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Identification of bacterial and fungal agents of clinical endometritis in dairy heifers and treatment by metronidazole or cephapirin

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ABSTRACT

This study was aimed to identify fungal and bacterial causes of clinical endometritis as the first study in one hundred thirty dairy heifers by culture and PCR methods, and also to evaluate intrauterine cephapirin and/or metronidazole treatments. Although there were no growth of bacteria and fungi agents in all samples but PCR results showed the presence of *E.coli* and *Trueperella pyogenes* in six heifers. Conception rate of the treated heifers with metronidazole and cephapirin was significantly higher than that of untreated heifers. Heifers in metronidazole-treated group had higher conception rate compared to cephapirin group but the difference was not significant. A greater conception rate was observed in heifers that had clear discharge at time of insemination, compared to heifers with mucopurulent or opaque discharges. The results of this study showed that the pregnancy rate of treated heifers was significantly higher than that of untreated heifers. It could be concluded that treatment of affected heifers and the clear discharges at the time of AI could improve conception rate.

Keywords: Heifer, Endometritis, PCR, *E. coli, T. pyogenes,* Metronidazole

Bacterial contamination is extremely common in the uterus in the early postpartum period (Elliott *et al.,* 1968; Miller *et al.,* 1980).80 to 100% of cows are found to have bacterial contamination of the uterus in the first 2 weeks postpartum(Földi et al. 2006). Many of these bacteria are eliminated during the first 5 weeks after parturition, but the remaining bacteria cause uterine disease in some cows(Noakes DE 2009, Sheldon et al. 2006). Inflammation limited to the endometrium is termed endometritis (Sheldon et al. 2006). Many complicationshave beenfound in pathogenic factors of endometritis. It is argued that pathogenic bacteria have an important role in occurrence of endometritis (Sun et al. 2011). *Fusobacterium necrophorum,Trueperella pyogenes, Prevotella melaninogenicus* and*Escherichia coli*are recognized as uterine pathogens associated with uterine endometrial lesions (Sheldon and Dobson 2004b, Williams et al. 2005b); moreover,*today, there appears to be mounting concern* over mycotic endometritis in cows (Ramsingh, Murali, & Sadasiva Rao, 2013). The increase in prevalence of mycotic endometritis has been attributed to several reasons, including regular and indiscriminate use of intrauterine(IU) antibiotics, postpartum uterine contamination, and concessions inhygiene during AI procedures (Sharma and Singh 2012). Mould and yeast have been isolated from endometritic cows (Vivek, 1989; Dascanio *et al*. 2000).Yet, there is no evidence supporting that bacterial and fungal agent in dairy heifers causes clinical endometritis. Although intrauterineinfusion with antibiotics has been found to be effective for the treatment of clinical endometritis (Drillich *et al*. 2005), there is no report on treatment of endometritis by intrauterine infusion of metronidazole. Metronidazole has activity against most obligate anaerobic bacteria including *Bacteroides* spp. (including *B. fragilis*), *Fusobacterium*, *Veillonella*, *Clostridium* spp., *Peptococcus*, and *Peptostreptococcus*. *Actinomyces*is frequently resistant to metronidazole (Plumb 2005). Metronidazole topical gel, cream, and lotion are used for treating and metronidazole vaginal gel is used for the treatment of bacterial vaginitis (Plumb 2005).Therefore, this study aimed to identify fungal and bacterial causes of clinical endometritis in dairy heifers by culture andpolymerase chain reaction (PCR)in order to detect inflammation in cervical discharge and to evaluate intrauterine metronidazole treatments.

Materials and Methods

Animals

The study was carried out in a large commercial dairy farm around Shiraz, Fars province, in the south of Iran (29° 58′ 34″ N, 52° 40′ 45″ E). One hundred and thirty repeat-breeder dairy heifers (weight >320kg and height >120cm) were

examined. The heifers were housed in open sheet barns and received corn silage, alfalfa hay and concentrates (containing corn meal, soybean meal, vitamins and minerals). All heifers were artificially inseminated by sex-sorted frozen semen after 13 months. Fifteen heifers with clinical signs of endometritis in last estrus periodwere selected for sampling, of which 87 heiferswere assigned to the experimental group and 43 heifers with opaque discharges (≤E1) to the control group. The experimental group received two different treatment protocols and the control heifers were inseminated without any treatment.

Clinical examination

All heifers were examined in luteal phase. During examination, the vulva of heifers was thoroughly cleaned with a dry paper towel, a clean Metricheck was inserted through the vulva, and the mucus contents of the cranial vagina were withdrawn for examination. The vaginal mucus was assessed in terms of its proportion of pus. Endometritis was classified into three categories: clear mucus with flakes of pus (E1), mucopurulent discharge or fluctuating contents in the uterus (E2) and purulent discharge with or without palpable contents in the uterus (E3) (Williams *et al.,* 2005). Then, the heifers were classified into two groups: healthy (vaginal dischargesscore ≤ E1) and endometritis affected (vaginal discharges score≥E2) (Dubuc *et al*. 2010).

Uterine samples collection

Uterine secretions of 15 heifers were collected; heifers were restrained and the perineum area was cleaned and disinfected using Savlon (chlorhexidine and cetrimide) solution. Sterile covered plastic infusion pipettes (pipettes were first autoclaved and then put on inside plastic sheaths, UV light was used to sterilize covers) were inserted into the cranial vagina and passed through the caudal cervix. The sheath was subsequently ruptured and the sterile pipette tip was manipulated through the cranial cervix into the uterus. A total of 40 ml of sterile saline was injected into the uterus and a sample of the fluid was aspirated. The volume of recovered fluid wasabout 10 ml. Prior to laboratory processing, samples were stored on ice. (Santos *et al.* 2010; Aghamiri *et al.* 2014).

DNA extraction from uterine fluid

The samples were centrifuged for 10 min at 5,000 *g*. The supernatant was discarded and the residue was transferred into 2 mL microtube. DNA extraction was performed from 200 µL of the suspension by AccuPrep® Genomic DNA Extraction Kit (Bioneer, South Korea), according to the manufacturer's instructions.

Identifying the presence of E. coli, T. pyogenes, F. necrophorum and P. melaninogenicus by PCR

Amplification reactions were carried out in a 20 µl reaction mixture containing 10 pmol of each primer (Table 1), 0.25 mM of each deoxynucleotide triphosphate, 0.75mM of $MgCl_2$, 1× PCR buffer, 1 U of Taq polymerase (Pars Toos Co, Iran), and 4 µl of sample DNA as template. PCR conditions used for each pathogen are listed in Table 1.

The selected genes (16S rRNA gene for *E. coli*, *plo* gene for the pyolysin toxin of *T. pyogenes*, *lktA* gene for leukotoxin of *F. necrophorum* and *phyA* gene for hemolysin of *P. melaninogenicus*) were specific and among the most popular genes used for molecular identification and confirmation of the isolated bacteria.

Table 1. Primer pairs used to amplify each target gene.

Species	Primer sequence	Annealing (°C)	Program*	Product (bp)	Reference
E. coli	gttaataccttgctcattga accagggtatctaatcctgtt	55	1	340	(Bicalho et al. 2010b)
T. pyogenes	ggcccgaatgtcaccgc aactccgcctctagcgc	55	2	270	(Silva et al. 2009)
F. necrophorum	aatcggagtagtaggttctg tttggtaactgccactgc	59	3	402	(Bennett et al. 2009)
P. melaninogenicus	cgtcatgaaggagattgg atagaaccgtcaacgctc	54	4	389	(Yoshida et al. 2005)

*PCR Program 1: ×1 (94°C, 600 sec), ×30 (94°C, 60 sec, 55°C, 60 sec, 72°C, 60 sec), ×1 (72°C, 600 sec). 2: ×1 (95°C, 600 sec), ×30 (95°C, 60 sec, 56°C, 60 sec, 72°C, 60 sec), ×1 (72°C, 300 sec). 3: ×1 (94°C, 300 sec), ×30 (94°C, 30 sec, 59°C, 30 sec, 72°C, 30 sec), ×1 (72°C, 300 sec). 4: ×1 (95°C, 300 sec), ×25 (95°C, 15 sec, 54°C, 30 sec, 72°C, 60 sec), ×1 (72°C, 300 sec).

E. coli ATCC 35218, *P. melaninogenicus* ATCC 25845, native *T. pyogenes* and *F. necrophorum* were used as positive controls. Amplification products were separated by electrophoresis through a 1% (w/v) agarose gel, stained with 0.5 µg/mL ethidium bromide, and visualized under ultraviolet trans-illuminator.

Bacterial and Fungal culture

Following the standard isolation method, the uterine discharge of 15 heifers was aspirated and transferred on ice to the laboratory for bacteriological and mycological investigation. .

The samples were cultured on sheep blood agar and MacConkey agar (MERCK), and incubated at 37°C for 48 h. The same culture on sheep blood agar (MERCK) was incubated anaerobically for up to 7 days. Standard biochemical tests were used for the isolation and identification of the isolates (Quinn *et al.* 2013).

For fungal isolation, Sabouraud's Dextrose agar (SDA) spot inoculation technique was employed. The plates were incubated at 28°C for 3 weeks and checked every 24 hours for mycological growth. Chloramphenicol was used in the agar media for initial fungal isolation. Duplicate culture was used for each sample.

Cervical cytology

Cervical samples were collected from cervical external os mucus by a plastic uterine pipette and aspirated by suction with a 50 ml syringe(Ahmadi et al. 2006) {Ahmadi, 2006a #139;Ahmadi, 2006 #238}.

Thin smears were prepared for cytological examination by smearing a drop of cervical mucus on a clean slide. The smears were then allowed to dry at room temperature for 30–35 minutes. Slides were examined within two hours of collection. A differential cell count of each smear was done on Giemsa-stained slides. Hundred cells were counted for each of 20 microscopic fields (□900) (Jain 1986).

Treatment protocols

The heifers with clinical endometritis ($\geq E_2$) were treated by two treatment protocols during estrous phase.

Protocol 1: Thirty one heifers with endometritis signs on the day of estrus were treated by 2 grams of third-generation cephalosporin solution(Ceftriaxone, Dana Pharmcy Co., Iran).20 ml normal saline syringe was introduced into the lumen of the uterus by using a disposable catheter.Estrus was detected by monitoring of visible estrous signs, according to farm procedure. All heifers in this estrous period with clear mucus received AI.

Protocol 2: Fifty six heifers with endometritis signs were treated by intrauterine metronidazole jell (Metrovage %0.75, Parsdarou Co, Iran)on the day of estrus.

One tube of metronidazole jell was supplied in 50ml disposable syringes with disposable transcervical catheters.Estrus was induced by $PGF_{2\alpha}$ injection on day 10 after the treatment in all cows in protocol one and two.

Protocol 3: Forty three heifers with opaque discharges (≤E1), like the control group, did not receive treatment, and were, therefore, inseminated artificially during the first estrous period.

Finally, after examination of the estrous discharges, the studied animals were inseminated by AI. The estrous discharges were classified in three categories: clear, opaque and mucopurulent discharges. Pregnancy diagnosis was performed by ultrasonography at 30-35days after insemination.

Statistical analysis

Conception rate of heifers with different types of vaginal discharge at the time of insemination was statistically analyzed with the Chi-square test using SPSS (SPSS for Windows, version 11.5, SPSS Inc, Chicago, Illinois). The conception rates in different groups of treated heifers were compared by Chi-square test.

Possible effects of risk factors on the conception rate of heifers were explored using logistic regression analysis. The data from all treatment groups of heifers were compared by logistic regression analysis using pregnant status as the dependent variable (0 denotes not-pregnant and 1denotes pregnant). Also, type of treatment, time of observed vaginal discharges, different types of vaginal discharge at the time of insemination, and number of artificial insemination were considered as independent variables. Probability values of $P \leq 0.05$ were considered statistically significant.

Results and Discussions

Micrococcus spp. was found in 5heifers affected by endomeritis. Other bacteria were *Staphylococcus lentus*, *Corynebacterium kutscheri*, *Enterobacter aerogenes* and *Moraxella* spp(Table 2). No bacteria were found in 7 heifers. PCR results showed the presence of *E.coli* and *Trueperella pyogenes* in 6 heifers concurrently; only *E. coli*in 2 heifers; and in one case only *Trueperella pyogenes was observed*. No bacteria were detected in 2 heifers by PCR (Table 2) (Figure 1). There was no growth of fungal agents in all samples taken from heifers.

Table 2. The number (%) of bacterial isolates from heifers affected by endometritis by culture and PCR.

Bacteria	Method	Number (%)
Micrococcus spp	Culture	5 (20.83%)
Staphylococcus lentus	Culture	1 (4.16%)
Corynebacterium kutscheri	Culture	1 (4.16%)
Enterobacter aerogenes	Culture	1 (4.16%)
Moraxella spp	Culture	1 (4.16%)
E.coli	PCR	8 (33.33%)
Trueperella pyogenes	PCR	7 (29.17%)
Fusobacterium necrophorum	PCR	0 (0%)
Prevotella melaninogenicus	PCR	0 (0%)

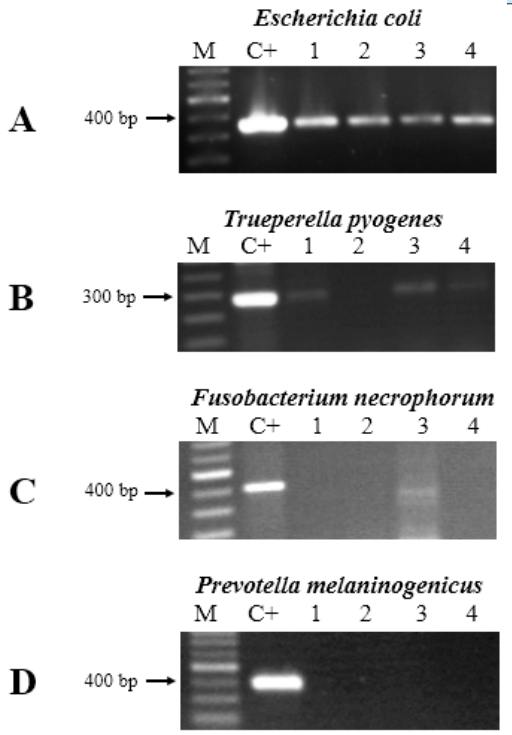

Figure 1. Single PCR amplification products of genomic DNA of uterine discharges. First panel: *E. coli* **(340 bp); Second panel:** *T. pyogenes* **(270 bp); Third panel:** *F. necrophorum* **(402 bp); Fourth panel:** *P. melaninogenicus* **(389 bp); M: molecular weight marker, C+: positive control, Lane1-4: samples from uterine discharges. The DNA fragments were visualized on 1% agarose gels stained with ethidium bromide.**

In cytological examination of cervical mucus, the mean (± SD) percentages of different cells were 41.93 ± 34.35, 13.93 ± 22.12, 43.93 ± 29.69 and 0.57 ± 1.50 for epithelial, large vacuolated epithelial, neutrophiles, and macrophages cell, respectively.

Conception rate of the treated heifers with cephapirin and metronidazole was significantly greater than that of untreated heifers (as the control group) ($P < 0.01$, see Table 3). As appeared in Table 3, heifers in metronidazole treated group had higher conception rate compared to cephapirin group; however, this difference was not significant ($P = 0.1$). There was a greater conception rate of heifers that had clear discharge at time of insemination, compared to heifers with mucopurulent or opaque discharges ($P <0.01$, see Table 4).

Table 3. Comparison of conception rate (pregnant /total animals) between different groups of treated heifers

	Treatment Groups % (N)		
	Control	**Cephapirin**	**Metronidazole**
Pregnantheifers	37.2 (16)[a]	77.4 (24)[b]	89.3 (50)[b]
Non pregnantheifers	62.8 (27)	22.6 (7)	10.7 (6)
Total	100 (43)	100 (31)	100 (56)

[a,b] Different superscript letters indicate significant difference in the same rows ($P<0.01$)

Table 4. Comparison of conception rate (pregnant cows/total cows) between heifers with different types of vaginal discharge at the time of insemination

Heifers	Vaginal discharge % (N)		
	Clear	**Opaque**	**Mucopurulent**
Pregnant heifers	84.3 (70)[a]	44.7 (17)[b]	33.3 (3)[b]
Non pregnant heifers	15.7 (13)	55.3 (21)	66.7 (6)
Total	100 (83)	100 (38)	100 (9)

[a,b] Different superscript letters indicate significant difference in the same rows ($P<0.01$)

Table 5. Odds ratios of the variables included in the final logistic regression model for conception rate of heifers

Variables	Class	Odds ration	95% CI	P value
Treatment	Control	Reference		
	Cephapirin	7.58	2.31 - 24.84	0.001
	Metronidazole	15.78	4.99 - 49.85	< 0.001
Time [a]	At treatment	Reference		
	At AI	3.23	1.03 – 10.09	0.044
Number of AI	Continuous	0.53	0.39 – 0.75	< 0.001

Likelihood ratio test = 48.37, 4 df, P < 0.0001; Hosmer and Lemeshow goodness-of-fit test = 8.54, 7 df, P = 0.30; the model fits

[a] Time of observed vaginal discharges

The likelihood of conception increased in heifers treated with cephapirin and metronidazole by 7.58 and 15.78 respectively. Yet, this increase was not observed in untreated heifers (control group, see Table 5). The likelihood of conception decreased in the studied heifers by 0.53 for each 1 time increase in number of artificial insemination (Table 5). No significant relationship was observed between time of observed vaginal discharges and type of treatment or number of artificial insemination using the logistic regression analysis.

Discussion

The objective of this study was to evaluate the relationships between clinical endometritis in heifers and uterine bacteriology, mycology and cervical cytology findings. In this paper, several nonpathogenic bacterial agents were observed, but no pathogen agent was found in routine bacterial culture. *E.coli* and *Trueperella pyogenes* were found as pathogen agents by PCR. It could be concluded that PCR is more sensitive than culture in the detection of pathogenic bacteria in similar cases.

Infections with *E. coli* and *Trueperellapyogenes* are associated with both metritis and postpartum vaginal discharge of cows. In the early postpartum period, infections of the uterus with *E. coli* pave the way for subsequent infection with other bacteria (Westermann *et al*. 2010). The most prevalent bacteria in the late postpartum period are *T. pyogenes* (Yavari *et al*., 2007; Bicalho *et al*. 2010a). In the current research, when *T. pyogenes* was isolated from uterine fluids approximately 21days postpartum, cows developed severe endometritis and were usually infertile

at first service (Bicalho *et al*. 2012). *T. pyogenes* were strongly associated with clinical endometritis when detected at the 34 to 36 days postpartum (Bicalho *et al*. 2012). Bicalho *et al*. (2012) found that *E. coli* at 34 to 36 days postpartum was not associated with clinical endometritis or reproductive failure (Bicalho *et al*. 2010a). Aghamiri *et al*. (2014) found three bacteria, *E. coli*, *T. pyogenes* and *F. necrophorum,* as uterine infection pathogens by PCR in same farm(Aghamiri *et al.* 2014). The studies reported above, however, did not investigate endometritis in heifer.

There are many reports of clinical endometritis with no bacterial growth (Ahmadi *et al*., 2007; Westermann *et al*., 2010). The detection of bacteria by culture presents some deficiencies: such as its costly and time consuming procedure and anaerobic fastidious bacteria (Aghamiri *et al*., 2014). Some authors have proposed detection of bacteria with other methods (Westermann *et al*., 2010). Few studies have detected bacteria of uterine discharges by applying PCR methods (Santos *et al*., 2011; Bicalho *et al*., 2012; Aghamiri *et al*., 2014).

The recognized uterine pathogens, most often associated with the clinical disease, are *T. pyogenes*, *F. necrophorum,P. melaninogenicus*and *E. coli* (Azawi 2008, Sheldon and Dobson 2004a). Several studies indicate that *T. pyogenes*is one of the most common isolates (Ahmadi *et al.* 2007, Bonnett *et al.* 1991, Mateus *et al.* 2002, Westermann *et al.* 2010, Williams *et al.* 2005a)and is usuallyassociated with anaerobic bacteriasuch as *F. necrophorum* and *P. melaninogenicus* (Sheldon *et al.* 2006, Williams *et al.* 2005a). *T. pyogenes*, *F. necrophorum* and *P. melaninogenicus* act synergistically to enhance the likelihood and severity of uterine disease (Bonnett *et al*. 1991, Sheldon and Dobson 2004a).

The cervical cytology of these heifers showed a high rate of infection in uterus. The cytological evaluation of cervical smear at 4 phase of estrus cycle in dairy heifers reported byAhmadi *et al*. (2006). Hormonal changes in different phases of estrous cycle affect neutrophil presence in cervical mucosa. The neutrophils count in heifer during diestrous period was reported as 0.60±0.30%. The results of fungal culture showed that the heifers were not affected by any fungal agents. We argue that the heifers had no postpartum disasters or disorders and it is a side effect of intrauterine antibiotic treatments.

The results of the treatment in this study were similar to those of Ahmadi and Dehghan (2007). They reported that uterine lavage plus PGF2α with cephapirin caused 70% pregnancy in first AI in repeat breeder dairy cows (Ahmadi and Dehghan 2007). Also, LeBlanc *et al.* (2002a) found that endometritic cows treated with cephapirin i.u. had a shorter time to pregnancy, than untreated cows

.Yet, such improved fertility after use of cephapirin in dairy cows have not been reported by other studies too. The use of ceftiofur hydrochloride intrauterine reduces the prevalence of uterine infection in cows with clinical endometritis, and the prevalence of *T. pyogenes*, but could not improve the fertility of dairy cows (Galvão *et al.* 2009). Treatment of cows affected by subclinical endometritis with one infusion of cephapirin or cloprostenol at 20–33 DIM significantly improves their reproductive performance (Kasimanickam *et al*. 2005). Post-TAI intrauterine cephapirin administration could not improve conception rate in repeat breeder dairy cows (GÜMEN *et al*. 2012).

Metronidazole has been used in human medicine for about 30 years. The clinical use covers thetreatment of anaerobic bacterial infections, amoebiasis, trichomoniasis, giardiasis and Crohn's disease. Metronidazole belongs to the group of 5-nitroimidazoles. It is used in veterinary and human medicine for the treatment of infections with protozoa (*Trichomonas, Treponema, Histomonas*) andwith obligate anaerobic bacteria (*Bacteroides, Fusobacterium, Campylobacter*) (Brogden *et al*. 1978).

The use of metronidazole in large animal reproductive treatment is notcommon. Metronidazole has been used as treatment of vaginitis (Elad *et al*. 2004) and pyometra (Stephens and Slee 1987) in cows. This study reports the first time in which Metronidazole is used in treatment of endometritis in heifers.

The results of this study showed that the pregnancy rate of treated heifers was significantly higher, compared to that of untreated heifers. The type of vaginal discharges at the time of insemination could be responsible for impairing the pregnancy rate, as the present results associated higher pregnancy rate with clear discharge in heifers. Research suggests that first degree of endometritis (the presence of mucus with flecks of pus) has no effect on reproductive records, such as pregnancy rate (LeBlanc *et al*. 2002b). So, the purulent or mucopurulent uterine discharges have been defined as clinical endometritis in many studies ((LeBlanc *et al*. 2002b; Dubuc *et al*. 2010). In this study, mildly purulent uterine discharges were associated with reduced pregnancy rate. The presence of mildly purulent uterine discharge (i.e., mucus with flecks of pus) until 33 DIM was not associated with reduced pregnancy rate.

In conclusion, we believe that post AI endometritis in heifer is probable. So, it is necessary to teach appropriate hygienic AI method to insemination technicians in order to improve dairy herd fertility. This paper represents the first report of endometritis in dairy heifers. The endometritis in heifers may occur in post AI, so it is essential to control hygiene during AI in dairy heifers and use double sheath

cover on AI gun. Treatment of the affected heifers and the clear discharges at the time of AI could improve conception rate.

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