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Clitoral Isolated Bacteria from Problem and Pregnant Mares in Iran

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Summary

In order to determine the kind of clitoral and uterine bacteria, 41 pure or crossbred Iranian mares were selected and 20 pregnant mares were encountered as control and 21 barren and/or repeated breeder mares as a test group. Clitoral bacterial samples were collected from pregnant and problem mares and uterine swabs and cytology smear samples were collected only from problem mares to determine the existing bacteria. The kind and numbers of clitoral bacteria were compared in control and test group with Chi–square and Fisher exact test. Findings showed that *E. coli* were the most frequent isolated bacteria in 80.9% of clitoral samples of barren, 68% of clitoral samples of pregnant mare and 61.9% of uterine samples of barren mares. However, they would be a secondary contamination. The most important isolated bacteria were β–hemolytic *Streptococci*, which were isolated from uterine and clitoral samples of problem mares and were not isolated from pregnant mares. There were important correlation between the rate of *Streptococcus zooepidemicus* and *Streptococcus equisymilis* (ksc= 0.691, p= 1.00) isolations from clitoral samples and the rate of them in uterine cultures of barren mares. It is concluded that examination of clitoral bacteria prior to breeding could be an accurate substituting for uterine culture and cytology, if β- hemolytic *Streptococci* have been isolate.

Introduction

Non-pregnant mares which have three subsequent and successful breeding with a fertile stallion in a breeding season has been assumed as a repeat breeder mare and, since she has left breeding season considered as a barren mare. Problem mares consist of both, barren and/or the most currently, repeat breeder mares (Albihn, 1998). Breeding aspects, quality and quantity of feeding, teasing manners, anti-parasitic programs, veterinary history of animals, systemic and especially chronic diseases and the most important ones, estrous cycle problems (sub estrous, anestrous, pseudo-pregnancy, and persistent corpus luteum) would be evaluated in problem mares. However, the most problem mares are accompanied by non-treated, unknown, and/or chronic uterine infections (endometriosis) (McKinnon and Voss, 1993; Noakes et al., 2001). Bacterial endometritis is the most common cause of sub fertility in mares (Asbury, 1992; Card, 1997; Nikolakopoulos and Watson, 1999; Watson,

1988). Two uterine swabs samples for bacterial culture and three slide smears of each sample for evaluating inflammatory cells prior to breeding would be suitable indicator of uterine conditions (Blance et al., 1994). Many researchers have found *Taylorella equigenitalis* as one of the most current venereal bacterial cause of uterine infections and infertility of mares. Simpson and Eaton-Evans (1978) showed that the clitoral fossa was a golden site to isolate *Taylorella 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 and Paccamontim, 2004). The findings conducted the hypothesis that clitoral bacteriologic samples might be a representative of uterine infections in problem mares, at least in existing conditions in Iran. In an effort to test this hypothesis, and determine the kind of reproductive pathogens of problem mares, a series of experiments were conducted using uterine and clitoral bacterial isolations and uterine cytology examinations in problem mares, comparing with the obtained data from pregnant mares.

Materials and methods

From 2000 Turkman, Turkman×Thoroughbred and Arab crossbred mares in Shohada jockey club (300 mares, related to National Iranian horse association, Tehran province), Gonbad e Kavoos, Bandar e Turkmen and Ag Galla, 41 mares were selected and divided in the test (21 barren and /or problem mares) and control (20 pregnant mares) groups during the breeding seasons of 2005. Pregnancy was examined by a probe of 5 MHZ, B-mod trans-rectal ultrasonography. The animals assumed a problem mare in the test group with a history of fertility failure at least after three successful breeding with a fertile stallion. Bacteriologic samples were obtained from 1- clitoral median sinuses and fossa in animals of tow groups and 2- uterine discharge in non-pregnant animals of test group. Clitoral samples were transferred to Amies charcoal transport media (Medical wire and Equipment co., Ltd.Corsham, Wilstoshire, UK) as soon as possible to support of *Taylorella 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. Bacterial cultures were followed in laboratory as soon as possible. The kind and the rates of clitoral bacterial infections were compared in control and test group with Chi–square, Fisher exact (two tailed) and McNemar tests.

Results

The rate of problem mares was about 28.4% in evaluated animals. *Taylorella equigenitalis* was not isolated from any animals of control and test groups. Many pure and mixed bacteria especially in combination with *E. coli* were isolated from clitoral and except one from all uterine samples. *E. coli* was the most frequent pure isolated bacteria in 80.9% of clitoral samples of barren, 68% of clitoral samples of

pregnant mares and 61% of uterine samples of barren mares. *Streptococcus zooepidemicus* was the most important mixed bacteria with *E. coli* (n =2) or *Klebsiella pneumonia* (n = 1), mixed *Klebsiella pneumonia* (n =3), mixed isolations of *E. coli* with *streptococcus equines* (n =1), *Staphylococcus intermedius* (n =1), and *Streptococcus equisymilis* (n=1) and mixed *Corynebacterium spp*. (n =2) which isolated only from clitoral samples of problem mares and do not from pregnant mares (Table 1). The most current pure bacteria isolated from uterine samples of non-pregnant animals of test group were *E. coli* (n=12) and *Streptococcus zooepidemicus* (n= 2) (Table 2). Fisher exact test showed any significant correlation between infertility and the rate of clitoral isolation of *E. coli* in the test and control groups (P= 0.4238). As well, the rate of clitoral isolation of *Klebsiella pneumonia* (P= 0.1.0) and beta hemolytic *streptococci* (P = 0.1069) were not significantly varied between the test and control groups. On the other hand, beta hemolytic *Streptococci* were isolated only from high PMN uterine samples. However, *E. coli* was isolated form 4 samples of high PMN and eight samples from low PMN uterine discharge (Table 3). Fisher exact test showed any significant correlation between the rate of PMN in uterine samples and presences of *E. coli* (p= 0.1576) and beta hemolytic *Streptococci* (p= 0.1166). Furthermore, McNemar test and kappa statistic calculation (KSC; agreement between two approaches) showed that there were important correlation between the rate of *E. coli* (ksc= 0.553, p= 0.125), *Streptococcus zooepidemicus* and *Streptococcus equisymilis* (ksc= 0.691, p= 1.00) isolations from clitoral samples and the rate of them in uterine cultures of barren mares. However, the rat of *Klebsiella pneumonia* isolation from clitoral samples showed any significant correlation with the rate of its isolation from uterine cultures of barren mares (ksc= -0.068, P=1).

Discussion

Problem mares were about 28.4% of evaluated animals in existing conditions in present study. In an excellent retrospective study of 639 Thoroughbred mares on one farm with 2466 coverings in 1528 mare years, the fertilization failure and early embryonic death rate based on a return to estrus within 30 days of a previous cover was 31.7%. While the late embryonic death rate of 26.5% was based on a return to estrus after 30 days after a previous cover (9.4%) and a diagnosis of no pregnancy at 42 days, often after the end of the breeding season (17.1%)(Badi et al.,1981). Endometritis is a major problem facing veterinarians treating studs and attempting to maximize conception and foaling rates. Recent advances have important understanding of the pathogenesis of endometritis and have resulted in more effective methods to minimize the affect on fertility. Diagnosis of infectious endometritis is based on one or more of following: Ultrasound detection of echogenic uterine luminal fluid and acute inflammatory changes on endometrial cytological examination or biopsy along with a positive endometrial culture (Perkins. 2004).

Table 1. The frequencies of clitoral isolated bacteria of barren as compared with pregnant mares.

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+Srep. 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. equinus*	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.

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

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

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	*Strep. zooepidemicus*	2	10	0	0	0	0	0	0	2	10
3	*Staph. aureus* + *K. pneumonia*	0	0	1	5	0	0	0	0	1	5
4	*Staph. albus*	0	0	0	0	0	0	1	5	1	5
5	*Strep. equisimilis*	1	5	0	0	0	0	0	0	1	5
6	*Strep. zooepidemicus* +*Bacillus cereus*	0	0	1	5	0	0	0	0	1	5
7	*Staph. 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

Whilst the response to bacterial challenge has been used in research studies, history is perhaps the most useful indicator of a susceptible mare in practice. Demonstration of clearance failure using scintigraphic method based on charcoal clearance has been to make an accurate diagnosis of susceptible mares (Noakes et al., 2001). Trans rectal ultrasonography to detect uterine luminal fluid (more than 2 cm prior to breeding) has also proved useful in identifying mares with a clearance problem and would appear the most useful technique in practice (Brinsko et al., 2003). Dimock and Snyder (1923) and Dimock and Edwards (1928), studied isolated bacteria of mares' uterine infection and find a highly significant correlation between uterine infection and subsequent infertility. They also find that culturing the cervical discharge in estrus period was a suitable procedure to identify causative pathogens of uterine infections in involved mares.

Sexually transmitted disease include contagious equine metritis (*Taylorella equigenitalis), Pseudomonas aeruginosa* and *Klebsiella pneumonia* (Perkins, 2004) *Taylorella equigenitalis* was not isolated from any of animals of control and test groups in existing conditions in present study. It is in agreement with the other studies in Iran (Gasemzadeh-nava et al., 2004). However, Ricketts et al. (1993) found *Taylorella equigenitalis* as one of the most current venereal bacterial cause of uterine

infections and infertility of mares. Many reports has suggested that the lateral clitoral sinuses may be too shallow to support growth of *Taylorella equigenitalis.*[18]

In present study *E. coli* was the most frequent pure isolated bacteria in 80.9% of clitoral samples of barren, 68% of clitoral samples of pregnant mares and 61% of uterine samples of barren mares. However, *E. coli* was isolated form 4 samples of high PMN and eight samples from low PMN uterine discharge. On the other hand there was any significant correlation between infertility and the rate of clitoral isolation of *E. coli* in the test and control groups. It means that clitoral and /or uterine isolation of *E. coli* would not be spontaneously an important finding, and it might be a non-pathogen strain and secondary contamination. The most important bacteria in present study, which isolated only from clitoral samples of problem mares, were *Streptococcus zooepidemicus, Klebsiella pneumonia*, *Streptococcus equines*, *Staphylococcus intermedius* and *Streptococcus equisymilis* and *Corynebacteriu* spp. On the other hand, the most current pure bacteria isolated from uterine samples of non pregnant animals of test group accompanying with high PMN in uterine cytology were beta hemolytic *Streptococci.*

It is reported that beta hemolytic *Streptococci* are the pathogens that are most frequently isolated in mare endometritis ,of which *Streptococcus zooepidemicus* is the dominant pathogen(Watson, 1988; Asbury and Lye, 1993; Miller and Francis, 1974). There are some reports indicating that other microorganisms such as *E. coli* (Albihn, 1998) and *Corynebacterium* spp. (Ball et al., 1988) 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 age and old endometritis involved mares in Iran, and beta-hemolytic *Streptococci* (6.3%) and *Klebsiella pneumonia* (0.9%) were the second and third important isolated pathogens, respectively.

E. coli (72.4%), *Klebsiella pneumonia* (0.6%) and *Taylorella equigenitalis* isolated as three major isolate bacteria from mares' uterine infection by Ricketts et al. (1993). Despite there were any significant variations between the test and control groups in the rate of clitoral isolation of *klebsiella pneumonia* and beta hemolytic *Streptococci,* the latter were clearly more in problem mares and the most current isolated bacteria in combination with high PMN in uterine samples of no pregnant animals. Beta hemolytic *Streptococci* might be the most current isolated bacteria of clitoral samples of problem mares in a study with more examined samples.

Despite an important correlation between the rate of *E. coli*, *Streptococcus zooepidemicus* and *Streptococcus equisymilis* isolations from clitoral samples and the rate of them in uterine cultures of barren mare, any important correlation was encountered for *Klebsiella pneumonia.* Negative correlation between isolation of *Klebsiella pneumonia* from clitoris and uterine infection in barren mares indicates agreement lees than is expected by chance and would be due to inadequate numbers of samples and/or speed expelling ability of uterus in comparison with clitoris. The ability to isolate bacteria in normal mares decreases progressively from the clitoral fossa (94%) to the vestibule (69%), cranial vagina (42%), and uterus (31%) (Hinrichs

et al. 1989). Because of this negative correlation, clitoral samples are not a reliable representative of uterine cultures**.**

It is concluded that beta-hemolytic *Streptococci* are the most common microorganisms to cause mare uterine and clitoral infections in Iran and despite common isolation of *E. coli;* it might be a secondary contamination. Examination of clitoral bacteria prior to breeding could be an accurate substituting for uterine culture and cytology, if beta - hemolytic *Streptococci* have been isolate.

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Reference

1)Albihn, A., 1998: Microbiology of uterine infections in Sweden, Equine Veterinary Data. **18**, 511.

2)Asburg, A. C., 1992: How effective is your treatment of bacterial endometritis Equine veterinary Journal **24,** 416-7.

3)Asbury, A. C., and S. K. Lye, 1993: In: Equine Reproduction. McKinnon, A. O, Voss, J. L., Lea and Febiger Company. First ed. 381-91.

4)Badi, A. M., T. M. Byrne, and E. P. Cunningham, 1981: An Analysis of Reproduction performance in Thoroughbred Mares. Irish Veterinary Journal. **35**, 1:1.

5)Ball, B. A., S. J. Shin, V. H. Patten, D. H. Lein, G. H. Wood, 1988: Use of a low-volume uterine flush for microbiologic and cytological examination of the mares' endometrium. Theriogenology. **29**, 1269-83.

6)Blance, M. M., J. Dugan, J. Manning, D. McInnis, and C. Wood, 1994: Reproductive management in the mare. Part 1: Equine Practice. **16**, 15-19.

7)Brinsko, S. P., S. L. Rigby, D. D. Varner and L. Blanchard, 2003: A practical method for recognizing mares susceptible to post-breeding endometritis (Last Updated) in: 49th annual convention of the American association of equine practitioners. New Orle and Louisianan Internet Publisher: International Veterinary Information Service, Ithaca NY; P0657.1103.

8)Card, C., 1997: Current therapy in large animal theriogenology. Youngquist R. S. editor 1st ed. WB Saunders Co, PP. 151-3.

9)Dim mock, W. W. and P. R. Edwards, 1928: Pathology and bacteriology of reproductive organs of mares in relation to sterility. Research Bulletin of Kentucky Agricultural Experimental Station. Lexington. 286.

10)Dim mock, W. W., and E. M. Snyder, 1923: Bacteria of the genital tract of mares and the semen of stallions and their relation to breeding efficiency. Journal of American Veterinary Medicine Association. **64**, 288-289.

11)Gasemzadeh-nava, H., F. Ghasemi, P. Tajik, and A. Shirazi, 2004: A review of mare endometritis in Iran. J. Equine Veterinary Science **24,** 188-192.

12)Hinrichs, K., M. R. Cummings, P. L. Sentich, and R. M. Kenney, 1989: Bacterial removal from the reproductive tracts of normal mares. Proc. Am. Assoc. Equine Pract. 11-16.

13)McKinnon, A. O., and J. L. Voss, 1993: Equine Reproduction. Lea and Febiger, Philadelphia. PP.196-258 and 368-381.

14)Miller, R. and J. Francis, 1974: The relation of clinical and bacteriological findings to fertility in thoroughbred mares**.** Australian Veterinary Journal**, 50**, 351-5.

15)Nikolakopoulos, E., and Watson, E.D., 1999: Uterine contractility is necessary for the clearance of intrauterine fluid but not bacteria after bacterial infusion in the mare. Theriogenology. **52**, 413-23.

16)Noakes, D. E., T. E. Parkinson, G. C. W England, 2001: Arthur's Veterinary Reproduction and Obstetrics.WB Saunders Co. PP. 609-612.

17)Perkins, N. R., 2004: Endometritis and Uterine therapy .In: Equine Internal Medicine. Reed S. M., W. M. Bayly and D. C. Sellon, 2nd ed. WB Saunders Co. PP. 1049–1058.

18)Pinto Carlos, R. F., and D. L. Paccamontim, 2004: Mare Reproductive Pathology. In: Equine Internal Medicine. Reed, S. M., W. M. Bayly, and D. C. Sellon 2nd ed. WB Saunders Co. PP. 1039–1048.

19)Reed, S. M., W. M. Bayly, and D. C. Sellon, 2004: *Equine Internal Medicine.* 2nd ed. WB Saunders Company. PP. 1039–1058.

Ricketts, S.W., A. Young, and E. B. Medici, 1993: Uterine and clitoral cultures. Equine reproduction, 1st ed. Vol. 1, Lea and Febigur, Philadelphia. PP. 234-224.

20)Simpson, D. J., W. E. Eaton-Evans, 1978: Developments in contagious equine metritis.Veterinary**.** Records. **102.** 19-20.

21)Simpson, D. J., W. E. Eaton-Evans, 1978: Sites of contagious equine metritis infection. Veterinary Records. **102**. 488.

22)Watson, E. D.,1988: Uterine defense mechanisms in mares resistant and susceptible to persistent endometritis : A Review. Equine Veterinary Journal. **20**, 397-400.