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Microbial Flora of Normal and Abnormal Cervical Mucous Discharge Associated with Reproductive Performance of Cows and Heifers in Estrus

Ayhan Ata*, Hülya Türütoğlu[1], Mehmet Kale[2], Mehmet Şükrü Gülay[3] and Faruk Pehlivanoğlu[1]

Mehmet Akif Ersoy University, Faculty of Veterinary Medicine,
Departments of Theriogenology and Artificial Insemination, 15100-Burdur, Turkey

ABSTRACT : The aim of the present study was to describe whether abnormal cervical mucus discharge (A-CMD) or pathogens in cervical mucus discharge (CMD) have effects on reproductive performance of cows and heifers in estrus. Animals having clear discharges (68 cows, 38 heifers) with normal viscosity and without bad odor were grouped as normal cervical mucous discharge (N-CMD) group. The other animals (84 cows, 32 heifers) were grouped as A-CMD group. Microorganisms isolated from samples were divided into three groups as uterine pathogens (UP), potential uterine pathogens (PUP) or opportunistic uterine pathogens (OUP). Presence of PUP was associated with A-CMD for both cows ($p<0.01$) and heifers ($p<0.02$). First service conception rates (FS-CR) were lower in cows positive for PUP ($p<0.01$). Moreover, presence of PUP and OUP affected FS-CR in heifers ($p<0.01$). Although A-CMD significantly affected FS-CR in cows ($p<0.04$), it did not affect FS-CR in heifers. Differences in average open day for cows ($p<0.02$) and first service age for heifers ($p<0.01$) were significant between N-CMD and A-CMD groups, respectively. The current study suggested that CMD should be evaluated more carefully when there are infertility problems. In addition to the known microorganism that causes sterility and infertility in the UP group, pathogens in the PUP group should be considered for their potential to cause infertility. (**Key Words :** Discharge, Cow, Heifer, Fertility, Bacteria)

INTRODUCTION

Reproductive performance (RP) is essential for well-managed and profitable dairy farms (Nebel and Jobst, 1998). With the trend of decreasing profit in dairy farming reported worldwide, it is necessary to identify where efficiency improvements can be made (Bishop, 1964). There are many factors that directly or indirectly influence the RP of cows. Cow infertility is affected by many specific and non-specific pathogens of the genital tract. The cervix and its secretions play a pivotal role in the reproductive performance of mammals (Hafez and Kanagawa, 1972; Matner, 1973). Cervical mucous discharge (CMD) is a mechanical barrier against pathogen of the uterus. Normally a cow in estrus discharges a viscous liquid from the vulva. The healthy liquid is clear, originates from the cervix and has no bad odor. Clear CMD on the artificial insemination (AI) gun following insemination was positively associated with an increased first service conception rate (FS-CR) (Loeffler et al., 1999). CMD of cows and heifers with abnormal appearance in estrus cycle is one of the factors that farmers or artificial inseminators consider it as a RP suppressor (Mahmoudzadeh et al., 2001). For example, endometritis in cows, characterized clinically by the presence of pus in the vagina (Dohmen et al., 1995; Sheldon and Noakes, 1998) is associated with lower FS-CR, increased open days (OD), and more culls for failure to conceive (Studer and Morrow, 1978; LeBlanc et al., 2002). Overall, it is often assumed that pus in the vagina reflects uterine infection but microbiological confirmation is also required. Therefore, the aim of the present study was to describe whether abnormal cervical mucus discharge (A-CMD) or pathogens such as aerobic bacteria and fungi in

* Corresponding Author: Ayhan Ata. Tel: +90-248-2344500, Fax: +90-248-2344505, E-mail: ayhana@mehmetakif.edu.tr
[1] Mehmet Akif Ersoy University, Faculty of Veterinary Medicine, Departments of Microbiology, 15100-Burdur, Turkey.
[2] Mehmet Akif Ersoy University, Faculty of Veterinary Medicine, Departments of Virology, 15100-Burdur, Turkey.
[3] Mehmet Akif Ersoy University, Faculty of Veterinary Medicine, Departments of Physiology, 15100-Burdur, Turkey.
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CMD have effects on RP of cows and heifers in estrus.

MATERIALS AND METHODS

Animals

Two hundred twenty two Holstein-Frisian cows and heifers, (cows between 2 and 11 year old; heifers between 15 and 24 months old) kept at 137 different dairy farms in the province of Burdur, Turkey, was assigned to the trial. Both cows and heifers brought to The Holstein Breeding Association of Burdur for AI was included in the trial. Annual average milk yields normal cervical mucous discharge (N-CMD) and A-CMD group cows were 5.201± 190.2 and 5.075±221.8 kg, respectively. To exclude the possible effects of reproduction problems related to nutrition deficiency, cows and heifers with lower body condition score (BCS) than 2.5 were not included in the study (Ferguson et al., 1994). Mean BCS for cows and heifers in N-CMD and A-CMD groups were 3.21±0.18 and 2.95±0.22 and 3.01±0.25 and 3.18±0.16, respectively. All dairy cows and heifers were examined by experienced veterinarian vaginally per rectum, and were healthy and free of anatomical abnormalities of the reproductive tract. For vaginal examination, the sterilized vaginal speculum (Kruuse, Marslev, Denmark) was inserted into the vagina and vagina was examined with the help of an external light source for any abnormalities such as vaginitis, pustule or vesicular lesion on the mucosa. Animals with detectable vaginal lesions were excluded from the study.

Collection of cervical mucous discharge

The CMD swab from each animal was taken 5 to 30 min before AI. Animals having CMD with clear and translucent mucus with normal viscosity and without bad odor were grouped as N-CMD (68 cows and 38 heifers) group. Animals with opaque mucus or mucus containing flecks of pus and purulent or mucopurulent material were grouped as A-CMD (84 cows and 32 heifers) group. After restraining the animal and securing its tail, the perineal region was washed, cleaned with soap 3-4 times and dried out. Swabs for CMD were obtained using a sterile double-guarded swab device (Kruuse, Equivet, Marslev, Denmark). The swab was inserted through the vagina into the lumen of the cervical canal, guided by palpation per rectum. The inner rod of the catheter was then pushed forward to expose the swab and rotated four times against the mucosa while moving the swab backward and forward, and then withdrawn within the catheter under rectal guidance by means of the conventional AI technique. All CMD swabs transported to the laboratory in the appropriate media for bacteriology (Cary Blair Transport Medium, Oxoid Ltd. Hampshire, UK) at 4°C, within 3 h and immediately processed.

Data collection and artificial insemination

Artificial insemination dates were recorded by the inseminator and pregnancy diagnosis was performed by rectal palpation 6-8 weeks after AI. The records of AI, pregnancy diagnosis and calving dates were obtained from the Holstein Breeding Associations data base. The AI coincided with middle of estrus, as evidenced by CMD, high myometrial tone and contractility. In addition to secondary signs of estrus, the stage of estrus cycle was confirmed by the presence of fluctuant Graafian Follicles and the absence of corpus luteum by rectal palpation for all animals prior to AI. The AI was performed for the first time on all unmated heifers and was the first time after postpartum in all cows. AI was performed regardless of normal or abnormal appearance of CMD on the day of spontaneous estrus. AI was performed using frozen-thawed semen containing at least ten million of motile spermatozoa (Consorzio Semenzo-Italy Via Masaccio, 11-42010 Mancasale, Italy) from a single bull (Donal Patron IAKUT ET) with proven fertility. Semen was placed at the corpus uteri for all cows and heifers. Breeding day (day 0) was accepted as equal to the day of onset of strong estrus signs.

Pregnancy diagnosis and calculations of first service conception rate

Pregnancy was diagnosed by rectal palpation of heifers and cows (at 40 to 55 days and 50-65 days post AI, respectively). When AI led to positive pregnancy check, it was defined as successful. If an animal declared non-pregnant by rectal palpation or returned to heat, the AI was coded as unsuccessful. First service conception rate (FS-CR) was calculated as the percentage of conception at 8 weeks after AI.

Microbiological examination

Swabs were streaked onto 7% sheep blood agar (Oxoid Ltd, Hampshire, England), MacConkey agar (Oxoid), Sabouraud dextrose agar (Oxoid), *Campylobacter* selective agar, and *Brucella* selective agar plates. *Brucella* selective medium was prepared by adding 5% inactivated horse serum, 20% dextrose solution at final concentration 1% and *Brucella* selective supplement (SR 209E, Oxoid) to *Brucella* medium base (Oxoid). *Campylobacter* selective medium was prepared by adding 5% defibrinated horse blood and Skirrow's antibiotic mixture (SR 069 E, Oxoid) to brain heart infusion agar (Oxoid). Blood agar and MacConkey agar plates were incubated at 37°C for 1-4 days in air. Sabouraud dextrose agar plate that was plated for fungus and yeast isolation was incubated at 37°C for 7 days. *Brucella* and *Campylobacter* selective agar plates were incubated at 37°C under microaerophilic condition with an Anaerojar system and a Campygen atmosphere-generating system (Oxoid) for 5 or 7 days. After presumptive

identification based on colony morphology and microscopic morphology, biochemical and growth characteristics of the isolates were determined (Koneman et al., 1988). Isolated pathogens were categorized according to known pathogenicity within the uterus (Farin et al., 1989; Bonnett et al., 1993; Sheldon et al., 2002; Williams et al., 2005). Microorganisms, isolated from samples, were divided into 3 groups: i) microorganisms that frequently cause endometritis (UP = uterine pathogens), ii) microorganisms that were an infrequent cause of endometritis (PUP = potential uterine pathogens), and iii) microorganisms transiently isolated from the uterine lumen and not associated with endometritis (OUP = opportunistic uterine pathogens).

Statistical analysis of data

The differences in average OD for cows and first service age (FSA) for heifers in N-CMD and A-CMD groups were compared by *Proc Mixed* procedure of SAS. All other parameters were compared by FREQ and LOGISTIC procedure of SAS.

RESULTS

During the study period none of the cows exhibited any overt clinical signs of diseases**.** The most frequently isolated bacteria were coagulase negative staphylococci (CNS) and α-hemolytic streptococci (AHS) in all groups of heifers and cows. Although CNS and AHS were isolated in N-CMD and A-CMD groups, *Arcanobacterium pyogenes, Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Serratia marcescens* and *Aspergillus* spp. were only isolated from A-CMD group (Table 1). Moreover, *Brucella* spp. and *Campylobacter* spp. were not detected in any CMD samples. The microorganism profiles of N-CMD and A-CMD groups were different and presence of UP and OUP in A-CMD was not significantly different for either group (Table 2). The presence of total microorganism only associated with A-CMD cow group ($p<0.01$). However, the presence of PUP was associated with A-CMD for both cows ($p<0.01$) and heifers ($p<0.02$) (Table 2).

Overall, appearance of bacteria did not affect the FS-CR for cows or heifers (Table 3). On the other hand, FS-CR were lower for cows positive for PUP (Table 3; 4.7 vs. 21.6%; $p<0.01$) and heifers positive for PUP (0 vs. 27.1%; $p<0.01$) and OUP (43.2 vs. 78.8%; $p<0.01$; Table 3). Although A-CMD significantly decreased FS-CR in cows ($p<0.04$), it did not affect FS-CR in heifers (Table 2). Average OD in N-CMD cow group was significantly lower

Table 1. The distribution of microorganisms isolated from cows and heifers with N-CMD and A-CMD

Microorganisms*	Cow				Heifer			
	A-CMD		N-CMD		A-CMD		N-CMD	
	n	%	n	%	n	%	n	%
A. pyogenes (1)	3	3.57	-	-	-	-	-	-
E. coli (1)	7	8.33	2	2.94	-	-	-	-
E. coli (1)+BHS (2)	4	4.76	-	-	-	-	-	-
E. coli (1)+AHS (3)	4	4.76	7	10.29	-	-	-	-
E. coli (1)+CNS (3)	-	-	5	7.35	-	-	2	5.26
BHS (2)	9	10.71	-	-	-	-	2	5.26
S. aureus (2)	3	3.57	-	-	4	12.50	-	-
M. haemolytica (2)+AHS (3)	4	4.76	2	2.94	4	12.50	-	-
CNS (3)	15	17.85	18	26.47	8	25.00	12	31.57
CNS (3)+*S. marcescens* (3)	2	2.38	-	-	-	-	-	-
CNS (3)+AHS (3)	3	3.57	2	2.94	-	-	2	5.26
CNS (3)+AHS (3)+*Aspergillus spp.* (3)	4	4.76	-	-	-	-	-	-
CNS (3)+AHS (3)+*Acinetobacter spp.* (3)	2	2.38	2	2.94	-	-	-	-
Candida spp. (3)	-	-	2	2.94	-	-	-	-
K. pneumoniae (3)	6	7.14	-	-	-	-	-	-
Morganella morganii (3)	-	-	2	2.94	-	-	-	
AHS (3)	8	9.52	2	2.94	4	12.50	6	15.78
AHS (3)+*S. marcescens* (3)	-	-	-	-	4	12.50	-	-
AHS (3)+ *P. aeruginosa* (3)	4	4.76	-	-	-	-	-	-
No bacteria and fungi	6	7.14	24	35.29	8	25.00	14	36.84
Total	84	100	68	100	32	100	38	100

* UP = Uterine pathogen, PUP = Potential uterine pathogen, OUP = Opportunistic uterine pathogen; categories were shown as 1, 2 and 3, respectively.
CNS = Coagulase negative staphylococci, AHS = α-hemolytic streptococci, BHS = β-hemolytic streptococci.

Table 2. Rates for Holstein cows and heifers with or without abnormal CMD[1]

Parameters[2]	Number of cows[3]			Number of heifers[3]		
	N-CMD (n = 68)	A-CMD (n = 84)	p<	N-CMD (n = 38)	A-CMD (n = 32)	p<
MO (n[4](%)[5])	44 (64.7)	78 (92.9)	0.01	24 (63.2)	24 (75.0)	NS
UP (n(%))	14 (20.6)	18 (21.4)	NS*	2 (5.3)	0 (0.0)	NS
PUP (n(%))	2 (2.9)	20 (23.8)	0.01	2 (5.3)	8 (25.0)	0.02
OUP (n(%))	42 (61.8)	52 (61.9)	NS	22 (57.9)	20 (62.5)	NS
FS-CR (n(%))	35 (51.5)	29 (34.5)	0.04	22 (57.9)	15 (46.9)	NS

[1] CMD = Cervical mucus discharge.
[2] MO = Total microorganism; UP = Uterine pathogens; PUP = Potential uterine pathogens; OUP = Opportunistic uterine pathogens; FS-CR = First service conception rate.
[3] Number of cows or heifers with the associated parameters. [4] n = Number of cases. [5] Percentage of cows or heifers showing cases in a group.
* NS = Not significant.

Table 3. Effects of presence of microorganisms on first service conception rates (FS-CR) of Holstein cows and heifers

Parameters[1]	Number of cows			Number of heifers		
	Pregnant (n = 64)	Open (n = 88)	p<	Pregnant (n = 37)	Open (n = 33)	p<
MO (n[2](%)[3])	48 (75.0)	74 (84.1)	NS*	22 (59.5)	26 (78.8)	NS
UP (n(%))	17 (26.6)	15 (17.1)	NS	0 (0.0)	2 (5.4)	NS
PUP (n(%))	3 (4.7)	19 (21.6)	0.01	0 (0.0)	10 (27.1)	0.01
OUP (n(%))	37 (57.8)	57 (64.8)	NS	16 (43.2)	24 (78.8)	0.01

[1] MO = Total microorganism; UP = Uterine pathogens; PUP = Potential uterine pathogens; OUP = Opportunistic uterine pathogens.
[2] n = Number of pregnant or open cows or heifers. [3] Percentage of cows or heifers in a group. NS = Not significant.

compared to A-CMD cow group (115.2±8.4 vs. 154.3±14.1 d; p<0.02; Table 4). Even though presence of microorganisms in A-CMD increased the OD following calving (p<0.01), presence of microorganisms in N-CMD did not increase the OD in cows (Table 4). In heifers, differences in FSA were significant between N-CMD (501.5±15.6 d) and A-CMD groups (576.5±18.9 d; p<0.01; Table 4). Moreover, presence of microorganisms in A-CMD

Table 4. Least squares means and SE for insemination intervals of Holstein cows and heifers

	Parameter	n =	Time (day)	p<
Average open day (OD) for cows	N-CMD	68	115.2±8.4	0.02
	A-CMD	84	154.3±14.1	
	MO-	30	125.5±15.2	0.26
	MO+	122	143.9±6.3	
	N-CMD&MO-	24	107.4±13.6	NS
	A-CMD&MO-	6	122.9±10.0	NS
	N-CMD&MO+	44	143.7±27.2	NS
	A-CMD&MO+	78	165.0±7.5	*
First service age (FSA) for heifers	N-CMD	38	501.5±15.6	0.01
	A-CMD	32	576.5±18.9	
	MO-	22	503.5±20.5	0.01
	MO+	48	574.5±13.4	
	N-CMD&MO-	14	483.0±24.7	NS
	A-CMD&MO-	8	519.9±18.9	NS
	N-CMD&MO+	24	520.0±32.7	NS
	A-CMD&MO+	24	633.0±19.0	**

N-CMD = Normal cervical mucus discharge; A-CMD = Abnormal cervical mucus discharge; MO+ = Positive for microorganism; MO- = Negative for microorganism; N-CMD&MO- = Normal cervical mucus discharge with no microorganism; A-CMD&MO- = Abnormal cervical mucus discharge with no microorganism; N-CMD&MO+ = Normal cervical mucus discharge with microorganism; A-CMD&MO+ = Abnormal cervical mucus discharge with microorganism.
NS = Not significant.
* A-CMD&MO+ group is significantly different (p<0.01) from A-CMD&MO- and N-CMD&MO- group.
** A-CMD&MO+ group is significantly different (p<0.01) from A-CMD&MO-, N-CMD&MO- and N-CMD&MO+ group; OD = Open days; FSA = First service age.

increased FSA intervals in heifers ($p<0.01$). However, presence of microorganisms in N-CMD did not affect FSA intervals (Table 4).

DISCUSSION

Bacterial species isolated in the present study agreed with previous reports (Griffin et al., 1974; Dohmen et al., 1995; Sheldon et al., 2002). Parallel to the findings of Panangala et al. (1978) our findings indicated that the most frequently isolated bacteria were CNS and AHS in all groups. This may prove that CNS and AHS present in normal flora of animals but they may also play a role in genital tract infections. However, similar rates of bacterial and fungal agents were not isolated in cows and heifers with N-CMD. In the current study, *A. pyogenes*, *S. aureus*, *P. aeruginosa*, *K. pneumonia*, *S. marcescens* and *Aspergillus* spp. were isolated only in the samples collected from A-CMD group. Although the agents considered as pathogen (Sheldon et al., 2002) were present in N-CMD cow group, there were only 2 incidences in N-CMD heifer group. Thus, the presence of higher incidences of uterine pathogens in N-CMD cow group could be explained by the fact that cows may frequently expose to pathogens during delivery and AI.

Griffin et al. (1974) reviewed a series of studies in which uterine swabs were collected prospectively from cows. They concluded that metritis caused by non-specific bacteria had no effect on RP. On the contrary, our findings indicated that A-CMD had a negative affect on FS-CR in cows, but not in heifers. Similarly, Mahmoudzadeh et al. (2001) reported that difference between the CR after AI of heifers with normal and abnormal CMD was not significant and A-CMD during estrus was associated with low fertility in cows but not heifers. Moreover, the rate of A-CMD with microbes was 5.1%, and FS-CR was lower for cows with A-CMD (22.6%) than normal groups (38.3%). Our findings suggest that, microorganism profile, rather than presence of A-CMD, appeared to be more important for affecting CR in heifers. On the other hand, both A-CMD and type of microorganisms isolated are important for FS-CR in cows. Therefore, bacteria and fungi may be changed the characteristics of CMD and it may lead to infertility by prolonging the necessary time for conception in cows.

The results of the current study indicated that appearance of A-CMD was associated with PUP for both cows and heifers. There was a negative affect of A-CMD on FS-CR for cows. Moreover, FS-CR was lower for cows with PUP and heifers with PUP and OUP. In addition to negative effect of pathogens on folliculogenesis, corpus luteum and $PGF_2\alpha$ secretion, they also change the characteristics of mucous and cause negative effect on ovum, spermatozoa and embryo (Peter and Bosu, 1988b). It is also thought that when bacteria and fungi are present in CMD, they can change the normal appearance of CMD that can lengthen OD. Uterine bacterial infection or bacterial products suppress pituitary LH secretion, and perturb postpartum ovarian follicle growth and function, which disrupts ovulation in cattle (Peter et al., 1989; Sheldon et al., 2002).

One of the unique features of the present study was that the swabs were taken when animals were brought for AI and animals were not inseminated until they showed spontaneous estrus signs, whereas most previous studies isolated the bacteria during the first month following calving and used estrus synchronization. Therefore, the OD for cows in N-CMD and A-CMD groups was longer in our study. Gilbert et al. (2005) also reported a significant lower overall conception rate (CR) between cows with or without clinical endometritis. Similar to our results, median days open was long and was 206 versus 118 for cows with or without subclinical endometritis (Gilbert et al., 2005). There are probably two reasons for such long time: i) too long voluntary waiting period, and ii) missed heats prior to the first service. Furthermore, presence of microorganisms in A-CMD could increase the OD following calving. On the other hand, both presence of microorganisms and A-CMD affected FSA in heifers. In addition, the increase of FSA in heifers was apparent only when A-CMD was associated with presence of microorganism. The increased OD or FSA in A-CMD may be due to pathogens and toxins stimulate the uterus to secrete abnormally higher levels of prostaglandins which delay the onset of cyclicity until the infection is cleared and the prostaglandin levels are low (Peter and Bosu, 1987a ; Bonnett et al., 1993; Sheldon et al., 2002;). Another possibility is that uterine infection may delay the initiation of folliculogenesis and suppress the rate of follicular growth in dairy cows during the early puerperium (Peter and Bosu, 1988b) by inhibiting LH release. Uterine infection and reproductive performance is also influenced by the presence of a suitable uterine environment, genetic factors, and the animal's innate and acquired immunity.

Findings of the current study suggested that cervical mucus discharge should be evaluated more carefully when there are infertility problems. In addition to the known microorganism that causes sterility and infertility in UP group, pathogens in PUP group should be considered as potential to cause infertility. Thus, future researches need to be conducted about PUP microorganisms, their effects on the reproductive performance and their clinical treatments in larger herds.

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