



# The Association of Intrauterine Antimicrobial Resistant *Trueperella pyogenes* with Cytological Endometrial Inflammation Status in Repeat Breeder Cows

Murat Onur Yazlık<sup>1</sup>, İnci Başak Müştak<sup>2\*</sup>, Gültekin Ünal<sup>3</sup> and Ufuk Kaya<sup>4</sup>

<sup>1</sup> Department of Obstetrics and Gynecology, Faculty of Veterinary Medicine, Ankara University, Ankara, Turkey

<sup>2</sup> Department of Microbiology, Faculty of Veterinary Medicine, Ankara University, Ankara, Turkey

<sup>3</sup> Bioeksen Ar-Ge Technologies Co. Istanbul Technical University, ARI Technopark, Istanbul, Turkey

<sup>4</sup> Department of Biostatistics, Faculty of Veterinary Medicine, Hatay Mustafa Kemal University

## ABSTRACT

This study describes the presence of *Trueperella pyogenes* from routine bacteriological examination, sequence analysis for antimicrobial resistance of the identified bacteria, and the association of *T. pyogenes* with cytological endometrial inflammation status in 32 repeat breeder cow enrolled in the study. Intrauterine samples for bacteriological examination were collected with cotton swabs; samples for subclinical endometritis were collected with cytotope technique. Antimicrobial resistance to commonly used antibiotics was evaluated by disc diffusion method. Presence of the tetW gene responsible for tetracycline resistance was detected by PCR. Subclinical endometritis, breed, parity, calving history, early postpartum uterine infection, ovarian tissue findings, and body condition score were analyzed as potential factors for *T. pyogenes* presence in repeat breeder cows. A total of 15 isolates were identified as *T. pyogenes*. These were combined to analyze the results by both biochemical and sequence analysis of the 16s rDNA gene. Subclinical endometritis was detected in 37.5% cows. The *T. pyogenes* strains were highly resistant (100%) to bacitracin, tetracycline, neomycin, and oxytetracycline. All tetracycline-resistant *T. pyogenes* strains were positive for the tetW gene. The variables had no effect on the presence of *T. pyogenes* in the repeat breeder cows. In conclusion, intrauterine *T. pyogenes* presence was not associated with subclinical endometritis, although *T. pyogenes* was detected in the later stages of lactation.

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## Authors' Contribution

MOY and IBM designed methodology, the investigation, performed sampling and wrote the original draft. IBM validated sequencing data. GÜ conducted the research and performed analyses. UK performed statistical analysis and contributed to writing and reviewing of the manuscript.

## Key words

Cow, Cytological endometritis, Repeat breeder, *Trueperella pyogenes*

## INTRODUCTION

The key to economic success in dairy farming is good fertility. Postpartum uterine disorders reduce the reproduction success of high yielding cows (Huszenicza *et al.*, 1999; Williams *et al.*, 2007; Sheldon *et al.*, 2009). Uterine disorders such as metritis, and clinical and subclinical endometritis have short and long-term effects on fertility (Gilbert *et al.*, 2005; LeBlanc, 2008). Management style can also reduce fertility after parturition (Yazlik *et al.*, 2018). After a successful postpartum period, repeat breeding is the most important fertility disorder in dairy cows (Yusuf *et al.*, 2010). Repeat breeding is defined as a cow that has a normal estrus cycle free from palpable

clinical abnormalities and with no vaginal discharge that has failed to conceive after at least three or more inseminations (Heuwieser *et al.*, 1997; Gustafsson and Emanuelson, 2002). Repeat breeding affects 10%-14% of dairy cows (Gustafsson and Emanuelson, 2002; Båge *et al.*, 2002). Repeat breeding can be caused by estrus detection problems (Lopez-Gatius *et al.*, 2004), prolonged estrus, delayed luteinizing hormone peak, rising progesterone after ovulation, genetic factors (Kendall *et al.*, 2009) and infections (Pérez-Marin and España, 2007).

Endometrial cytology is a diagnostic technique used in repeat breeder cows. Cytotope is a novel diagnostic method for determining subclinical endometritis in cows (Pascottini *et al.*, 2015), measured by the proportion of polymorph nuclear neutrophils (PMNs) in endometrial cells. The PMN threshold for defining subclinical endometritis varies according to the study from  $\geq 3$  to  $\geq 18\%$  (Sheldon *et al.*, 2006). The prevalence of subclinical

\* Corresponding author: [ibkaya@ankara.edu.tr](mailto:ibkaya@ankara.edu.tr)  
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endometritis in repeat breeder cows varies from 12.7% to 52.7% in dairy cows, *i.e.* subclinical endometritis is the main reason for becoming a repeat breeder (Salasel *et al.*, 2010). The presence of bacteria in the uterus in the early postpartum period causes inflammation, histological lesions, and the delay of uterine involution (Sheldon *et al.*, 2006). Intrauterine bacterial contamination is a physiological process that occurs during the early postpartum period (Kundsen *et al.*, 2016). However, previous studies have shown that bacteria could be present for much longer period. Karstrup *et al.* (2017) reported opportunistic pathogenic bacteria in a pregnant cow uterus. There is little information about repeat breeding and intrauterine pathogens in dairy cows (Pothmann *et al.*, 2015). McDougall (2005) concluded that further research is needed regarding bacteriological findings of repeat breeding cow uteri. Repeat breeding is also associated with ovarian dysfunction or impaired function (Kendall *et al.*, 2009). Repeat breeding has also been interpreted as cyclic in cows (Heuwieler *et al.*, 1997). However, ovarian activity around insemination is also not been clearly known (Pothmann *et al.*, 2015).

In the present study, we hypothesize that uterine contamination by *T. pyogenes* in the early postpartum period may persist in later stages. Consequently, subclinical endometritis may lead to repeat breeder syndrome. This study aims to describe the presence of *T. pyogenes* in uterus, their status of antimicrobial resistance and association with cytological endometrial inflammation in repeat breeder cows. The results may be helpful in treatment or prevention of repeat breeding in dairy cows.

## MATERIALS AND METHODS

### *Animals*

The present study used the reproductive organs of 32 cows culled from various herds and slaughtered in one abattoir in Ankara, Turkey.

Age, breed, parity, calving assistance, and postpartum uterine disorders (*i.e.* metritis, endometritis, or pyometra), interval from calving to first artificial insemination (AI), and number of inseminations were recorded. The body condition score (BCS) before slaughtering (Ferguson *et al.*, 1994). Metritis was marked by as inflammation of the uterus within the first 10 days postpartum with the presence of vaginal discharge and systemic signs of illness, while endometritis was marked by inflammation of the endometrium later in lactation with vaginal discharge, without any systemic signs. Pyometra was identified by accumulation of purulent material within the uterine lumen in the presence of a persistent corpus luteum and a closed cervix without systemic signs (Sheldon *et al.*, 2006).

Cows with at least one corpus luteum were considered cyclic, whereas, those with no active or regressed corpus luteum and with incidental small follicles were considered anovulatory (Polat *et al.*, 2015).

Samples were divided into two groups according to intrauterine bacteria. Cows showing the presence of *T. pyogenes* from the uterine samples were included in the infected group, while cows with no bacterial presence in the uterine samples were labelled as control group.

### *Uterine cytology and bacteriologic culture*

Endometrial samples were collected with cytotape technique for cytological examination (Pascottini *et al.*, 2015). Cytological slides were prepared by rolling the tape onto a clean glass microscope slide, drying, fixing and staining by laboratory procedure. The percentage of PMN was evaluated by counting 300 cells under a microscope at 400x magnification. A proportion of 3% or greater PMN was defined as subclinical endometritis (Salasel *et al.*, 2010). For the bacteriological sampling the uterine surface was washed with sterile saline solution and sterilized with iodine solution. The serosa of uteri was incised with a scalpel, and aseptic and sterile swabs passed through the incision.

### *Bacterial isolation and identification*

A total of 32 uterus swabs obtained from the cows were investigated. After bacterial cultivation on brain heart infusion agar (Oxoid), the isolates were grown on agar supplemented with 5% sheep blood and MacConkey agar (Oxoid) at 37°C for 48h under aerobic and anaerobic conditions. This was followed by Gram staining and biochemical tests for catalase, oxidase, urease, nitrate reduction, fermentation of glucose, lactose, maltose, mannitol, sucrose, gelatin, and esculin hydrolysis.

For molecular identification of *T. pyogenes* isolates and sequence analysis of the 16S rDNA gene. DNA was extracted with genomic DNA isolation kit (QIAamp DNA Mini Kit; Qiagen). The universal primers 27-F AGAGTTTGATCMTGGCTCAG and 1492-R GGTTACCTTGTTACGACTT were used in the sequence analysis (Lane, 1991). A total volume of 25 µl PCR mix, consisting of 3 mM MgCl<sub>2</sub> (Thermo Fischer Scientific, USA), 0.2 mM dNTPs (10 mM dNTP mix; Thermo Fischer Scientific, USA), 0.2 µM of each primer, 2.5 µl PCR reaction buffer, 2U Taq DNA polymerase (Thermo Fischer Scientific, USA), and 2 µl template DNA was used. The PCR amplification conditions were as follows: 94°C for 4 min., 35 cycles of 94°C for 30 sec, 58°C for 30 sec, 72°C for 1 min, and a final extension step of 72°C for 6 min. The amplified PCR products were visualized on 1.5% agarose gel (Promega Corporation, USA) with

SafeView Classic (Applied Biological Materials, Canada) in gel electrophoresis apparatus.

After amplification, the PCR products were purified using ExoSAP-IT PCR Product Cleanup reagent (Thermo Scientific, USA). A BigDye Direct Cycle Sequencing Kit (Thermo Scientific, USA) was used for the sequence analysis according to the instructions. Sephadex G-50 (Sigma-Aldrich) was used to purify the amplicons and the sequence analysis was performed in an Applied Biosystems 3500 Genetic Analyzer (Applied Biosystems). CLC Main Workbench software version 7 was used for analyzing the sequences.

#### Antimicrobial susceptibility tests

Disc diffusion was used to detect antimicrobial resistance in the strains based on the CLSI 2019 standard protocol. The following discs were used: ampicillin (AMP: 10 µg), bacitracin (B: 10 U), ceftiofur (EFT: 30 µg), cefotaxime (CTX: 30 µg), chloramphenicol (C: 30 µg), erythromycin (E: 15 µg), gentamicin (GM: 10 µg), lincomycin (MY: 15 µg), neomycin (N: 30 µg), oxytetracycline (OT: 30 µg), ofloxacin (OFX: 5 µg), penicillin G (P: 10 U), streptomycin (S: 10 µg), tetracycline (TE: 30 µg), ceftazidime (CAZ: 30 µg). *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were used as positive controls in all tests.

#### PCR amplification for tetracycline-resistance gene

All tetracycline-resistant isolates were investigated for the presence of *tetW* gene by PCR as described by Billington *et al.* (2006). The protocol was as follows: a 25 µl PCR mixture containing 3 mM MgCl<sub>2</sub> (Thermo Fischer Scientific, USA), 0.2 mM dNTPs (10 mM dNTP mix; Thermo Fischer Scientific, USA), 0.2 µM of each primer (tetW-F GACAACGAGAACGGACACTATG and tetW-R CGCAATAGCCAGCAATGAACGC), 2.5 µl PCR reaction buffer, 2U Taq DNA polymerase (Thermo Fischer Scientific, USA), and 2 µl template DNA. PCR amplifications were as follows: 95°C for 5 min, 35 cycles of 95°C for 30 sec, 55°C for 30 sec, 72°C for 30 sec, and a final extension step of 10 min at 72°C. PCR products were analyzed under a UV illuminator as described above.

#### Statistical analysis

Before performing the statistical analysis, the data were examined using the Shapiro-Wilk test for normality to meet parametric test assumptions. Student t test and Mann-Whitney u test were used to evaluate the between-group differences for BCS, number of inseminations, pregnancy to first AI interval, and pregnancy to culling interval. Fisher exact test was used to compare the group frequencies for SE, breed, and pp uterine infection. Logistic regression was used to determine which risk factors may influence the in utero presence of *T. pyogenes* in repeat breeder cows. The model included subclinical endometritis (0%, ≥3%), parity, BCS, calving (i.e. no assistance), uterus infection (no, yes), calving to first artificial insemination interval, calving to culling interval, and number of inseminations, with odds ratios and 95% confidence intervals were calculated for all variables. P<0.05 was considered as significant in all analyses. Data were analyzed with Stata 12/MP4 statistical software program.

## RESULTS

Fifteen isolates identified as *T. pyogenes* from 32 repeat breeder animals were biochemically analyzed and nucleotide of mitochondrial (16) rDNA gene. Half of the animals were crossbred (Native Black x Brown Swiss) while the other half were Holsteins in the present study. 21.8% of the cows were primiparous while the others were multiparous. The mean BCS was within the ideal range; 46.8% of all cows had calving assistance while the other half had normal parturition; 43.7% of all cows had early postpartum uterine infection; 59.3% of the cows had the largest follicles (regressing) in the presence of a CL; 31.2% had preovulatory follicles; 9.3% had anovulatory follicles. 60% of cows which isolated *T. pyogenes* had corpus luteum; on the other hand, 76.4% of cows had corpus luteum in non-presence of *T. pyogenes*. The proportion of subclinical endometritis was 37.5% for all animals in the present study. The mean calving to first insemination interval was 102.1 days with a range of 45 to 174 days and the average calving to culling interval was 291.4 days with a range of 160 to 450 days for cows that presence of *T. pyogenes*. Table I shows

**Table I. Proportion of variables and occurrence of *T. pyogenes* in repeat breeder cows.**

Group	SE	Cross breed	BCS	PP uterine infection	Pregnancy to first AI interval	Pregnancy to culling interval	No. of inseminations
Infection	40%	47.0%	3.06 (2.0-4.0)	47%	102.1 (45-174)	291.4 (160-450)	7.8(5-11)
Control	35.3%	53.3%	3.15 (4.25-2.0)	40%	98.1 (62-150)	241.2 (169-420)	7.2(4-14)
P value	0.647	0.993	0.662	0.735	0.708	0.08	0.551

Abbreviations: SE, subclinic endometritis; BCS, body condition score; PP, postpartum; AI, artificial insemination.

the distribution of parameters according to the presence of *T. pyogenes*. The logistic regression model revealed no effect of breed, parity, BCS, calving assistance, or postpartum uterine diseases on presence of *T. pyogenes* in repeat breeder cows (Table II).

**Table II. Effect of variables included in a binary logistic regression model on presence of *T. pyogenes* in repeat breeder cows.**

Variables	OR	95% CI	P value
Breed	0.41	0.06 – 2.56	0.338
Subclinical endometritis	0.28	0.04 – 1.95	0.197
Parity	1.22	0.52 – 2.87	0.647
BCS	2.01	0.36 – 11.22	0.426
Calving	3.14	0.34 – 28.77	0.312
Pp uterine infection	2.27	0.27 – 19.20	0.451
Calving-first AI interval	1.02	0.98 – 1.06	0.282
Calving-culling interval	0.98	0.96 – 1.00	0.104
No. of inseminations	1.19	0.70 – 2.03	0.526

Abbreviations: BCS, body condition score; PP, postpartum; AI, artificial insemination; OR, odds ratio; CI, confidence interval. Variables: Breed (reference-Native Black, 1- Holstein), subclinical endometritis (reference- 0 %, 1-  $\geq$  3%), parity (reference- primiparous, 1- multiparous), BCS (reference-  $>3.5$ , 1-  $\leq$  3.5), Calving (reference- no assistance, 1- assistance), and Pp uterine infection (reference- no, 1- yes).

**Table III. Antimicrobial susceptibility of 15 *T. pyogenes* isolates.**

Antimicrobial agent	Number of isolates (%)		
	Susceptible	Intermediate	Resistant
Ampicillin (AMP)	15 (100)	0 (0)	0 (0)
Bacitracin (B)	0 (0)	0 (0)	15 (100)
Ceftiofur (EFT)	15 (100)	0 (0)	0 (0)
Cefotaxime (CTX)	15 (100)	0 (0)	0 (0)
Ceftazidime (CAZ)	15 (100)	0 (0)	0 (0)
Chloramphenicol (C)	15 (100)	0 (0)	0 (0)
Erythromycin (E)	15 (100)	0 (0)	0 (0)
Gentamicin (CN)	15 (100)	0 (0)	0 (0)
Lincomycin (MY)	13 (86.6)	0 (0)	2 (13.3)
Neomycin (N)	0 (0)	0 (0)	15 (100)
Ofloxacin (OFX)	15 (100)	0 (0)	0 (0)
Oxytetracycline (OT)	0 (0)	0 (0)	15 (100)
Penicilline (P)	15 (100)	0 (0)	0 (0)
Streptomycine (S)	15 (100)	0 (0)	0 (0)
Tetracycline (TE)	0 (0)	0 (0)	15 (100)

The S-typed, beta-hemolytic colonies on blood agar were observed after 48 h incubation under 5% CO<sub>2</sub>. Under microscopic investigation, Gram-positive, small, pleomorphic bacteria were detected. Since all *T. pyogenes* strains had the same 16S rDNA sequence, only one strain was selected and deposited in the NCBI under GenBank accession number MN907639.

#### Antimicrobial susceptibility tests

The susceptibility of *T. pyogenes* strains to 15 antibiotics is shown in Table III. The strains were most resistant to bacitracin, tetracycline, neomycin, and oxytetracycline, two isolates (13.3%) were resistant only to lincomycin. The strains resistant to at least three or more antibiotics were defined as multiple drug-resistant (MDR). Fifteen multiple-drug resistance strains were detected (100%). Fifteen strains were resistant to B, N, OT, TE while 2 strains were resistant to MY.

The *tet(W)* gene was observed phenotypically in all tetracycline-resistant strains (100%).

## DISCUSSION

Previous studies have shown varying prevalence of subclinical endometritis in cows (LeBlanc, 2008; Plöntzke *et al.*, 2010; Senosy *et al.*, 2012). However, these studies focused on the early postpartum period. According to Pothman *et al.* (2015), there is little information about subclinical endometritis prevalence in repeat breeder cows. Salasel *et al.* (2010) and Pothman *et al.* (2015) reported subclinical endometritis prevalence of 52.7% and 12.7%, respectively, which suggests that subclinical endometritis might be a risk factor for repeat breeder cows. The different reported percentages depend on the number of animals used and methodology. The main methodological difference is the PMN threshold definition for diagnosing subclinical endometritis. While Salasel *et al.* (2010) used 3%, Pothman *et al.* (2015) used two different categories: between 0% and 5% and greater than 5%. In the same study, 48.2% of cytological samples from repeat breeder cows met the threshold of 0-5% prevalence. In the present study, the incidence of subclinical endometritis was 37.5%. Our sample size was smaller than in other studies whereas the percentage of subclinical endometritis cows was slightly greater. A possible explanation for this could be the PMN threshold value defined for cytological samples. Calving to first AI interval was also greater than in Salasel *et al.* (2010) and Pothman *et al.* (2015). More cows in the present study had histories of previous uterine infections than in Pothman *et al.* (2015). Early postpartum uterine infection may increase the incidence of subclinical endometritis during the later stages of lactation.

There were no significant interactions in the risk factors analyzed for the presence of *T. pyogenes* in repeat breeder cows, which may be due to the number of isolates. In contrast to Pothman *et al.* (2015), we only sampled a few cows. This limitation was because we only evaluated 2 groups of cows with detected *T. pyogenes* in uterine samples and no bacterial growth in uterine samples. Previous studies showed that *T. pyogenes* is less likely to be detected because it is an anaerobic organism. Previous studies also compared cows detected with different bacteriological cultures. The bacteriological tests of the uterine samples with or without endometritis and in early or late postpartum periods showed that the frequently isolated bacteria are *T. pyogenes*, *E. coli*, *Pseudomonas* sp., *Streptococcus* sp., *Staphylococcus* sp., *Pasteurella multocida*, *Clostridium* sp., *Fusobacterium* sp., and *Bacteroides* sp., According to the postpartum time and interaction of bacteria, *E. coli* is the predominant etiological factor of uterine infection in the first 10 days after parturition (Földi *et al.*, 2006). An over-presence of *E. coli* coupled with depressed immune system resulted in metritis in dairy cows in the early postpartum period. *E. coli* is also a promoter microorganism for the development of uterine infection caused by *T. pyogenes* 15 days after parturition. *T. pyogenes* cooperates with *Fusobacterium necrophorum* and *Prevotella melaninogenicus*. *T. pyogenes* produces cholesterol-dependent cytotoxin (forming pores into the cell membrane) and a growth factor, pyolisin, for *F. necrophorum*. Both these factors affect the endometrial epithelial cell and possibly phagocytic cells (Miller *et al.*, 2007; Sheldon *et al.*, 2010). *F. necrophorum* and *P. melaninogenicus* produce substances that impair the local immune factors. Thus, the main factor *T. pyogenes* persists in the uterus for a long time even in pregnant cows (Miller *et al.*, 2007; Karstrup *et al.*, 2017). This mechanism may help explain the isolation of *T. pyogenes* at later stages of lactation in this study.

Antimicrobial resistance may prevent the elimination of *T. pyogenes*. As a result, *T. pyogenes* can persist in the uterine environment and be isolated at later stages of lactation. Tetracycline resistant *T. pyogenes* strains have been reported in several studies. Trinh *et al.* (2002) reported 70.6% tetracycline resistant *Arcanobacterium pyogenes* strains in pigs. Guérin-Faubleé *et al.* (1993) showed that 67% of *T. pyogenes* strains were resistant to tetracycline, doxycycline, and minocycline. Similarly, 74.5% *T. pyogenes* strains isolated from cows with mastitis were resistant to tetracycline (Zastempowska and Lassa, 2012). Santos *et al.* (2010) and Liu *et al.* (2009) also reported tetracycline-resistant *T. pyogenes* strains isolated from the uterus of postpartum dairy cows with endometritis. Zhang *et al.* (2017) reported 62.5% and 68.7% resistance rates

against oxytetracycline and tetracycline, respectively, among 32 *T. pyogenes* strains isolated from dairy cows with endometritis. In our study, 15 *T. pyogenes* (100%) strains were resistant to both oxytetracycline and tetracycline, which is a higher frequency than in previous studies.

Tet(W) is a globally distributed tetracycline resistance gene. Studies have shown that it is associated with tetracycline resistance in *T. pyogenes*. Zhang *et al.* (2017) found tet(W) genes in 53.1% of tetracycline-resistant *T. pyogenes* strains while Zastempowska and Lassa (2012) found tet(W) gene in 85.5% of tetracycline-resistant *T. pyogenes*. Billington *et al.* (2006) demonstrated that all tetracycline-resistant *T. pyogenes* isolates carry the tet(W) gene. Similar results were obtained in this study: all tetracycline-resistant strains had this gene.

## CONCLUSION

In conclusion, the presence of intrauterine *T. pyogenes* was not associated with cytological endometrial inflammation status in repeat breeder cows in this study. On the other hand, the presence of *T. pyogenes* can be detected in the later stages of lactation. This information may be useful in developing a treatment strategy for repeat breeder cows. However, further research is needed to determine the molecular role of the presence of intrauterine bacteria and subclinical endometritis in repeat breeder cows.

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### Statement of conflict of interest

The authors have declared no conflict of interest.

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