessarily caused by FPT, it can be caused by the infection itself consuming immunoglobulin (Robinson *et al.*, 1993).

In a review of neonatal foal infections, Knight (1978) stated that: "The foal's environment is not, as some fear, an armed camp of hostile microbes waiting to pounce. For the most part, the vast majority of bacteria are impartial and, if anything, are interested in a symbiotic relationship". This harmonious state is only disrupted when host defence mechanisms fail.

Foal immunology

Ontogeny

The immune system develops early in foetal life (Osburn *et al.*, 1982, Dudan *et al.*, 1990, Tizard, 1996), with the cellular and humoral aspects appearing sequentially. Lymphoid tissue and circulating lymphocytes appear in the first trimester, functional T lymphocytes are present at about week 14 and functional B lymphocytes at week 29 (Perryman *et al.*, 1980), but not all antigens are equally capable of stimulating foetal lymphocytes (Tizard, 1996). Production of antibody in response to some viral antigens has been demonstrated in lymphocytes from equine foetuses (Martin and Larson, 1973, Morgan *et al.*, 1975). Complement effects, such as haemolysis and coagulation, can be induced at 4 months' gestation. Although foals are essentially agammaglobulinaemic at birth, it is sometimes possible to detect IgG in the foetus in the last trimester or in neonates that have not suckled. This is thought to be caused by intrauterine antigenic stimulation (Osburn *et al.*, 1982, Dudan *et al.*, 1990).

The neonate

The neonatal foal lacks specific immunity and the non-specific immune mechanisms are immature. At birth, the concentration of circulating B

lymphocytes is only one third that of the adult and it does not reach adult levels until the foal is 3 weeks old (Banks, 1982). In some animal species, a correlation between high levels of corticosteroids in the newborn and a reduced cell-mediated immune response has provided a plausible explanation for neonatal immunosuppression. The excessive activity of suppressor T lymphocytes or immature B lymphocytes may also account for an inability to produce antibody in response to some stimuli (Banks, 1982). Studies on neutrophils from neonatal foals have shown that there is an increased random migration and a decreased stimulated migration in foal neutrophils, as compared to neutrophils from their dams (Morris *et al.*, 1987), while the phagocytosis and killing capacity is similar (Morris *et al.*, 1987, Wichtel *et al.*, 1991). However, caution has been suggested in the interpretation of results from neutrophil studies, as there are many sources of variation in the *in vitro* models used (Morris *et al.*, 1987, Wichtel *et al.*, 1991). The serum of equine neonates appears to be deficient in some complement factors, and, consequently, is poor in its opsonic activity (Lavoie *et al.*, 1990, Bernoco *et al.*, 1994, Tizard, 1996). Complement levels increase rapidly in colostrum-deprived foals, indicating a response to antigenic stimulation (Bernoco *et al.*, 1994). In contrast, in foals given colostrum there is an initial decrease in serum complement, concurrent with an increase in IgG, possibly due to the utilisation of complement in the formation of immune complexes (Bernoco *et al.*, 1994). The response of neonatal foals to endotoxin infusion is similar to that of adult horses (Lavoie *et al.*, 1990), although it is somewhat diminished in colostrum-deprived foals (Allen *et al.*, 1993). This observation indicates that the neonatal immune system is fully capable of mounting an inflammatory response, but that some colostral factors may facilitate this.

Passive transfer of immunity

The mechanisms of passive transfer of immunoglobulins from mare to foal have been well studied and described (Jeffcott, 1971, McGuire and Crawford, 1973, Jeffcott, 1974a, Jeffcott, 1974b, Jeffcott, 1974c, Jeffcott, 1975, Banks, 1982, Norcross, 1982, Kohn *et al.*, 1989, Saikku *et al.*, 1989, Dudan *et al.*, 1990, Tizard, 1996). The equine placenta is epitheliochorial and therefore impermeable to macromolecules. Thus, apart from low levels of endogenous IgM, the foal is essentially agammaglobulinaemic at birth, and, in the absence of colostral Ig, any immune response will be a primary and slow one. The colostrum contains all Ig classes but is dominated by IgG and, to a lesser extent, IgA. The mammary gland synthesises IgA, but IgG is derived from serum and is actively concentrated in the mammary gland shortly before parturition by an as not yet fully understood mechanism. The transfer of serum IgG to colostrum causes a lowering of blood IgG levels in the mare. The Ig content of colostrum varies between different animal species and possibly also between breeds within the same species. Shortly after foaling, the mammary secretion changes from colostrum to milk, containing proportionally more IgA than IgG. Most of the secretory IgA appears to remain on the mucosal surfaces in the upper gastrointestinal tract, providing local protection in the oropharynx and the small intestine. Due to the low proteolytic

activity in the neonatal gut, and the presence of antitrypsin in colostrum, Ig molecules are mainly intact when they reach the small intestine, where they are pinocytosed by specialised cells in the epithelium that take up macromolecules. This uptake is non-selective and any large molecule will be absorbed. For example, the foal intestine will also absorb radiolabelled polyvinyl pyrrolidone, (Jeffcott, 1974c) or bovine Ig (Holmes and Lunn, 1991). The macromolecules accumulate as intracellular globules until the cell is full, then they are released via the base of the cell into the intercellular space and transported via the lymphatics to the blood. The specialised cells are quickly replaced by more mature epithelial cells. It takes some 3 h for an ingested macromolecule to reach the blood. Usually, the absorptive capacity of the neonatal gut lasts for approximately 24 h, but it is most effective just after birth. Starvation may prolong the period of capacity for macromolecule absorption. Other, as yet unidentified, colostral factors appear to favour the absorption of macromolecules. After the first 24 h of life, the foal has attained maximum levels of serum Ig. These levels gradually decline, as immunoglobulins are metabolised and are diluted when the blood volume increases as the foal grows. By the age of 5 months, all maternal Ig has disappeared. The presence of maternal Ig appears to exert a negative feed-back on endogenous Ig production and the foal does not attain sufficient levels of all Ig classes until 4 months of age. Apart from immunoglobulins, colostrum also contains cytokines, lymphocytes and some other, as yet unidentified, factors that appear to support the neonatal immune system. Maternal lymphocytes may be transferred from colostrum via transintestinal migration, but the biological significance of this remains unclear.
Although a great deal is known about the ontogeny and function of the immune system of the neonatal foal, most studies are hampered by the inherent dangers in investigating individual immune system components separately, when the interplay between various factors determine the result of an immunological challenge.

Bacterial virulence attributes

All bacteria that have a pathogenic potential possess some virulence attributes. Selective pressure exerted by host defence mechanisms benefits bacterial clones with a natural ability to resist those mechanisms. This ability may be due to extracellular or cell wall components, toxic metabolites, alternate metabolic pathways etc.

Capsule

Some actinobacilli, such as *A. pleuropneumoniae*, produce extracellular capsules that interfere with opsonisation, phagocytosis and complement-mediated killing (Fenwick, 1995). Capsules in the *Pasteurellaceae* are serotype-specific and appear to be correlated to virulence (Inzana, 1990). Equine actinobacilli are reported to lack capsule (Kim, 1976, Philips, 1984), but usually produce extracellular slime, the function of which is as yet unknown.

LPS

Lipopolysaccharide (LPS), also known as endotoxin, forms part of the outer cell membrane of all Gram-negative bacteria. LPS is not a classic virulence attribute, although it is often mentioned as one, as its main contribution to pathogenicity is the activation of host defence systems that ultimately cause clinical signs and tissue damage. The pro-inflammatory and antigenic nature of LPS plays a critical role in the elimination of the infection. The host response to LPS often seems to be in excess of what is necessary to clear the infection, and it has been suggested that if this feature is consistent for a certain bacterial species or strain, LPS might be regarded as a true virulence factor (Fenwick, 1990). It has been noted that the LPS structure can be correlated with pathogenic potential and host specificity in the *Pasteurellaceae* (Fenwick, 1995). The structure may vary between strains and between different growth stages of the same strain. LPS that is released from bacteria can bind to neutrophils and alter their adhesiveness and locomotive properties, which partly explains the neutropenia seen in experimental endotoxin infusion and infections with Gram-negative bacteria (Wilson, 1985). LPS can also affect the microbicidal properties of neutrophils. However, some effects of LPS on neutrophils may be secondary and linked to complement activation. There are differences in LPS between different bacterial strains, but LPS prepared by different methods from the same bacterial strain can also act differently in an experimental situation (Wilson, 1985, Fenwick, 1990). The biological activity of LPS released *in vivo* is also different from that of purified LPS, due to interactions with serum proteins. Thus, the toxic effects of LPS on leukocytes, platelets and the vascular endothelium that are observed *in vitro* should be interpreted with caution.

Adhesins

A variety of adhesins, with receptors on a variety of cell types in different host animals, have been described for the *Pasteurellaceae* family (van Alphen, 1995), but not, so far, for equine actinobacilli. Electron microscopy of *Actinobacillus* spp. isolated from swine showed no presence of fimbriae (Fodor *et al.*, 1990). In addition, no fimbriae were observed by electron microscopy of two Swedish strains of *Actinobacillus* spp. from horses (Sternberg, unpublished results).

Iron acquisition

Iron is an essential growth factor for bacteria and a continuous iron supply is necessary for electron transport, energy metabolism and DNA synthesis. Free Fe^{3+} is not readily available in the bacterial environment. Most of the host's iron supply is intracellular and unavailable, and the extracellular iron is bound to host glycoproteins such as transferrin and lactoferrin. Some bacteria produce siderophores, compounds that chelate iron, but equine actinobacilli are apparently incapable of siderophore production. Instead, they possess receptors that bind host transferrin to the bacterial cell surface (Kirby *et al.*, 1995).

Leukotoxin

A common feature among the *Pasteurellaceae* is the ability to produce leukotoxin. The leukotoxins in question belong to the RTX group (repeats in toxins) of which the *E. coli* haemolysin (Hly) is regarded as the prototype (Welch *et al.*, 1992, Menestrina *et al.*, 1994). These toxins contain highly conserved regions of glycine-rich Ca^{2+}-binding tandem repeats of amino acid peptide sequences in the N-terminal portion of the structural toxin molecule (Clinkenbeard *et al.*, 1992, Welch *et al.*, 1992, Menestrina *et al.*, 1994, Czuprynski, 1995). The toxic mechanism is similar to that of the complement membrane attack complex, insertion of the hydrophobic portion of the toxin molecule into the target cell membrane forms transmembrane pores, resulting in colloid osmotic imbalance with subsequent swelling and cytolysis (Clinkenbeard *et al.*, 1992, Welch *et al.*, 1992, Menestrina *et al.*, 1994, Czuprynski, 1995). The binding of the toxin molecule to the leukocyte is thought to be mediated by a protein receptor (Brown *et al.*, 1997) and once it is inserted into the bacterial membrane, the toxin behaves as an intrinsic protein and cannot simply be washed off (Menestrina *et al.*, 1994). The lysis of leukocytes leads to the release of lysosomal enzymes and subsequent tissue damage. Tissue damage may enhance bacterial growth by providing a ready source of bacterial nutrients. The cytolytic activity of RTX leukotoxins appears to be dose-dependent, with smaller concentrations activating neutrophils and larger concentrations killing them (Czuprynski *et al.*, 1991, Maheswaran *et al.*, 1992), and the existence of more than one signal pathway responsible for activation and lysis, respectively, has been suggested (Welch *et al.*, 1992, Brown *et al.*, 1997). Similar effects have been observed with other leukocytes, but these have been less studied (Czuprynski, 1995). However, it has been proposed that at least some of the stimulatory effects observed in some experiments were caused by LPS present in the toxin preparation, exerting its effect alone or in combination with leukotoxin (Stevens and Czuprynski, 1995). A relationship between the production and biological activity of RTX toxin and LPS has been suggested (Czuprynski, 1995). The genes encoding the various RTX toxins are somewhat similar, with most of the operons consisting of an A gene encoding the structural toxin, a C gene encoding the protein required for the modification and activation of the A protein product, and B and D genes encoding proteins required for transport and excretion of the toxin molecule (Lo, 1990, Welch *et al.*, 1992, Menestrina *et al.*, 1994, Van Ostaaijen *et al.*, 1997). RTX genes and their toxin products have been identified in various members of the *Pasteurellaceae* family, such as *Mannheimia (Pasteurella) haemolytica* (Baluyut *et al.*, 1981, Lo *et al.*, 1987, Strathdee and Lo, 1989), *Actinobacillus pleuropneumoniae* (van Leengoed *et al.*, 1989, Rycroft *et al.*, 1991, Devenish *et al.*, 1992, MacDonald and Rycroft, 1992, Frey, 1995), *Actinobacillus actinomycetemcomitans* (Tsai *et al.*, 1984, Kolodrubetz *et al.*, 1989) and *A. suis* (Burrows and Lo, 1992, Van Ostaaijen *et al.*, 1997). Some of these toxins, such as the *M. haemolytica* leukotoxin, are species specific and only affect leukocytes from the usual host animal, while others, such as the 3 toxins of *A. pleuropneumoniae*, affect leukocytes from other

animals as well (Czuprynski, 1995). Comparisons of the RTX genes from different bacterial species have shown that they are related (Strathdee and Lo, 1987, Lo, 1990, Welch *et al.*, 1992) and known RTX genes have been used as probes to identify RTX determinants in the genomes of other bacterial species (Kuhnert *et al.*, 1997). Such studies have demonstrated the presence of RTX genes in equine actinobacilli (Lo, 1990, Burrows and Lo, 1992, MacDonald and Rycroft, 1992), but so far no further attempts to clone, sequence and characterise these determinants have been published.

Antimicrobial resistance

As many actinobacilli are susceptible to β-lactams, penicillin treatment has been recommended when *Actinobacillus* infection is suspected (Bredin, 1973). Usually, as in the case of foal septicaemia, when therapy must be initiated before culture results are available, penicillin is given in combination with an aminoglycoside (Knight, 1978, Liu, 1980, Carter and Martens, 1986, Clabough, 1988, Brewer and Koterba, 1990). However, it should be kept in mind that the temporal and geographic variations in antimicrobial susceptibility patterns are substantial. For example, in 1949, the hope was expressed that streptomycin would soon be available for the treatment of equine *Actinobacillus* infections (Farrelly and Cronin, 1949) and by 1986, it was noted that aminoglycoside resistance was becoming more and more common among bacterial isolates from horses (Brewer and Koterba, 1986). Geographic variations in susceptibility patterns may be due to differences in antimicrobial usage, but also differences in movement and contact patterns of horses in different areas. A lot of variation is seen in the reported susceptibility data of equine actinobacilli (Jang *et al.*, 1987, Snyder *et al.*, 1987, Vaissaire *et al.*, 1988, Lavoie *et al.*, 1991, Ensink *et al.*, 1993, Golland *et al.*, 1994, van Duijkeren *et al.*, 1995). However, it is often impossible to compare these data due to the great variation in laboratory techniques used and the way that results are presented.

Although generally susceptible to β-lactams, the production of β-lactamase has been observed in several species of *Pasteurellaceae*, such as *M. haemolytica* (Azad *et al.*, 1992, Wood *et al.*, 1995), *P. multocida* (Philippon *et al.*, 1986), *A. pleuropneumoniae* (Juteau *et al.*, 1991, Chang *et al.*, 1992), and *Haemophilus influenzae* (Medeiros *et al.*, 1986). The enzymes have been identified as being of the ROB-1 type, with the encoding genes commonly residing on transferable plasmids. There are various other types of β-lactamases, which vary in structure, spectrum of activity, and stability (Livermore, 1996). The encoding genes appear to vary in their expression in different bacterial hosts, resulting in different minimum inhibitory concentrations (MIC) when they are transferred from one bacterial species to another (Brive *et al.*, 1977), something that might interfere with the interpretation of transfer experiments.

Most figures regarding the prevalence of antimicrobial resistance concern bacteria that are commonly found in clinical samples. Little information is available on the resistance patterns in the predominant normal bacterial microflora. However, in the case of opportunistic pathogens such as equine

Actinobacillus spp. the strains that are present in the normal flora are the potential disease-causing agents and therefore the susceptibility patterns of the isolates from the normal flora may be of equal clinical interest. Due to the high risk of side effects caused by disturbances of the intestinal microflora in this animal species, only a limited range of antimicrobial substances are available to horse practitioners. In view of this circumstance, it is important to monitor the antimicrobial susceptibility patterns of horse pathogens.

Aims of the present study

The overall aim of this work was to generate information regarding various aspects of equine *Actinobacillus* spp. in general and, in particular, *A. equuli* as a cause of foal septicaemia. This goal can be further specified as:

- To study the prevalence and epidemiology of *A. equuli* in the oral flora of healthy horses.
- To investigate the molecular epidemiology of *A. equuli* and, by comparing clinical isolates to isolates in the normal flora, to evaluate the phylogenetic differences between *A. equuli* strains causing disease and those found in the normal flora.
- To study the effects of culture supernatants of equine actinobacilli on equine neutrophils, as an indicator of possible leukotoxin production in these bacteria.
- To determine if *Actinobacillus* spp. present in the oral cavity of healthy horses provoke an immune response in the host animal and if this immune response is transferred from mare to foal.
- To demonstrate the patterns of antimicrobial susceptibility of Swedish isolates of equine *Actinobacillus* spp., not only in order to provide a basis for therapeutic choices, but also to emphasise the importance of resistance monitoring of opportunistic bacteria.

Comments on material and methods

Further details on the methods used are given in papers I-V.

Animals (Paper I, III, and IV)

The horses in the study all came from the Uppsala region and exhibited no signs of clinical disease. The breed varied but the majority were either Swedish Warmblood or Standardbred. Sampling was performed by the author or, in the case of some of the newborn foals and their mothers, the horse owners who were veterinarians. Blood samples were taken from the jugular vein with a Vacutainer® tube and culture samples were taken using a cotton swab applied to the buccal part of the oral cavity. Colostrum samples were taken by milking into a sterile plastic tube. All experiments were approved by the Ethical Committee for Animal Experiments, Uppsala, Sweden

Bacterial culture and identification (Papers I-V)

The clinical isolates, (i.e. bacteria isolated from horses with clinical disease) were all derived from samples submitted to diagnostic laboratories and had been previously identified by standard biochemical tests. All isolates were tested and, if applicable, reclassified according to the categories shown in Table 1 in the introduction. Isolates not matching any of these categories were designated *Actinobacillus* sp. Oral samples from healthy horses were cultured on blood agar, with and without the addition of 0.5 mg/l of clindamycin. This concentration is far below the minimum inhibitory concentration of any clinical isolate of *Actinobacillus* spp. in this study, but enough to impair the growth of most Gram-positive bacteria, thereby facilitating the detection of *Actinobacillus* colonies. This culture medium did not appear to inhibit the growth of the bacteria selected for, in contrast to some of the more selective media used for other members of the *Pasteurellaceae* family (Holm *et al.*, 1987). Cultures were incubated at 37°C in an aerobic environment and examined after 24 and 48 h. Isolates matching *Actinobacillus* spp. (greyish, round colonies, small pleomorphic Gram-negative rods) were further identified according to Table 1. In papers I and II, only *A. equuli sensu stricto* and L-arabinose positive *A. equuli* strains were studied. The reason for this was that these two subtypes are more commonly associated with foal septicaemia in the Uppsala region, as compared to other *Actinobacillus* spp.

Pulsed-field gel electrophoresis (Paper I)

The characterisation of DNA by electrophoresis is limited by the difficulty in obtaining suitable fragments of chromosomal DNA. Large DNA molecules in solution are prone to shearing and agarose gel electrophoresis using constant fields cannot resolve DNA molecules in the appropriate size range between 40 and 4000 kbp (Maslow *et al.*, 1993). This limitation can be overcome by *in situ* lysis of bacteria embedded in agarose and the subsequent digestion of DNA directly in the agarose, with restriction enzymes that have rare recognition sites, and separation of the resulting fragments in agarose gels by pulsed-field gel

electrophoresis (PFGE). By PFGE, the total genomic DNA can be resolved into a limited number of restriction fragments with distinct migration speeds. For the PFGE of actinobacilli, fresh TSB broth cultures were washed, mixed with melted agarose and lysozyme and allowed to set in the wells of a plastic mould. The agarose plugs were then incubated overnight with lysozyme, followed by proteinase K for 48 h, and subsequently digested with *Sma*I (Life Technologies, Inchinnan, UK) and *Apa*I (Amersham Pharmacia Biotech, Uppsala Sweden), in two separate experiments. The restriction fragments were separated by PFGE in a 1% agarose gel in a Gene Navigator system (Amersham Pharmacia Biotech, Uppsala, Sweden). A separate program was used for each restriction enzyme, after which the gel was stained with ethidium bromide and photographed under ultraviolet light. Duplicate plugs were made after five repetitive subcultures on blood agar, with both sets of plugs being used in the analyses, in order to determine the reproducibility of the experiments.

Biochemical fingerprinting (Paper II)

Biochemical fingerprinting is a phenotyping method in which the kinetics of several biochemical reactions are recorded in order to give a specific pattern for each bacterial strain. The method used in this study was described by Kühn (1985) and has since been developed into a commercial system with 7 different sets of tests for different bacterial species (Möllby *et al.*, 1993). This so-called Pheneplate (PhP) system (BioSys inova, Stockholm, Sweden) consists of microtitre plates containing dehydrated biochemical substrates. The results from the biochemical tests are obtained as spectrophotometric absorbance values that are transformed into biochemical fingerprints by using a computer program. The biochemical fingerprints can then be used for calculations of similarities and clustering. In this study the PhP-48 plate, containing 48 biochemical tests (including two control wells), was used. Bacteria were suspended in proteose peptone substrate (PhP-CS), inoculated onto the plate and after incubating for 24 and 48 h, the absorbance was read at 620 nm. The results were analysed by using the computer software package supplied by the PhP manufacturer.

Ribotyping (Paper II)

Typing systems based on biochemical traits have limitations because these traits are often inconsistently expressed. Simple restriction fragment length polymorphism (RFLP) analysis may be useful, but interpretation is difficult due to the multitude of chromosomal fragments seen in the gel and subtle differences may be obscured by the large number of bands. Transfer of the DNA fragments to a nylon membrane and hybridisation with a probe for ribosomal RNA (rRNA) genes reduces the number of bands and provides a typing method that is fairly simple to evaluate. Ribosomal RNA genes are present in all bacteria and the universal regions are highly conserved between different species. Thus, the *E. coli* rRNA gene can be used as a probe for rDNA from other bacterial species. Other methods for ribotyping also use variations in chromosomal positions or the primary structure of rRNA genes, by PCR amplification of intergenic spacer

regions or RFLP of amplified regions of rRNA genes, in order to group isolates (Schmidt, 1994). DNA from cultures of *Actinobacillus* spp. was prepared by phenol-chloroform-isoamyl alcohol extraction, digested with *Bam*HI and *Pst*I (Amersham Pharmacia Biotech, Uppsala, Sweden), in separate experiments, and separated on a 0.7% agarose gel by electrophoresis at 60 V (3.3 V/cm) for 16 h. The *A. equuli* type strain ATCC 19392/NCTC 8529 was used as a standard and placed in both the left and right outer lanes of all gels. The DNA was transferred to a nylon membrane by Southern blotting and immobilised by baking at 80°C for 30 min. A digoxigenin (Boehringer Mannheim, Mannheim, Germany) labelled cDNA probe obtained by the reverse transcription of *E. coli* 16S and 23S rRNA as earlier described (Popovic *et al.*, 1993) was used for hybridisation. Detection was performed with antidigoxigenin conjugate and NBT/BCIP staining.

The choice of restriction enzymes was made after the evaluation of experiments with four different enzymes, namely *Eco*RI, *Hin*dIII, *Pst*I and *Bam*HI. *Pst*I and *Bam*HI were found to give the most reasonable number of clearly discernible bands and, therefore, these enzymes were used in the subsequent experiments. These enzymes have been used by some other authors who ribotyped *Pasteurellaceae* (Snipes *et al.*, 1989, Carpenter *et al.*, 1991, Alaluusua *et al.*, 1993, van Steenbergen *et al.*, 1994). The decision to use two different enzymes was based on earlier reports of variability in the clustering of *Pasteurellaceae* depending on the enzyme used (Snipes *et al.*, 1992), as well as the fact that both *Pst*I and *Bam*HI gave patterns with a fairly low number of bands.

Clustering methods (Papers I and II)

The PhP software package used for the biochemical fingerprinting measured phenotypic diversity using Simpson's index of diversity (Simpson, 1949). Dendrograms were constructed by UPGMA (unweighted pair group method with arithmetic mean) clustering, with the identity level set at 0.98, i.e. isolates with a correlation coefficient of 0.98 or more were classified as the same phenotype. Dendrograms for the strains subjected to PFGE and ribotyping were also constructed by UPGMA clustering, using the computer software package Gel Compar (Applied Maths, Kortrijk, Belgium). UPGMA is a frequently used clustering strategy that is fairly quick and simple (Sneath and Sokal, 1973). A matrix of correlation coefficients is used to generate a dendrogram in which the two, or more, strains with the highest correlation value are grouped into a unit with a connection, or branch point, corresponding to that value on the horizontal axis of the dendrogram. The process continues in the direction of lower correlation values by searching for the strain-strain, strain-unit or unit-unit with the next highest value. As units consist of two or more strains, branch points are determined by the average correlation value between members of a unit and the strain or unit with which it is paired. Thus, the larger the unit, the less accurate the pairing with a new strain, and it has been recommended that dendrograms for less closely related strains should be treated with considerable caution (Seward *et*

al., 1997). Different clustering methods give rise to substantially different dendrograms. Clustering should be regarded as a way of getting an overall view of the similarity between strains, not a definite answer to the question of strain relatedness.

Preparation of culture supernatants (Paper III)

Five strains of *A. equuli sensu stricto* and one haemolytic strain of Bisgaard's taxon 11 subtype 1 were used in the neutrophil experiments. Culture supernatants were prepared by culturing *Actinobacillus* strains in RPMI 1640 cell culture medium without L-glutamine. One control tube containing sterile RPMI was run in parallel to the inoculated tubes. The tubes were incubated with gentle shaking at 37°C for approximately 6 h, until the phenol red pH indicator of the medium began to change colour. In other members of the *Pasteurellaceae* family, the production of leukotoxin appears to reach maximum in early log phase (Burrows and Lo, 1992, Clinkenbeard *et al.*, 1992), after culturing for approximately 6 h (MacDonald and Rycroft, 1992). After centrifugation, supernatants were collected and passed through sterile filters with a pore size of 0.2 μm. The pH of the filtered supernatants was then adjusted to 7.2 ± 0.2 (equal to that of the control) with NaOH. This crude preparation was used in the neutrophil function assays.

The supernatant would be expected to contain various bacterial metabolites, and LPS, that may have interfered with the experiment. However, this simple method of preparation has been used previously, in studies of *M. haemolytica* leukotoxin (Baluyut *et al.*, 1981, Rosendal *et al.*, 1988, Czuprynski and Noel, 1990), and it was judged that it would suffice for the initial study of leukotoxic effects. The problem of obtaining toxin preparations free of LPS has been discussed (Czuprynski, 1995), as RTX toxins are labile *in vitro* (Clinkenbeard *et al.*, 1992, Devenish *et al.*, 1992), but it has been proposed that this is less important in neutrophil studies as LPS does not generally activate neutrophil effector functions (Czuprynski, 1995). In other studies on leukotoxins of *Pasteurellaceae*, heat inactivation at 60°C for 30 min (Baluyut *et al.*, 1981, Rycroft *et al.*, 1991) or 65°C for 2 h (Stevens and Czuprynski, 1995) has been used to determine the heat stability of leukotoxic effects. As LPS is heat stable, the elimination of leukotoxic effects by heat inactivation is usually interpreted as a heat labile leukotoxin having produced these effects. In this study, heat inactivation at 65°C for 2 h was used.

Flow cytometry (Paper III)

Manual analysis of the phagocytic capacity of neutrophils is laborious, time-consuming and not always reliable, as there is a limit to the number of neutrophils that can be microscopically evaluated objectively. Flow cytometry (fluorescent-activated cell sorting, FACS) can be used for the rapid analysis of the properties of individual cells in suspension. The cells are passed through a laser beam and the light scattered by the cells indicates their size and internal complexity. If neutrophils are exposed to fluorescent opsonized yeast cells, the

kinetics of attachment and ingestion can be studied. In this study, neutrophils from healthy foals and adult horses were exposed to *Actinobacillus* culture supernatants for 30 min at 37°C and subsequently allowed to phagocytose fluorescein labelled yeast cells, prior to analysis by flow cytometry. Neutrophils were prepared from whole blood with EDTA to which NH_4Cl solution had been added, in order to lyse erythrocytes, and subsequent washing in buffer. In the phagocytic assay, data from 50 000 events were collected for each sample. The proportion of phagocytic neutrophils was calculated as the percentage fluorescent granulocytes (i.e. granulocytes with engulfed yeast particles) out of all granulocytes. The total number of neutrophils was calculated as the total number of granulocytes among 50 000 events. To estimate the within-method variation a separate FACS assay was performed, with four separate controls (RPMI medium only) and cells from 2 different horses.

Chemiluminescence (Paper III)

The stimulation of phagocytosis of granulocytes results in degranulation and the production of H_2O_2 and free oxygen radicals, a process that can be measured by the chemiluminescence (CL) assay. By exposing neutrophils to opsonized zymosan particles in the presence of luminol and measuring the light emission in a luminometer, an estimate of the neutrophil respiratory burst can be obtained. The CL assay has been used for evaluating the effects of *M. haemolytica* leukotoxin on bovine neutrophils (Czuprynski and Noel, 1990). In the present study, neutrophils from healthy foals and adult horses were exposed to *Actinobacillus* culture supernatants for 30 min at 37°C and subsequently subjected to the CL assay. Light emission, measured in relative light units (RLU), was monitored at intervals of 45 s for 60 cycles at a temperature of 38°C, in a 96-well microtitre plate. The area under the curve (AUC) for the luminescence during the 60 cycles was recorded. All samples were run in triplicate, and the mean value for each triplicate was calculated. The within-method variation of the assay was determined by running duplicate controls in six experiments. Comparisons were also made between the effect of *Actinobacillus* spp. supernatants and heat inactivated supernatants, supernatants from the culture of a haemolytic *E. coli*, and purified *E. coli* LPS (Sigma Chemical Co., St Louis, USA).

Antigen preparation (Paper IV)

There are various methods for antigen preparation, such as heat extraction, sonication, LiCl extraction or the addition of a detergent such as Tween, Triton-X or sodium desoxycholate, and many of these have been used for actinobacilli (Vallee *et al.*, 1974, Kim, 1976, Rycroft and Taylor, 1987, Rycroft *et al.*, 1998). Neutral detergents such as Tween or Triton-X are somewhat less efficient than sodium desoxycholate, but too much sodium desoxycholate in the gel may interfere with the antigen-antibody reaction (Johansson and Wroblewski, 1983). In the present study, the sodium desoxycholate method was chosen, as it is easy

to perform and worked better than sonication or heat extraction when tested by immunodiffusion with equine *Actinobacillus* spp. In short, fresh culture material was dissolved in PBS without Ca^{2+} and Mg^{2+} and sodium desoxycholate was added to a final concentration of 1% (w/vol). After vortexing, the mixture was kept at 8°C for 6 h. After centrifugation, the supernatant was used directly for immunodiffusion.

Immunodiffusion (Paper IV)

Various methods have been used for the detection of antibodies against *Actinobacillus* spp. Double immunodiffusion was chosen because it is simple and informative, easy to interpret, and suitable for situations in which both the antibody and antigen concentrations are unknown (Johnstone and Thorpe, 1982). As concentration gradients of antibody and antigen are automatically formed by the diffusion process, a precipitate will form somewhere between the wells, provided that the equivalence concentration is obtained at some point in the overlapping gradients. Agar gel immunodiffusion (AGID) was performed in commercially available agarose plates (Auto I.D.®, Immunoconcepts, Sacramento, USA). Na-desoxycholate, at a final concentration of 1% (w/vol) was added to the colostrum samples before application, as this was found necessary to achieve diffusion of the colostrum. All isolates from each mare-foal pair were tested against the sera of both mare and foal, as well as the colostrum. AGID plates with serum samples were incubated at room temperature for up to 48 h and checked every 12 h for the presence of precipitation lines. Plates with colostrum samples were incubated at 37°C for the first 24 h, as this was found to improve the diffusion of colostrum from the wells, and subsequently at room temperature for another 24 h, with checking for precipitation lines every 12 h. Initially, for the first two mare-foal pairs, all analyses were performed in duplicate, but as no difference could be detected between the results from duplicate experiments, the subsequent analyses were generally performed only once. However, in the cases where differences between the mare and foal serum were detected, the entire analysis, including the antigen preparation, was repeated, to ensure that the detected difference was reproducible.

Determination of antimicrobial susceptibility (Paper V)

The most common methods used for determining antimicrobial susceptibility are disk diffusion and microdilution. Disk diffusion is a qualitative method and microdilution is a quantitative method, providing minimum inhibitory concentration (MIC) values. The tested organism is usually classified as either susceptible (S), intermediate (I) or resistant (R) to the substance in question. This classification is clinically useful, but provides little information about the distribution of MIC values within bacterial populations. Knowledge of this distribution is essential for evaluating the prevalence of acquired resistance. In a homogeneous bacterial population, the MIC values for each antimicrobial substance tested would be expected to follow a normal distribution. If the peak of the curve were close to the break-point value used, a certain proportion of the

population would end up on the "wrong" side of the break-point and, consequently, be classified in another group of the S-I-R system than the other bacteria sharing the same features. The microbiological break-point is the MIC value that separates the normal population from the bacteria with acquired resistance. Sometimes, susceptibility data is presented in the form of MIC_{50} and MIC_{90} values, i.e. the MIC values at which 50% and 90% of the isolates, respectively, are susceptible. This information is of little value for the comparison of resistance patterns and the prevalence of resistance. In the present study, MIC values were determined by use of a commercial microdilution system, SVA VetMIC®+/- (National Veterinary Institute, Uppsala, Sweden), Approximately 10^3-10^4 CFU (colony forming units) of bacteria in Mueller Hinton broth supplemented with 1% (vol/vol) IsoVitalex® enrichment (Becton Dickinson Microbiology Systems, New Jersey, USA) were inoculated into each well of the VetMIC® plate. This plate contains dried two-fold dilutions of 17 antibacterial substances: penicillin, ampicillin, oxacillin, cephalotin, enrofloxacin, erythromycin, neomycin, streptomycin, spiramycin, clindamycin, fusidic acid, gentamicin, oxytetracycline, trimethoprim-sulfa (1:20), sulfamethoxazole, chloramphenicol and nitrofurantoin, plus a set of control wells containing the (dried) buffers used to dilute the antimicrobials. After inoculation, the plate was covered with plastic tape and incubated at 37°C for 16-18 h. The results were read as either growth or no growth, where the lowest concentration found to impair growth was recorded as the MIC value.

Isoelectric focusing (Paper V)

Isoelectric focusing (IEF) is a method for protein separation in which the protein molecules become aligned as sharp bands at their isoelectric points in an electrophoretically produced pH gradient. β-lactamase proteins can be visualised by adding a substrate that changes colour after degradation by β-lactamase. A high degree of resolution is obtained, as focusing is caused by forces that act against diffusion and proteins are therefore concentrated in narrow bands during the separation. IEF has frequently been used for the analysis of β-lactamases (Matthew and Harris, 1976, Labia and Barthelemy, 1977, Olsson Liljequist *et al.*, 1980, Marre *et al.*, 1982, Saino *et al.*, 1982, Azad *et al.*, 1992). The presence of satellite bands is not uncommon and it has been suggested that the pattern of satellite bands changes if the β-lactamase gene is transferred to a different bacterial host (Labia *et al.*, 1976, Brive *et al.*, 1977) and therefore comparisons should always be made between enzymes from the same host strain, but other authors have disputed this statement (Matthew *et al.*, 1975). In the present study, the bacterial cells were sonicated and the sonicated preparations were centrifuged. The β-lactamase activity of the supernatants as well as the pellets was analysed by IEF, using a commercial system, Ampholine PAGplate, pH 3.5-9.5 (Amersham Pharmacia Biotech, Sweden). Detection of β-lactamase activity was performed by staining the gel with the chromogenic β-lactam nitrocefin.

Plasmid analysis (Paper V)

There are various methods for the preparation of plasmids (Aldrich Meyers *et al.*, 1976, Kado and Liu, 1981). Plasmid DNA can be separated from chromosomal DNA by methods based on its smaller size, or based on the fact that covalently closed circular DNA is not, like chromosomal DNA, denatured by SDS treatment at an elevated temperature. In the present study a commercially available system for rapid plasmid preparation, Wizard™ Miniprep (Promega, Madison, USA), was used. In this system, the bacterial cells are lysed and plasmid DNA is eluted from a minicolumn. Electrophoresis was used for the detection of plasmids within the size range 0.8-11 kb. The size range was selected based on findings in other studies of *Pasteurellaceae*, where the β-lactamase genes have been found on plasmids ranging in size between 2 and 7 kb (Juteau *et al.*, 1991, Azad *et al.*, 1992).

Results and discussion

Source of infection - prevalence and epidemiology of *A. equuli* and other equine *Actinobacillus* spp. in the oral flora of healthy horses (Papers I, II and IV)

From the results obtained in the investigations of the equine oral flora, it would appear that the oropharynx of healthy horses is the likely origin of the causative bacteria in most neonatal *Actinobacillus* infections and that all foals are exposed to this type of bacteria immediately after birth. The complexity of *Actinobacillus* spp. in the normal flora of the oral cavity was reflected in the culture results from mares and their newborn foals, where a variety of subtypes were isolated from most horses. The oral flora of the foal was usually very similar to that of the mare, indicating an immediate colonisation of the oropharynx of the foal shortly after birth. In the prevalence study, *A. equuli* could be isolated from horses on all farms, varying in frequency between 12 and 88% on different farms. All samples yielded growth of some type of *Actinobacillus* spp., and usually several different phenotypes could be isolated from the same horse. The overall prevalence of *A. equuli sensu stricto* and L-arabinose positive *A. equuli* was very similar, although there were some variations between the different farms. On one farm, where the horses were sampled repeatedly during one week, isolation frequencies varied substantially between different sampling occasions. This may be explained by the sampling of only a small part of the mouth on each occasion, as the composition of the microflora in this spot may change quite rapidly over time. Several samples taken from different parts of the oral cavity on every sampling occasion might have given a less variable result. However, it is highly likely that this sampling would have demonstrated a 100% prevalence of *A. equuli* in the oral cavity of the horses on most farms. Due to the complexity of the normal flora and the consequent likelihood of missing *A. equuli* in a single sample, as well as the very high prevalence of this bacterial species on most farms, it was not possible to identify herds or mares with a higher risk of *A. equuli* infections in their foals.
The PFGE analysis emphasised the complexity displayed in the culture results, indicating that there was no herd infection with *A. equuli*, but that many different strains were present in different horses within the same population and also in the same horse. Some strains that were phenotypically identical and isolated from the same horse were found to differ in their PFGE patterns. Horses that were in daily contact were sometimes found to carry the same strain, but no strain dominated on any particular farm. The clustering of different band patterns showed a great variability, and no clustering of strains from horses on the same farm was observed. Most strains displayed less than 50% similarity, and the most similar ones came out as a 70% match. No phylogenetic differences between clinical isolates and normal flora isolates could be detected by ribotyping and biochemical fingerprinting of isolates of *A. equuli sensu stricto* and L-arabinose positive *A. equuli* from clinical samples and from the oral cavity of healthy horses. This observation indicates that clinical disease might be caused by any

strain found in the normal flora providing there is no, as yet undiscovered, virulence gene distinguishing strains with pathogenic potential from less pathogenic strains.

Virulence factors - effects of culture supernatants on equine neutrophils (Paper III)

Bacterial culture supernatants of equine *Actinobacillus* spp. appeared to contain leukotoxic metabolites, as they caused an average decrease of 22% in the total number of granulocytes in the FACS assay ($p<0.01$), and an average decrease of 26% in light emission in the CL assay ($p<0.001$). Supernatants from the haemolytic taxon 11 strain appeared to contain either a higher amount or more potent leukotoxic metabolites as long as its haemolytic activity was expressed, causing a 44% decrease in the total number of granulocytes ($p<0.01$) and a 52% decrease in light emission ($p<0.01$). This effect was abolished by heat treatment. Heat treatment had a smaller effect on the leukotoxic activity of supernatant from one of the *A. equuli* strains and no effect at all on another. One of the RTX toxins from *A. pleuropneumoniae* is reportedly stable for 5 min at 60°C but inactivated at 100°C (Rycroft *et al.*, 1991). However, that would not necessarily be the case for other RTX toxins. The culture supernatants used in this study would be expected to have contained a substantial amount of LPS. LPS might, by stimulating adhesiveness, cause neutrophils to adhere to each other or to the walls of the test tubes and, consequently, cause a reduction in the number of free granulocytes in the cell suspension. However, it is possible that the manipulation and shifting of temperature during incubation already caused maximum stimulation of neutrophil adhesiveness, and that the addition of an unknown quantity of LPS might not have made any substantial difference in this respect. Purified LPS at a concentration of up to 30 mg/l reduced the AUC value in the CL assay, but the effect was less than that of *Actinobacillus* supernatants. Thus, at least some of the effect on the neutrophils can be explained by the presence of endotoxin in the culture supernatant. Halfway through the study, the haemolytic strain ceased to express haemolysis, which led to a reduction in the leukotoxic effect of the supernatant. The loss of haemolytic activity after subculture or storage of actinobacilli has been noted by other authors (Hughes, 1972, Carman and Hodges, 1982). Evaluation of the stability of the methods used revealed that within-method variation was substantial, but still far less than the observed effects. Despite these variations, the overall effect was a reduction in the total number of granulocytes and AUC values, which supports the theory that equine *Actinobacillus* spp. do produce one or more types of leukotoxic metabolites. The leukotoxic effects were mainly reflected in the decreased survival of neutrophils and not in neutrophil functions.

Further studies, such as the characterisation of different metabolites present in the culture supernatants from equine *Actinobacillus* spp., are required in order to explain the exact cause of the effects observed in this study. Examination of

bacterial isolates for the presence of RTX genes would be necessary in order to determine the possible existence of a leukotoxin and its presence in different strains. An initial screening of three of the strains used in this study, with various RTX gene probes under different hybridisation conditions, has demonstrated a signal for one of the *A. pleuropneumoniae* RTX toxin genes, with DNA from the haemolytic taxon 11 strain, while for the two *A. equuli* strains tested the results were doubtful.

Prophylaxis and treatment - immunoglobulin and antimicrobials (Papers IV and V)

The results of the AGID testing indicate that an immune response to *Actinobacillus* spp. colonising the oral cavity is present in many adult horses and that this immune response is transferred from mother to foal via colostrum. Thus, an adequate intake of colostrum would be expected to protect the foal from most of the *Actinobacillus* spp. present in the oral cavity of the dam, providing that the presence of specific antibody would suffice in protecting a normal foal from *Actinobacillus* infection. It has been suggested that a humoral immune response is the most important in the protection from infections by members of the *Pasteurellaceae* family (Biberstein, 1990), so the presence of antibody may be crucial to the foal. Antibodies against 48 out of the 77 *Actinobacillus* isolates from all horses in the study were present in the respective sera of 13 mares and 9 foals. In 11 out of all 15 mother-foal pairs, the antibody content of the foal serum was similar to that of the mare, although in some cases differing for 1-2 bacterial strains. In 9 cases this was reflected in the antibody content of colostrum from the mare. In 7 colostral samples, some of the antibodies found in the serum of the mare and foal could be detected, but many of the colostral samples were difficult to analyse due to auto-precipitation. Twenty-four of the 48 antibody types found in the serum of the mare and/or the foal were not detected in colostrum. This could be explained by methodological problems, such as auto-precipitation, that may have impaired the detection of antibodies present in some of the colostrum samples.

In one foal sample, taken only 1 h after intake of colostrum, the antibody detected in mare serum and colostrum was absent. This corresponds to the findings in other studies (Jeffcott, 1974c), in which it took about 3 h for molecules absorbed via colostrum to reach the blood of the foal. Other variations between the sera from mares and their foals may be due to a true difference in immune response, or merely a difference in antibody concentration, with some samples falling below the detection level of the AGID test. There was no subtype of *Actinobacillus* spp. that appeared more likely to provoke an antibody response. Apart from one strain of *Branhamella* sp. found in one horse, no immune response could be detected against any other bacteria. However, the failure to detect antibodies against other bacteria does not necessarily prove the absence of such antibodies. The AGID method has a limited sensitivity and the method used for antigen preparation may not have been optimal for other types of bacteria. However, as the focus of this study was on the *Actinobacillus* spp., this must be

regarded as a minor problem. It is not certain that all antigens expressed *in vivo* will be expressed by bacteria cultured *in vitro*, and vice versa. Until this question is answered, if ever, it must be assumed that antigens against which antibody can be detected *in vitro* are also expressed *in vivo*. However, structural and antigenic characteristics may vary between bacterial growth stages, as has been shown for *A. pleuropneumoniae* (Fenwick, 1995), and some equine IgG molecules may only be capable of precipitating when the antigen-antibody concentration is exactly right (Dudan *et al.*, 1990). Hence, some immune response may have remained undetected by the techniques used in this study.

The results of the antimicrobial susceptibility testing suggest that resistance against commonly used antimicrobial substances is present among Swedish strains of equine *Actinobacillus* spp., albeit so far only in a minority of the population. This emphasises the need for susceptibility testing, even though the antibiograms were very similar for most strains studied. When tested against penicillin, ampicillin, trimethoprim-sulfa and streptomycin, small groups of strains with what appeared to be acquired resistance could be identified. The frequent use of these substances in Swedish equine practice would be expected to exert a selective pressure for resistant strains among *Actinobacillus* spp. in the normal flora of the horse. Considering the fact that only two strains of the 150 included in this study were resistant to trimethoprim-sulfa, the practice adopted at the horse clinic of the Faculty of Veterinary Medicine in Uppsala, of using a combination of penicillin and trimethoprim-sulfa as the first-choice treatment for septicaemic foals, must still be deemed likely to be effective as far as the actinobacilli are concerned. However, it is worth noting that the two strains resistant to trimethoprim-sulfa were also resistant to penicillin, even though β-lactamase production was not detected in these two strains. Both of these strains were isolated from healthy horses. The majority of the penicillin-resistant strains produced β-lactamase, which may be of clinical value since β-lactamase can be quickly detected with the aid of a nitrocefin disc, before the results of a full susceptibility test are available. There was some correlation between penicillin resistance and resistance to other antimicrobials, with eight of the penicillin resistant strains also being resistant to either trimethoprim-sulfa or streptomycin. This observation suggests that the rapid detection of β-lactamase may give an indication as to the presence of other resistance traits. The six strains with acquired resistance to streptomycin were all found in the group with acquired resistance to penicillin. All these strains produced β-lactamase. The β-lactamases appeared to be bound tightly to the cell wall, thereby frustrating any further characterisation by IEF. Plasmids of approximately 3 kb were found in 4 out of 7 β-lactamase producing strains submitted to plasmid analysis, indicating the possible presence of plasmid-mediated β-lactamase in these strains. A rapid spread of β-lactamase genes among equine *Actinobacillus* spp. may lead to future problems, not only due to therapeutic failure. In areas where prophylactic treatment of neonatal foals with penicillin is common, selection for β-lactamase producing strains may lead to an increase in neonatal *Actinobacillus* infections.

Subtyping of equine *Actinobacillus* spp. - PFGE, ribotyping, biochemical fingerprinting and antibiograms (Papers I, II and V)

In the present study, various typing methods were used. The phenotypic methods, biochemical fingerprinting and antimicrobial susceptibility testing, were less discriminatory but biochemical fingerprinting could be useful for the characterisation of unrelated strains. The DNA-based methods, PFGE and ribotyping, had a high discriminatory power, which is useful in epidemiological investigations. With PFGE, the analysis of duplicate plugs made from different subcultures showed that the method gave highly reproducible results. The two restriction enzymes gave different band patterns, but strains that were identical with one enzyme were also identical with the other, indicating that the results were reliable and that PFGE worked well for the detection of isolates belonging to the same clone. By ribotyping, *Bam*HI digestion gave a total of 33 different ribotypes of which 14 contained more than one strain, with the lowest clustering level at 13%, while *Pst*I gave 48 different ribotypes of which 23 contained more than one strain, and the lowest clustering level at 18% similarity. Both enzyme digestions resulted in an average number of 5-6 bands. This low number of bands may have contributed to the low level of overall similarity obtained by ribotyping. If the total number of bands is small, each band difference will have a comparatively large impact on the overall similarity between the strains compared. Moreover, as has been previously discussed, the clustering of strains with a low similarity is not reliable. Most strains investigated were epidemiologically unrelated, which would also be expected to result in a dendrogram with many branches and a low overall similarity. There was no distinct clustering of epidemiologically related strains, such as the normal flora isolates from horses on the same farm. This data corresponds to the results obtained by PFGE analysis, but as PFGE has a higher resolution, ribotyping might have been expected to show more distinct clusters if some relatedness existed. The correlation between the band patterns obtained with the two different restriction enzymes was poor. Only a few groups of strains were characterised as the same type, i.e. belonging to the same ribotype, by both enzyme digestions. This further emphasises the great overall variability of the strains studied. By biochemical fingerprinting, a total of 26 different phenotypes were identified, 8 of which contained more than one strain, with the lowest clustering level at 83% similarity. The correlation between the phenotypes, obtained by biochemical fingerprinting, and the ribotypes was poor. This was not unexpected, as the use of typing methods based on different features, such as the location of rRNA genes together with the sequences of restriction enzyme recognition sites, and metabolic activity, would most likely result in the identification of a large variety of subtypes. Subtyping by antimicrobial susceptibility testing does not appear to be very useful for the purpose of investigating strain relatedness, mainly due to the frequent exchange of some resistance traits between different bacteria, making susceptibility patterns very variable over time. Moreover, the strains included in this study exhibited very similar antibiograms with little correlation to either the biochemical subtype or

the source of the isolates. A certain degree of discrepancy between the strains from clinical samples and strains from healthy horses might have been expected, as many clinical samples were from horses in an antibiotic-dense environment such as a clinic. However, the size of the material may have been too small for detecting subtle differences.

Conclusions

- The prevalence of *Actinobacillus* spp. in the normal flora of healthy horses is high, but may vary substantially over time, and most, if not all, horses are carriers.
- So far, there is no evidence of any difference in the pathogenic potential between various subtypes of *Actinobacillus* spp. or between different strains. The overall phenotypic and genotypic similarity between different clinical isolates, and between isolates from the normal flora, is no greater than the overall similarity between all types of isolates.
- Equine *Actinobacillus* spp. produce metabolites that exhibit toxic effects on equine neutrophils. The type and/or potency of such metabolites appear to vary between strains. Some metabolites may be related to the RTX toxins present in other species of the *Pasteurellaceae* family.
- *Actinobacillus* spp. carried in the oropharynx of healthy horses provoke a humoral immune response in the host animal, that can be transferred from mares to their foals via colostrum. This observation indicates that prompt and successive passive transfer of colostral antibodies is of specific importance in protecting the neonatal foal from *Actinobacillus* infections.
- *Actinobacillus* spp. from Swedish horses exhibit similar antimicrobial susceptibility profiles and are generally susceptible to substances used in equine practice. However, the possible presence of plasmid-mediated β-lactamase in this bacterial population may change this situation.

Suggestions for future studies

Further studies on equine *Actinobacillus* spp. should include the search for virulence attributes. The characterisation of a possible RTX gene that could be used as a probe may assist in the differentiation between strains with different pathogenic potential. The effects of the product of such an RTX gene on equine leukocytes as well as leukocytes from other animal species could be studied by specific staining, and by electron microscopy. The possible existence of other leukotoxic metabolites should also be investigated.

Comparison of LPS from equine *Actinobacillus* spp. and LPS from other bacteria may give an indication as to whether LPS could be regarded as a virulence attribute for *Actinobacillus* spp. Moreover, purified LPS, prepared by various methods, from equine *Actinobacillus* spp. should be included in leukocyte experiments.

Adhesive factors should also be investigated. For this purpose, study of the interaction between bacteria and cultured cells, and comparative investigations of known adhesins from related bacteria such as *A. actinomycetemcomitans* could be used. Adhesive factors might also be identified after the culture of bacteria under *in vivo*-like conditions, such as tissue chambers.

Further characterisation of the β-lactamase found in some strains in this study may clarify whether it is plasmid-mediated or transferable by some other mechanism, and if it is related to similar enzymes found in other members of the *Pasteurellaceae* family. This could be achieved by the identification of the encoding genes, by already established methods for probing and PCR, and by transfer experiments.

Further serological investigations, of foals with *Actinobacillus* infections and their dams, would be of particular interest if the results could be correlated to information regarding the time of colostrum intake, the Ig content of the colostrum and the presence of predisposing stress factors. The isolates from mares and foals that were found to provoke an immune response in the host animals, should be further investigated for the identification and characterisation of antigenic determinants. Such knowledge would be useful in the planning of prophylactic measures against *Actinobacillus* spp. infection.

The effects of different prophylactic and therapeutic measures could also be evaluated. This might involve field studies/clinical trials on farms frequently affected by foal septicaemia and/or experimental infection models.

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When the Creator wished to create the horse, he said to the Wind:
I shall have thee bear a being who shall carry my worshippers. This being shall be loved and regarded by all my slaves. It shall be feared by all who fail to heed my commandments. And he created the horse and said to it:
I have made thee without equal. All the treasures of the earth lie between thine eyes. Thou shall cast mine enemies beneath thy hooves, but my friends thou shalt bear on thy back. This shall be the seat from which prayers ascend to me. Over the whole earth thou shalt be fortunate and preferred before all other creatures. In thee shall be the love of the Lord of the Earth. Thou shalt fly without wings and conquer without sword.

The Koran

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