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Bacterial Flora of Semen Collected from Danish Warmblood Stallions by Artificial Vagina

By *M. Madsen*[1] and *P. Christensen*[2]

[1]Department of Veterinary Microbiology, Section of Bacteriology, and [2]Department of Anatomy and Physiology, Section of Anatomy, The Royal Veterinary and Agricultural University, Frederiksberg, Denmark.

Madsen, M. and P. Christensen: Bacterial flora of semen collected from Danish warmblood stallions by artificial vagina. Acta vet. scand. 1995, 36, 1-7. – Semen samples were collected from 21 Danish Warmblood stallions by the Colorado artificial vagina (Colorado AV, 14 samples) or by the Missouri artificial vagina (Missouri AV, 7 samples). The semen was examined bacteriologically by direct plating (DP) on blood agar plates, and by plating of semen swabs stored in Stuart's transport media (TM) at 4°C for 1-4 days. No significant differences were observed between results obtained by DP and cultures of identical TM samples. Of the 21 samples examined, only 1 TM (4.8%) and 2 DP samples (9.5%) were sterile, while the rest yielded a predominantly mixed flora comprising 1 to 4 bacterial genera. The natural flora was dominated by coagulase-negative staphylococci *(Staphylococcus lentus, S. capitis, S. haemolyticus, S. xylosus)* (16/21 = 76%), coryneforms (11/21 = 52%) and alpha-hemolytic streptococci and lactobacilli (7/21 = 33%). Potential venereal pathogens were isolated from 7 stallions (33%). Beta-hemolytic streptococci were found in 4 stallions used for natural service, whereas Pseudomonas aeruginosa serotype 6 (2 samples) and Klebsiella pneumoniae subsp. pneumoniae capsule type K5 (1 sample) were isolated from 3 stallions used exclusively for artificial insemination. The role of the stallion as a carrier of potential venereal pathogens, and the artificial vagina as a source of contamination, is discussed in the context of mare endometritis.

horse; artificial insemination; Klebsiella pneumoniae; Pseudomonas aeruginosa.

Introduction

With the successful development of equine artificial insemination in recent years, stallion fertility status has become an important consideration in horse breeding establishments, and satisfactory examinations with regard to semen quality has become mandatory *(Dowsett* 1988, *Colenbrander et al.* 1992, *Hurtgen* 1992).

Semen collected with the closed type artificial vagina usually contains numerous bacteria and fungi originating mainly from crypts in the surface of the penis and folds of the prepuce, an observation which has led many investigators to consider the presence of bacteria and fungi in semen to be a natural phenomenon *(Burns et al.* 1975, *Simpson et al.* 1976, *Bowen et al.* 1982, *Jones et al.* 1984, *Tischner & Kosiniak* 1992). However, venereal infections may be caused by microorganisms carried with semen, and stallions are usually asymptomatic carriers of these microorganisms *(Hughes et al.* 1975, *Merkt et al.* 1975, *Bowen et al.* 1982, *Kikuchi et al.* 1987, *Vaissaire et al.* 1987, *Blanchard et al.* 1992). For this reason, it becomes important to distinguish the indigenous microflora from the potentially pathogenic organisms which may

cause disease and inflammation in the male and female reproductive tract.

The aim of the present study was to evaluate the bacterial flora of semen, collected in general stud practice by 2 different artificial vaginas (AV) (the Missouri type and the Colorado type), from Danish Warmblood stallions as a part of the breeding soundness examinations at the start of the 1993 breeding season. The results of bacteriological examination by direct plating on blood agar plates at the stud farm (DP) and by plating semen swabs stored in Stuart's transport media (TM) for 1-4 days at 4°C were compared.

Materials and methods

Twenty-one Danish Warmblood stallions aged approximately 3 years were examined. All stallions were clinically healthy, and all were in use for breeding at the time of sample collections. Twelve stallions were used for natural service, while the remaining 9 were used for semen collection for artificial insemination.

Semen samples were collected at 4 breeding stations at the start of the reproductive season in 1993. Semen was collected from 14 stallions from 3 stations with the Colorado AV and from 7 stallions from 1 station with the Missouri AV (Table 1). Both AVs offer the possibility of using a disposable inner liner which limits the risk of contaminating the semen with bacteria from the AV. All semen was collected using a disposable inner liner, but other parts of the AV (e.g. collection bottles) were frequently reused after washing and disinfection with a quaternary ammonium base compound.

Following proper teasing the stallion mounted either a mare in oestrus or a phantom and the collector deflected the stallion's penis into the AV as quickly as possible. Often it was not possible to avoid the stallion's penis touching the mare or the phantom during this part of the collection but the deflection was always done with a sterile gloved hand. The penis and prepuce of stallions were not washed before collection.

Freshly obtained 0.05 ml semen samples for bacteriological examination were spread with a disposable inoculating loop on blood agar plates (Oxoid Blood Agar Base No. 2 (CM 271), supplemented with 5% calf blood) and either incubated at the station at 37°C for 18-24 h in atmospheric air, or taken to our laboratory, where they were incubated under identical conditions within 24 h of inoculation. In addition, a sterile swab was submerged in the semen sample at the time of collection, placed in Stuart's transport medium (Oxoid CM 111), held at 4°C for 1-4 days, and submitted to our laboratory. Semen swabs were inoculated on to blood agar plates on arrival by making a swab impression smear on the plate followed by spreading with a disposable inoculating loop, and incubated as described above.

Following incubation, the number of different colony types were recorded, and the amount of bacterial growth was scored on a scale from 0 to ++++, with 0 representing no growth, and +, ++, +++ and ++++ representing growth in respectively 1, 2, 3 or all quadrants of the plate. Typical bacterial colony types were isolated, and identified to genus or species level according to *Cowan* (1974) and *Krieg & Holt* (1986).

Results

The overall results of the bacteriological examinations are shown in Table 1. In only 2 of 21 samples (9.5%) there was no growth by direct plating (DP), one of which was obtained with the Missouri AV and the other with the Colorado AV, and in only 1 (4.8%) sample submitted as a swab in transport medium (TM). The majority of the remaining samples

Table 1. Results of bacteriological examinations of semen from 21 Danish Warmblood stallions from 4 breeding stations.

Station and sample no.	AV type	Previous use	Potential pathogens			Commensals						No growth
			BS	PSA	KL	CS	CF	AS	LB	MC	OTH	
1-1	CAV	N	+			+			+			
1-2		N	+			+	+					
1-3		N	+			+	+					
1-4		N	+				+					
2-1	CAV	N				+						
2-2		AI				+					+	
2-3		AI				+					+	
2-4		AI				+			+			+
2-5		AI										+
3-1	MAV	AI		+		+	+				+	
3-2		AI		+		+						
3-3		N					+	+		+	+	
3-4		AI				+	+		+			
3-5		N				+	+				+	
3-6		N					+				+	
3-7		N										+
4-1	CAV	AI			+	+						
4-2		N				+	+				+	
4-3		N				+			+			
4-4		N				+	+	+		+		
4-5		N				+		+				
% of samples			19	9.5	4.8	76	52	14	19	9.5	33	9.5
% of isolates			7.5	3.8	1.9	34	23	5.7	8	3.8	13	

AV: Artificial vagina; CAV: Colorado artificial vagina; MAV: Missouri artificial vagina; N: Natural service; AI: Artificial insemination; BS: Beta-haemolytic streptococci; PSA: Pseudomonas aeruginosa; KL: Klebsiella pneumoniae; CS: Coagulase-negative staphylococci; CF: Coryneforms; AS: Alpha-haemolytic streptococci; LB: Lactobacilli; MC: Micrococci; OTH: Other isolates (*Moraxella* sp. (2), *Bacillus* sp. (3), *Pasteurella* sp. (1), *Acinetobacter* sp. (1)).

yielded a mixed flora, comprising 2 to 4 bacterial genera, which was dominated by coagulase-negative staphylococci and coryneforms. Fifteen of 18 staphylococcal isolates (83%) were further identified as *Staphylococcus lentus,* the remaining 3 isolates as *S. capitis, S. haemolyticus* and *S. xylosus.* Other common isolates included alpha-hemolytic streptococci, lactobacilli, micrococci, bacilli and gram-negative rods. Potential venereal pathogens were less commonly encountered, and only in small numbers in mixture with other bacteria *(Klebsiella pneumoniae* subsp. *pneumoniae,* capsule type K5 in 1 (4.8%), and

Table 2. Growth intensity and diversity of bacterial flora from cultures of semen samples (N = 21) by direct plate culture (DP) as compared to culture of samples submitted as swabs in transport medium (TM).

DP:	Growth intensity*)					
No. of genera	Nil	+	++	+++	++++	Total
0	2	–	–	–	–	2
1	–	3	0	0	0	3
2	–	7	0	0	0	7
3	–	6	1	0	0	7
4	–	1	1	0	0	2
Total	2	17	2	0	0	21

TM:	Growth intensity					
No. of genera	Nil	+	++	+++	++++	Total
0	1	–	–	–	–	1
1	–	0	2	1	0	3
2	–	5	1	1	0	7
3	–	3	3	0	0	6
4	–	2	2	0	0	4
Total	1	10	8	2	0	21

*) See 'Materials and methods' for details.
DP: Direct plating.
TM: Stuart's transport media.

Pseudomonas aeruginosa serotype 6 in 2 (9.5%) of 21 samples). These 3 stallions were exclusively used for semen collection. All samples from the 4 stallions from Station 1, which only used natural service, grew beta-hemolytic, trehalose-positive streptococci in mixture with either coagulase-negative staphylococci or coryneforms. One of the beta-hemolytic streptococcal isolates belonged to Lancefield's serogroup C *(Streptococcus zooepidemicus),* whereas the other 3 isolates could not be referred to any of the serotypes (A,B,C,D,F,G) tested.

The intensity and diversity of bacterial growth obtained by the 2 sampling methods (DP versus TM) is shown in Table 2. Overall, a greater amount of bacterial growth was obtained from TM cultures, but without any significant influence on the diversity of the bacterial flora.

Discussion

The results presented in Table 1 demonstrate that semen collected by use of an AV must be expected to be contaminated with bacteria. There were no obvious differences in the amount and composition of the bacterial flora obtained from semen collected by either of the 2 AV types. In general, samples collected as swabs in transport media tended to yield a heavier growth as compared to samples spread directly on blood agar plates (Table 2). The observed difference in growth intensity is probably insignificant, and may be easily explained by the greater amount of semen collected by the swab, as compared to that con-

tained in an inoculating loop. The results indicate that semen samples may be safely collected by swabs and submitted in transport media for bacteriological examination at a later and more convenient time and place.

The normal bacterial flora of semen collected by AV may originate from the stallion's penis and prepuce, the inner liner of the AV, the handler's hands and the skin of the mounting mare, especially if several false mounts precede ejaculation *(Tischner & Kosiniak* 1986). Our results support this view and concur with earlier studies on the bacterial flora of semen *(Simpson et al.* 1976, *Klug & Sieme* 1992), as the bacterial isolates (Table 1) were dominated by coryneforms (23% of isolates) and coagulase-negative staphylococci (34% of isolates), which are typical members of the residential skin flora *(Krieg & Holt* 1986). The dominance of *S. lentus* within the staphylococcal isolates probably places this species as part of the natural flora of the stallion's penis and prepuce, which is interesting, as *S. lentus* has primarily been considered a part of the normal skin flora of ruminants, and has only occasionally been isolated from equids. *S. capitis, S. haemolyticus* and *S. xylosus* are all common members of the human skin flora *(Krieg & Holt* 1984). Alpha-hemolytic streptococci and lactobacilli are commonly found on mucous membranes of the equine genital tract *(Wingfield Digby & Ricketts* 1982, *Blanchard et al.* 1992).

The isolation of beta-hemolytic streptococci from stallion semen has been reported by several authors, and the prevalence of infected stallions has been shown to increase during the breeding season, with most stallions becoming negative after being withdrawn from service *(Mazurova & Mazura* 1988, *Klug & Sieme* 1992, *Clement et al.* 1993). Beta-hemolytic streptococci may regularly be recovered from cervical samples of breeding mares, and it is the general opinion that the genital tract of the mare is a natural reservoir for these organisms *(Wingfield Digby & Ricketts* 1982). The 4 stallions found positive for beta-hemolytic streptococci in this study were all exclusively used for natural service, a finding which support this view.

Pathological conditions such as seminal vesiculitis and epidymiditis caused by *P. aeruginosa* and *K. pneumoniae* have been reported. These conditions are easily diagnosed by the massive growth in pure culture of the causal organism *(Crouch et al.* 1972, *Merkt et al.* 1975, *Hamm* 1978, *Squires et al.* 1981, *Blanchard et al.* 1987a, 1987b, *Held et al.* 1990). In the present work, these organisms were isolated in small numbers from 3 stallions *(P. aeruginosa* (2), *K. pneumoniae* subsp. *pneumoniae* (1)). Although the stallions did not exhibit any signs of genital tract disorders, the presence of potential venereal pathogens is a cause of concern. Further characterization identified the *K. pneumoniae* strain as capsule type K5, which previously has been isolated from cases of equine metritis *(Crouch et al.* 1972, *Merkt et al.* 1975, *Kikuchi et al.* 1987). The 2 strains of *P. aeruginosa* were isolated from 1 breeding station, and the isolates belonged to the same serotype (serotype 6). Although the discriminatory power of the serotyping system is low, and a classification as identical serotypes does not necessarily indicate identity between the 2 isolates, it is tempting to speculate whether the isolates originated from the 2 stallions or from a common source of contamination. *P. aeruginosa* is very commonly found in moist and wet environments, and are furthermore renowned for its resistance to many antibiotics and antiseptics *(Squires et al.* 1981, *Blanchard et al.* 1987b). The isolation of *P. aeruginosa* from 2 stallions which were used for artificial insemination emphasizes the need for thorough

cleaning and disinfection of non-disposable equipment used for semen collection and insemination. Equine metritis caused by *P. aeruginosa* is feared by many horse owners and practitioners, as the condition is frequently refractory to treatment. Several authors have reported on *P. aeruginosa* metritis in mares and the differing virulence of different strains *(Hughes et al.* 1966, *Blanchard et al.* 1992, *Klug & Sieme* 1992), but only 1 study includes some information on the serotypes encountered, unfortunately using a different serotyping scheme which does not allow a comparison to the present findings *(Anzai et al.* 1991). Reliable information on the serotypes of *P. aeruginosa* causing endometritis in mares is thus lacking for epidemiological purposes, and at present we are unable to evaluate the risk of the presence of *P. aeruginosa* in clinically healthy stallions for transmitting the infection to mares.

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Sammendrag

Bakteriel flora i sæd opsamlet fra danske varmblodshingste med kunstig skede.

Sædprøver opsamlet fra 21 danske varmblodshingste med 2 typer af kunstig skede (14 prøver med »Colorado« type og 7 prøver med »Missouri« type) undersøgtes bakteriologisk dels ved direkte udsæd (DP) på blodagar på hingstestationen, dels ved parallel udsæd af sædsvaberprøver opbevaret i Stuart's transportmedium (TM) ved 4°C i 1-4 dage før dyrkning. Der observeredes ingen signifikante forskelle i den bakterielle flora mellem prøver udtaget med de 2 forskellige skedetyper, eller mellem de 2 bakteriologiske dyrkningsmetoder (DP kontra TM). Kun 1 (4,8%) af TM prøver, og kun 2 (9,5%) af DP prøver var sterile, medens der fra de resterende prøver dyrkedes en blandingsflora bestående af 1 til 4 bakterieslægter, domineret af koagulase-negative stafylokokker *(Staphylococcus lentus, S. capitis, S. haemolyticus, S. xylosus)* (16/21 = 76%), corynebakterier (11/21 = 52%) og alfa-hæmolytiske streptokokker og laktobaciller (7/21 = 33%). Fra 7 hingste (33%) isoleredes potentielle veneriske patogener, omfattende beta-hæmolytiske streptokokker fra 4 hingste, der brugtes til naturlig bedækning, samt *Pseudomonas aeruginosa* serotype 6 (2) og *Klebsiella pneumoniae* subsp. *pneumoniae* kapseltype K5 (1) fra 3 hingste udelukkende anvendt til kunstig sædopsamling. Hingstens rolle som bærer af potentielle veneriske patogener, og den kunstige skede som mulig smittekilde, diskuteres i relation til endometritis hos hopper.

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Reprints may be requested from: M. Madsen, Danish Veterinary Laboratory, Hangøvej 2, DK-8200 Århus N, Denmark.