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Full Length Research Paper

The important clitoral isolated bacteria of Iranian problem mares

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In this study, in order to determine the importance of clitoral isolated bacteria in problem mares and their correlation with the kind of uterine infections, 41 pure or crossbred Iranian mares were selected and divided into two groups. Twenty pregnant mares were encountered as control and 21 barren and/or repeat breeder mares were selected as test group. Clitoral bacterial samples were collected from pregnant (control) and problem (test) mares, but uterine swabs and cytology smear samples were collected only from problem mares. The kind and number of clitoral bacteria were compared in control and test groups with Chi–square or Fisher exact (two tailed) and McNemar tests. Findings showed that *Escherichia coli* was the most frequent isolated bacteria in 80.9% of clitoral and 61.9% of uterine samples of barren, and 68% of clitoral samples of pregnant mares. They should be a secondary contamination. The most important isolated bacteria were β–hemolytic *Streptococci*, which were isolated from uterine and clitoral samples of the problem mares but not from the clitoral samples of the pregnant mares. There were an important correlation between the clitoral isolation rates of *Streptococcus zooepidemicus* and *Streptococcus equisymilis* (ksc = 0.691, p = 1.00) with their uterine isolation rates in barren mares. It was concluded that the examination of clitoral bacteria prior to breeding could be considered as a useful screening test in problem mares, and the clitoral isolation of β- hemolytic *Streptococci* could be assumed as an important finding in the problem mares prior to breeding.

Key words: Clitoral bacteria, pregnant mares, uterine infections, crossbred Iranian mares.

INTRODUCTION

At the new breeding season, before the start of the breeding operation, swab samples are collected from the vaginal vestibule, clitoral fossa and sinuses (Ricketts, 1996) at any stage of the estrous cycle. These swab samples are cultured aerobically to screen for the presence of venereal bacteria, *Pseudomonas aeruginosa* and *Klebsiella pneumonia* (capsule types 1, 2, or 5) and microaerophilically for the presence of *Taylorella equigenitalis* (Reed, 2009). The involved mare is not present to the stallion until she has been specifically treated and a series of follow-up swab samples (usually three sets, 7 days apart) have confirmed successful elimination (McKinnon et al., 2011).

It is cleared that clitoral fossa and sinuses are potential areas of chronic symptomless CEM carriers, and clitoral swabbing have became incorporated into routine stud farm preventive medicine programs (McKinnon et al., 2011; Reed, 2009).

On the other hand, bacteriological examinations of clitoral fossa and sinuses are essential part of the complete gynaecological examination of a mare, and

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should be performed in mares that fail to conceive (repeat breeder), suffer from early fetal death or abortion, show signs of genital abnormality, or are being routinely examined as barren mares after the end of mating season.

Breeding aspects, quality and quantity of feeding, teasing manners, anti-parasitic programs, veterinary history of animals, systemic and especially chronic diseases, estrous cycle problems are evaluated in problem mares. Most problem mares are involved to non-treated, unknown, and/or chronic uterine infections, (McKinnon et al., 2011). Bacterial endometritis is the most common cause of sub-fertility in mares (Asbury and Lye, 1993; Wolfsdorf and Caudle, 2007; Nikolakopoulos, 1999; Noakes et al., 2009; Watson, 1988). Endometritis is a major problem facing veterinarians treating studs and attempting to maximize conception and foaling rates. Recent advances have increased the understanding of the pathogenesis of endometritis and have resulted in more effective methods to minimize the effect on fertility. Diagnosis of infectious endometritis is based on one or more of the followings: Ultrasound detection of echogenic uterine luminal fluid, acute inflammatory changes on endometrial cytological examination, or biopsy along with a positive endometrial culture (Perkins, 2004). Whilst the response to bacterial challenge has been used in research studies, its history is perhaps the most useful indicator of a susceptible mare in practice. Demonstration of clearance failure using scintiographic method based on charcoal clearance has been used to make an accurate diagnosis of susceptible mares (Noakes et al., 2009). Detection of uterine luminal fluid (more than 2 cm prior to breeding) with trans-rectal ultrasonography has also proved a useful technique in identifying mares with a clearance problem and appears to be the most useful technique in practice (Brinsko et al., 2003). Dim mock and Edwards (1928) studied the isolated bacteria of mares' uterine infection and found a highly significant correlation between uterine infection and subsequent infertility. They also found that culturing the cervical discharge in the estrus period was a suitable procedure to identify causative pathogens of uterine infections in the involved mares. Two uterine swabs for bacterial culture and three slide smears of each sample for evaluating inflammatory cells prior to breeding would be suitable indicators of uterine conditions (Nielson, 2005).

Many researchers have found *T. equigenitalis* to be one of the most current venereal bacterial causes of uterine infections and infertility in mares. Simpson and Eaton-Evans (1978) showed that the clitoral fossa was a prime site to isolate *T. equigenitalis* from infected mares. Even in contagious equine metritis-free areas, one must keep in mind that the clitoral sinuses may function as nidi for uterine infection, especially infection iatrogenically induced during diagnostic procedures of the reproductive tract or artificial insemination (Pinto Carlos and Paccamontim, 2004). The aim of this study was to determine the importance of clitoral isolated bacteria in mares prior to breeding season and their correlation with uterine infections.

MATERIALS AND METHODS

From 2000 Turkmen, Turkmen×Thoroughbred and Arab crossbred mares, 4 to 10 years of age, in Shohada Jockey Club (300 mares belonging to the National Iranian Horse Association, Tehran province), Gonbad-e-Kavoos, Bandar-e-Turkmen and Aggalla, 41 mares were selected and classified in the study (21 barren and /or problem mares as test group and 20 pregnant mares as control group) during the breeding seasons (spring and summer). In the control group, pregnancy was diagnosed, using B-mode trans-rectal ultrasonography, 5 MHz (Ibex, Elmedical, USA). The animal was assumed as a problem mare in the test group when she had a history of fertility failure, after at least three unsuccessful breedings with a fertile stallion. Bacteriologic samples were obtained from 1- clitoral median sinuses and fossa in the animals of the two groups, and 2- uterine sample by a double-guarded swab in non-pregnant animals of the test group. Clitoral samples were transferred to Amies Charcoal Transport Media (Medical Wire and Equipment Co., Ltd. Corsham, Wilstoshire, UK) as quickly as possible to support *T. equigenitalis* and other possible bacteria. Cytological smears of uterine samples were obtained on slides before transferring the samples to Amies Transporting Media and stained by Diff quick (American Scientific Product, McGraw Park, IL) to evaluate the presence of polymorph nuclear (PMN) and other leucocytes. The samples were divided to low (<0.5%) and high (>0.5%) polymorph nuclear (PMN) according to Derek (1993). Bacterial cultures were followed in the laboratory as soon as possible. The kind and rates of clitoral bacterial infections were compared in the control and test groups with Chi–square, Fisher exact (two tailed) and McNemar tests.

RESULTS

Our findings showed that the incidence rate of problem mares was about 28.4% (568 from 2000 evaluated mares). *T. equigenitalis* was not isolated from the animals of the control and test groups. Many pure and mixed bacteria, especially in combination with *Escherichia coli*, were isolated from the clitoral and uterine samples. *E. coli* was the most frequent pure isolated bacteria in 80.9% of the clitoral and 61% of uterine samples of barren mares, and 68% of clitoral samples of pregnant mares (Tables 1 and 2).

Streptococcus zooepidemicus was the most important bacteria mixed with *E. coli* (n = 2) or *K. pneumonia* (n = 1). Mixed *S. zooepidemicus,* mixed *K. pneumonia* (n = 3), mixed *E. coli* with *S. equines* (n = 1), *Staphylococcus intermedius* (n = 1), and *Streptococcus equisimilis* (n = 1) and mixed *Corynebacterium spp*. (n = 2) were isolated only from the clitoral samples of the problem mares and were not isolated from the pregnant mares (Table 1). The most current pure bacteria isolated from the uterine samples of the non-pregnant animals in the test group were *E. coli* (n = 12) and *S. zooepidemicus* (n = 2) (Table 2). Fisher exact test did not show any significant correlation between infertility and the rate of clitoral

Table 1. The frequencies of clitoral isolated bacteria of barren and pregnant mares.

S/N	Clitoral isolated bacteria	Barren mares		Pregnant mares		Total	
		N	%	N	%	N	%
1	*E. coli*	6	28.6	5	25	11	26.8
2	*E. coli+Staph. albus*	2	9.5	4	20	6	14.6
3	*E. coli+proteus*	1	4.8	2	10	3	7.3
4	*E. coli*+yeast	1	4.8	2	10	3	7.3
5	*E. coli+Strep. zooepidemicus*	2	9.5	0	0	2	4.9
6	*Bacillus cereus*	1	4.8	1	5	2	4.9
7	*Corynebacterium spp.*	0	0	2	10	2	4.9
8	*Strep.zooepidemicus+K. pneumonia*	1	4.8	0	0	1	2.4
9	*E. coli+ K. pneumonia*	1	4.8	0	0	1	2.4
10	*K. pneumonia*	0	0	1	5	1	2.4
11	*E. coli+ Corynebacterium spp.*	1	4.8	0	0	1	2.4
12	*Strep. equines*	0	0	1	5	1	2.4
13	*Staph. aureus+Corynebacterium spp.*	1	4.8	0	0	1	2.4
14	*E. coli+ Strep. equines*	1	4.8	0	0	1	2.4
15	*Proteus+ Bacillus cereus*	1	4.8	0	0	1	2.4
16	*Staph. intermedius*	0	0	1	5	1	2.4
17	*E. coli+ Staph. intermedius*	1	4.8	0	0	1	2.4
18	*E. coli+Strep. equisimilis*	1	4.8	0	0	1	2.4
19	*Staph. aureus*	0	0	1	5	1	2.4

Table 2. Frequencies of uterine isolated bacteria in barren mares.

S/N	Uterine isolated bacteria	N	%
1	No growth	1	4.8
2	*E. coli*	12	57.1
3	*S. zooepidemicus*	2	9.5
4	*S. equisimilis*	1	4.8
5	*S. aurous +K. pneumonia*	1	4.8
6	*S. zooepidemicus+ K. pneumonia*	1	4.8
7	*E. coli* + *Corynebacterium* spp.	1	4.8
8	*S. albus*	1	4.8
9	*S. intermedius*	1	4.8
Total	Pure isolations	17	81
	Mixed isolations	3	14.3
	Total	21	100

isolation of *E. coli* in the test and control groups (P = 0.42). Also, the rate of clitoral isolation of *K. pneumonia* (P = 0.10) and beta hemolytic *streptococci* (P = 0.10) did not vary significantly between the test and the control groups. On the other hand, beta hemolytic *Streptococci* were isolated only from high PMN uterine samples of non-pregnant mares, and *E. coli* was isolated from 4 samples of high PMN and eight samples from low PMN uterine discharge (Table 3; Figure 1). Fisher exact test did not show any significant correlation between the rate of PMN in the uterine samples and the presence of *E. coli* (P = 0.15), or beta hemolytic *Streptococci* (P = 0.11). Furthermore, McNemar test and kappa statistic calculation (ksc; agreement between two approaches) showed that there were important correlations between the rate of *E. coli* (ksc = 0.55, P = 0.12), *S. zooepidemicus* and *S. equisymilis* (ksc= 0.69, P = 1.0) isolations from the clitoral samples and their isolation rate in the uterine cultures of the barren mares. However, the isolation rate of *K. pneumonia* from the clitoral samples showed no significant correlation with the rate of its isolation from the uterine cultures of the barren mares

Table 3. The frequencies of mares uterine isolated bacteria of high PMN as compared with low PMN.

S/N	Uterine isolated bacteria	PMN ≥ 0.5 %				PMN < 0.5%				Total	
		Pure isolations		Mixed isolations		Pure isolations		Mixed isolations			
		N	%	N	%	N	%	N	%	N	%
1	*E. coli*	4	20	1	5	7	35	0	0	12	60
2	*S. zooepidemicus*	2	10	0	0	0	0	0	0	2	10
3	*S. aureus* + *K. pneumonia*	0	0	1	5	0	0	0	0	1	5
4	*S. albus*	0	0	0	0	0	0	1	5	1	5
5	*S. equisimilis*	1	5	0	0	0	0	0	0	1	5
6	*S. zooepidemicus*+ *B. cereus*	0	0	1	5	0	0	0	0	1	5
7	*S. intermedius*	1	5	0	0	0	0	0	0	1	5
8	*E. coli* + *Corynebacterium* spp.	0	0	1	5	0	0	0	0	1	5
Total		8	40	4	20	7	35	1	5	20	100

(ksc = -0.06, P = 1.0).

DISCUSSION

There are several reports in the literature concerning the field of diagnosis and treatment of endometritis in the mare. The combined use of cytology and bacteriological culture as an aid in the diagnosis of mare endometritis was initially alluded to almost 20 years ago, and is currently the most commonly used procedure (Liu and Troedsson, 2008). Problem mares were about 28.4% of the evaluated animals in the existing conditions of the present study. In an excellent retrospective study of 639 Thoroughbred mares on a farm with 2466 coverings in 1528 mares, the non return rate, that is, 30 days after a previous cover was 9.4% and the rate of non pregnant mares after the end of the breeding season was 17.1% (Badi et al., 1981). Isolation of venereal bacteria including *T. equigenitalis* (contagious equine metrits), *P. aeruginosa,* and *K. pneumonia* (Perkins, 2004) can be considered an important event in low PMN samples (McKinnon, 2011) but, the interpretation of isolation of the others depends on the number of PMNs, purification of culture and the number of bacterial colonies (Mckinnon, 2011). In the present study, *T. equigenitalis* was not isolated from the mares of the two groups. This is in agreement with another study conducted in Iran (Ghasemzadeh-nava et al., 2004) but, Ricketts et al. (1993) have found *T. equigenitalis* to be one of the most current venereal bacterial causes of uterine infections and infertility in mares. Many reports have suggested that the lateral clitoral sinuses may be too shallow to support the growth of *T. equigenitalis (*Perkins, 2004). In the present study, *E. coli* was the most frequent pure isolated bacteria in 80.9% of clitoral and 61% of uterine samples of barren mares, and 68% of clitoral samples of pregnant mares. *E. coli* was isolated from 4 samples of high PMN and 8 samples from low PMN uterine discharge. On the other hand, there were no significant correlations between infertility and the rates of clitoral isolation of *E. coli* in the test and control groups. This means that the clitoral and/or uterine isolation of *E. coli* is not an important event, and it seems to be a secondary contamination and non-pathogen strain. The most important bacteria in the present study were isolated onlyfrom the clitoral samples of the problem mares, were *S. zooepidemicus, K. pneumonia*, *S. equines*, *S. intermedius* and *S. equisimilis* and *Corynebacterium* spp.

On the other hand, the most current pure bacteria isolated from uterine samples of the non pregnant animals of the test group accompanied by high PMN in uterine cytology were beta hemolytic *Streptococci.* It has been reported that beta hemolytic *Streptococci* are the most frequently isolated pathogens in mare endometritis, and *S. zooepidemicus* is the dominant pathogen (Simpson and Eaton-Evans, 1978; Asbury and Lye, 1993; McKinnon and Voss, 1993; Nielson, 2005). Recently, Leblanc et al. (2007) reported that *E. coli* followed by beta hemolytic streptococcus were the most frequently isolated microorganisms from the positive uterine flush cultures, alone or in combination with another microorganism. There are some reports indicating that other microorganisms such as *E. coli* and *Corynebacterium* spp. (Ball, 1998) have been the most commonly recovered microorganisms from the uteri of selected sub-fertile mares. Ghasemzade-nava et al. (2004) showed that *E. coli* (64%) was the most frequently isolated pathogen from young, middle aged and old endometritis involved mares in Iran, in which beta-hemolytic *Streptococci* (6.3%) and *K. pneumonia* (0.9%) were the second and third most important isolated pathogens, respectively. *E. coli* (72.4%), *K. pneumonia* (0.6%) and *T. equigenitalis* were the three major isolated bacteria from mares' uterine infection by Ricketts et al. (1993). Despite the lack of any significant variations between the test and control groups in the isolation rate of clitoral *K. pneumonia* and beta hemolytic

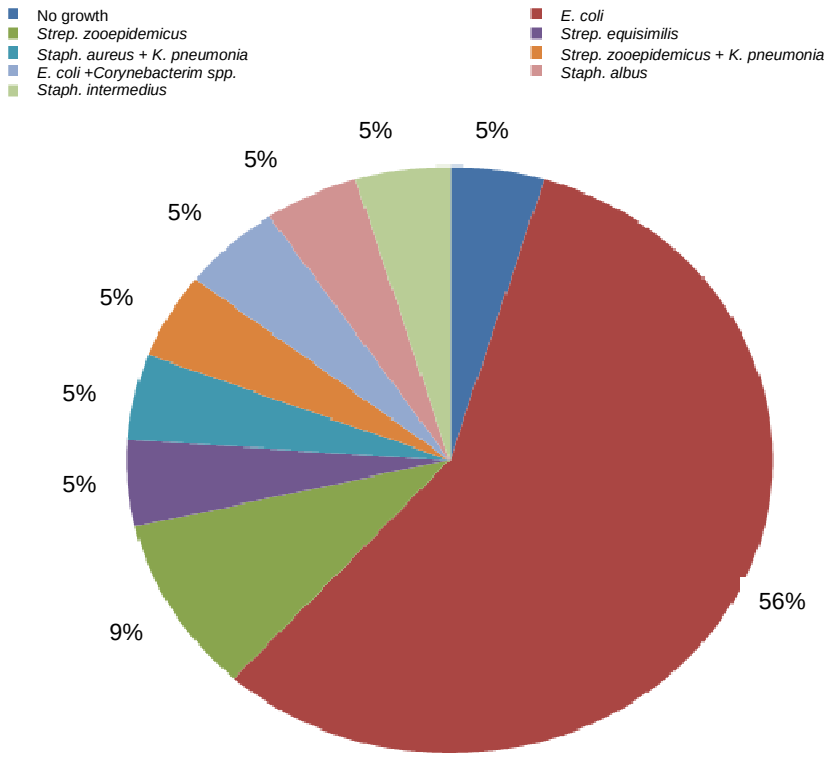

Figure 1. The isolation rates of uterine isolated bacteria in barren mares.

Streptococci in the present study, the latter were clearly more common in problem mares and were the most current isolated bacteria in combination with high PMN in uterine samples of non-pregnant mares. There were an important correlations between the isolation rates of *E. coli*, *S. zooepidemicus* and *S. equisimilis* in the clitoral samples and their isolation rate in the uterine cultures of the barren mares, but there were no important correlations between the isolation rates of *K. pneumonia* in the clitoral samples and its isolation rates in the uterine cultures of the barren mares. Negative correlation between the isolation rates of *K. pneumonia* in the clitoral samples with the uterine samples in the barren mares indicates that, the agreement is less than that expected by chance and may be due to inadequate number of samples and/or the speed of expelling ability of the uterus in comparison with the clitoris. So, on the base of this negative correlation, clitoral samples may not be a reliable representative of uterine cultures. The ability of bacterial isolation in normal mares has been decreased progressively from the clitoral fossa, 94%, to the vestibule, 69%, cranial vagina, 42%, and uterus, 31% (Hinrichs et al., 1989).

It is concluded that beta-hemolytic *Streptococci* are the most common cause of uterine and clitoral infections in mares, and despite the common isolation of *E. coli*; it may be a secondary contamination. The examination of clitoral bacteria prior to breeding can be considered as a useful screening test in problem mares.

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