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Short communication

DETECTION OF *MYCOPLASMA BOVIGENITALIUM* AND *MYCOPLASMA TAURI* IN HOLSTEIN FRIESIAN DAIRY COWS WITH SUBCLINICAL ENDOMETRITIS

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Mycoplasma spp. is the cause of serious cattle health disorders that lead to poor reproductive efficiency. Chronic seminal vesiculitis, infertility, vulvovaginitis and dystocia were observed in *Mycoplasma* spp. infection. The objectives of the present study were to investigate the frequency of Mycoplasmas in the uterus of postpartum dairy cows and its potential role in the occurrence of subclinical endometritis. Our study included 102 Holstein Friesian dairy cows 22-32 days postpartum. Uterine samples for cytological, bacteriological, and molecular analysis were provided using Cytoprint AI®. Metricheck® was used for the assessment of cervicovaginal mucus. After detection of *Mycoplasma* spp. genome in uterine samples, the swabs of the vulva, vagina, nasal swab, and samples of milk were sampled in order to exclude possible contamination with other microorganisms as potential trigger of subclinical endometritis. The genome of *Mycoplasma* spp. in the uterus was confirmed in 4 cows (2.40%). Sequencing of the 16S RNA revealed that detected mycoplasmas belonged to *Mycoplasma bovigenitalium* and *Mycoplasma tauri* species, with a prevalence of 1.80% and 0.6%, respectively. The cytological evaluation showed a 69.90%, 54.87% and 48.33% of polymorphonuclear cells for *M. bovigenitalium* positive cows, and 61.64% for *M. tauri* positive cow. Bacteriological examination revealed *Trueperella pyogenes* in uterine samples in all 4 observed cows. This is the first study where *M. tauri* was detected in the uterus of a live cow with subclinical

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enodmetritis. In this paper we hypothesized that *M. bovigenitalium* and *M. tauri* may have a certain role in the etiology of subclinical enodmetritis.

Keywords: dairy cows, *Mycoplasma bovigenitalium*, *Mycoplasma* spp, *Mycoplasma tauri*, subclinical endometritis.

INTRODUCTION

The *Mycoplasma* genus belongs to the class of *Mollicutes,* which is characterized by the lack of the cell wall [1]. *Mycoplasma* spp. are reported in cattle worldwide, causing severe health disorders including pneumonia, mastitis, arthritis, and reproductive failures [2]. Prevalence of *Mycoplasma* spp. in cattle with pneumonia ranges between 72% and 86% depending on the region and production systems [3-5]. After *Mycoplasma bovis*, *Mycoplasma bovigenitalium* is the second most frequently isolated from cattle [6]. From the point of reproductive failure, mycoplasmas have been associated with vulvovaginitis, infertility, endometritis, and dystocia of cows [7,8]. The highly contagious nature of some mycoplasmas and the antibiotic resistance makes controlling of this infection very difficult [9].

M. bovigenitalium infection is commonly clinically reported as pustular vaginal discharge (PVD), but it also can be unapparent [10]. However, *M. bovigenitalium* causes various cattle reproductive disorders including chronic seminal vesiculitis, infertility, mastitis, and vulvovaginitis [2,11-13].

M. tauri was reclassified in January 2022 from *Mycoplasma* sp. *Zaradi* to the present name *Mycoplasma tauri* sp. nov. after retrospective comprehensive taxonomic study [14]. Reclassified strains were originated from a collection of *M. Zaradi* isolates from Austria, Germany, and Slovakia. Strains were isolated from the prepuce of a bull, the uterus of a cow with endometritis, semen of healthy bulls, and bulls with orchitis collected from 2006-2016. *Mycoplasma tauri* was recovered only from samples derived from cattle genital tract, which indicates tropism to the genital epithelium [14].

Subclinical endometritis is associated with reduced conception rate after the first artificial insemination, prolonged service period and consequent increase in on-farm costs [15]. Research conducted in Serbia showed its prevalence of 62.74% [16]. Pathogenic bacteria are considered to play a minor role in the etiology of subclinical endometritis, thus, etiology of subclinical endometritis origin is still discussed and has not yet been elucidated [17].

Data about the influence of mycoplasmas on subclinical endometritis (SCE) in literature are sparse, except one study where a correlation between *M. bovigenitalium* and SCE was noticed [8]. So far, there are no published papers in Serbia related to the role of mycoplasmas in the development of reproductive failures in cows. Therefore, this study aims to contribute to the scientific knowledge and to investigate the frequency of *M. bovigenitalium* and *M. tauri* in the uterus of postpartum (PP) cows and their potential role in the etiology of SCE.

MATERIALS AND METHODS

Animals

The farm was located in the central Serbia region and housed 2200 cows in 4 different free-stall barns. All animals were vaccinated with Bovilis BVD® (MSD Animal Health, Wellington, New Zealand), Bovilis IBR Marker live® (MSD Animal Health, Wellington, New Zealand), and Bravoxin 10® (MSD Animal Health, Wellington, New Zealand). Within the State Animal Health Control Program, animals were tested negative for brucellosis, enzootic bovine leucosis, and tuberculosis. Cows were fed an identical diet formulated for lactating cows, milked three times a day. Animals were selected based on the day of starting the double ovsynch fertility protocol. The histories of lameness and mastitis of cows included in the experiment were obtained from Dairy Comp 305 (Valley Agricultural Software, Tulare, California). Semen was not tested for the presence of *Mycoplasma* spp.

Study design

In total, 102 lactating primiparous and multiparous Holstein-Friesian cows with a mean live weight of 530±21.2 kg were enrolled in this study during 2022. After the specimens from the uterus were sampled using Cytoprint AI®, cytological evaluation was performed and the cows were divided into 2 groups (SCE positive and SCE negative). Cytoprint AI® tips (brushes) from the SCE positive group of cows (n=60; 58.82%) were submitted to PCR for detection of *Mycoplasma* spp. *Mycoplasma* spp. positive cows were further tested for *Ureaplasma* spp. by PCR as well as using conventional bacteriological methods. In *Mycoplasma* spp. positive cows vulvar, vaginal, and nasal swabs were additionally sampled to detect *Ureaplasma* spp, *Mycoplasma* spp, and bovine herpes virus 1 (BoHV-1). Also, a milk sample was taken to detect *Mycoplasma* spp. and *Ureaplasma* spp.

Cervicovaginal mucus samples

To collect cervicovaginal mucus, the sampling was performed using Metricheck® (Metricheck®, Simcro, New Zealand). The cervicovaginal mucus was collected by administrating the device through the vulval lips to the cranial extent of the vaginal fornix. Then the handle of the device was slightly elevated, and the device was retracted caudally for the evaluation of the content [18].

Uterine samples for cytology

Uterine samples for cytological evaluation were obtained at 22-32 PP by using the Cytoprint AI® technique. After introducing the Cytoprint AI® in the uterus, rotation was performed clockwise, after which the brush was retracted into the sterile plastic tube and extracted caudally. Then the brush was rolled down the clean glass microscope slide (Menzel Gläser, Braunschweig, Germany), air-dried and sent to the laboratory.

Cytoprint AI® tip was cut into the sterile plastic tube and refrigerated until arrival at the laboratory for bacteriological and molecular diagnostics.

Swabs and milk samples

To detect *Mycoplasma* spp, *Ureaplasma* spp. and BoHV-1, vaginal, vulvar, and nasal swabs were collected using cotton swabs and by standard technique. The milk samples were collected using a standard technique [19].

Cytological evaluation

For the cytology of uterine samples, slides were stained with MGG Quick Stain (Bio Optica, Milano, Italy) according to the manufacturer's instructions. Determination of the percent of polymorphonuclear cells (%PMN) was carried down by counting a minimum of 200 cells per cow on 400× magnification [20]. The diagnostic criteria for evaluating subclinical/cytological endometritis was 18% or more at day 21-33 PP [21]. The mean %PMN in cows that were negative for *Mycoplasma* spp. and positive to SCE was 52.14%.

Molecular investigation of targeted pathogens

Cytoprint AI® tip, vulvar, vaginal and nasal swabs were immersed in 2 ml of sterile PBS and thoroughly vortexed. The suspensions were centrifuged for 10 min at 4,000 rpm and the decanted supernatants were used for DNA extraction (IndiSpin Pathogen Kit, Indical, Germany). The milk samples were processed as described by Sachse [22], PCR was completed using a commercial kit, HotStarTaq Master Mix Kit (Qiagen, Germany). The reaction mix was composed of 2 µl template DNA, 10 µl 1x HotStar Master Mix, 0.6 µl of each primer (10 µM) as previously published [23] and 6.8 µl RNase-free water.

Amplification of partial 16S rDNA was accomplished using Mastercycler, Eppendorf (Germany), and the temperature profile was as follows: initial denaturation at 95 °C for 15 min, 40 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min and elongation at 72 °C for 2 min, and single step of final extension at 72 °C for 10 min. PCR products were analyzed in 2% agarose gel stained by ethidium bromide and visualized under UV light after electrophoreses at 60 V for 1 hour. PCR products showing specific amplification of 1029 bp were purified using GeneJET PCR Purification Kit (ThermoFisher Scientific) and sequenced in Macrogen Europe, The Netherlands, using the Sanger method. The consensus sequence was constructed using the Staden package 2003 and submitted to NCBI GenBank under accession numbers OM818656-OM818659. Phylogenetic analysis was performed using Mega X, the Neighbor-Joining method, and the bootstrap test (1000).

Detection of *Ureaplasma* spp, and BoHV-1 was done by previously described protocols [24,25].

Bacteriological examination

Bacteriological examination of swabs of the uterus was done presumably by culture-base analyses [26,27]. In brief, all samples were inoculated onto Columbia blood agar base supplemented with 5% ovine blood, McConkey agar, and Sabouraud agar (HiMedia, India). After incubation, according to colony morphology, oxygen requirements, Gram staining, hemolytic properties, catalase, oxidase tests, and CAMP synergistic hemolytic characteristics, the isolates were identified. Further confirmation of selected colonies was done by the MALDI-TOF analysis (VITEK® MS, BioMerieux).

The research related to use of animals has been complied with all the relevant national regulations and institutional policies for the care and use of animals (the experiment was approved and by Ethical Committee of Faculty of Veterinary Medicine, University of Belgrade and the Ministry of Agriculture, Forestry and Water Management-Veterinary Directorate, Republic of Serbia; Permit number: 323-07-04903/2022-05.

RESULTS AND DISCUSSION

The Metricheck® score was 3 in all 4 Mycoplasma positive cows. Cervicovaginal discharge was composed of more than 50% white or yellow pus with a slight presence of blood (Figure 1a).

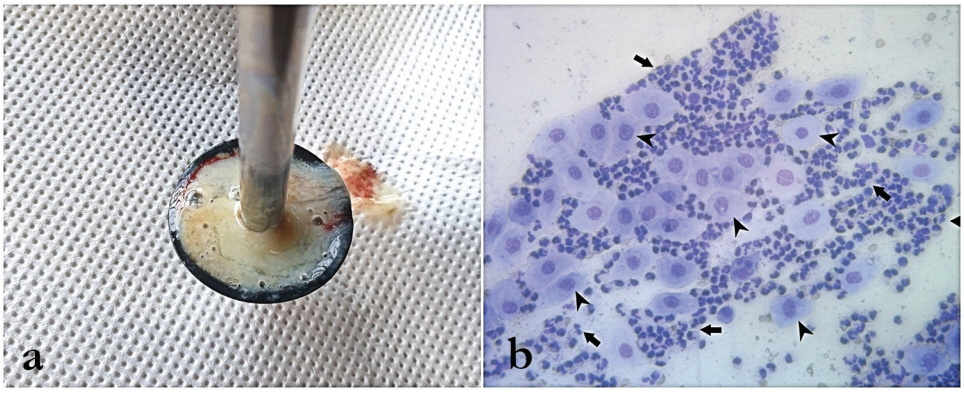

Figure 1. a) Metricheck® cup filled with pus mixed with a slight presence of blood; **b)** polymorphonuclear cells (arrow) and endothelial cells (arrowhead), Cytoprint technique, MGG Quick Stain®, ×400.

Out of total 4 positives for *Mycoplasma* spp, 3 samples (1.80%) were confirmed as *M. bovigenitalium* and 1 sample (0.6%) as *M. tauri*. Analyzing nucleotide sequences by subjecting to BLAST searches against reference genomes in the NCBI GeneBank, it was confirmed that OM818656-OM818658 matches with high scores with *Mycoplasma bovigenitalium*, and OM818659 with *Mycoplasma tauri* (Figure 2). Uteri samples were tested negative for *Ureaplasma* spp. Milk samples tested negatively for *Mycoplasma* spp.

and *Ureaplasma* spp. The genome of *Mycoplasma*, *Ureaplasma* spp, and BoHV-1 were not detected in the vaginal, vulvar, and nasal swabs.

Mycoplasmas role in the occurrence of respiratory tract disorders and the development of mastitis is well established [5,6]. However, *M. bovigenitalium* can be found in the vaginal mucus of both healthy cows [31] and cows with reproductive disorders, even infertility [13]. *M. bovigenitalium* was isolated from vaginal swabs of dairy cows with different pathological conditions [32]. *Mycoplasma bovigenitalium* was also isolated from semen, and it was shown that can be transmitted by natural breeding and by artificial insemination [33]. Since *M. bovis* and *M. bovigenitalium* can colonize the reproductive tract and produce severe salpingo-oophoritis, they were also isolated from the oviducts [34]. Additionally, *M. bovigenitalium* was implicated in several outbreaks of granulopapular vulvovaginitis, which was characterized by granulopustular lesions of the vagina [12]. However, the information about the isolation of mycoplasmas from the uterus of cows, and the association between mycoplasma infection and subclinical endometritis based on PMN are rather scarce [8]. In the present study %PMN in *Mycoplasma* spp. negative cows was lower (52.14%) compared to the *Mycoplasma* spp. positive cows (58.82%). This fact further confirms the possible role of mycoplasmas in etiology of SCE.

This is the first study in which *M. tauri* was confirmed in the uterus of a live cow and associated with SCE. To the best of our knowledge, there is no data about the prevalence of *M. tauri* and its detection from the uterus of live cows till this study.

In our study the prevalence of *Mycoplasma* spp. was 2.4% that was lower compared to the other study [8] which reported a 7.4% prevalence of *Mycoplasma* spp. in the uterus of early PP dairy cows from week 5 to week 7.

Of the seven mycoplasma species, so far only *M. bovigenitalium* was detected in uterine swabs [8]. In our study two *Mycoplasma* spp. were detected: *M. bovigenitalium* in 3 cows

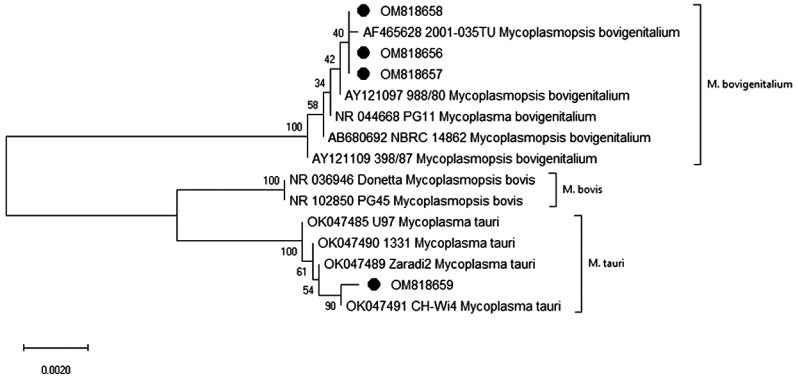

Figure 2. Phylogenetic tree was inferred using the Neighbor-Joining method. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. Sequences from this study are labeled with node.

(1.8%) and *M. tauri* (0.6%) in one SCE positive cow. Prevalence of *M. bovigenitalium* was obtained in 9.29% of tested vaginal swabs [35]. Also, in another study mycoplasmas were isolated from the uterine secretion after transrectal uterine horns massage at a slaughterhouse [34]. However, due to different sampling techniques and diagnostic methods prevalence of genital mycoplasmas cannot be fairly compared.

The %PMN in *M. bovigenitalium* positive cows was 69.90%, 54.87%, and 48.33% and in *M. tauri* positive cows 61.64% (Figure 1b). Mean %PMN in *Mycoplasma* spp. positive cows was 58.68%. Uterine cytology in the present study showed a high percentage of PMN in all 4 cows. The same results were provided in Ghanem´s [8] research, also it was proved that the incidence of dystocia was higher in mycoplasma positive compared with mycoplasma negative cows. Additionally, the incidence of cytologic endometritis was higher in mycoplasma positive than in mycoplasma negative cows at week 7 PP. For these reasons, the authors concluded that *M. bovigenitalium* infection in the uterus might be associated with recent dystocia and with cytologic endometritis in PP dairy cows. In the present case by analyzing data from Dairy Comp 305, there was no history of distocia in tested cows. However, in Ghanem´s [8] study was doubted that mycoplasmas may have been from the vagina due to the opened cervix at the time of sampling. The factor that favors contamination is the open cervix, which makes the contamination between the vagina and the uterus possible through drainage. As a result, the uterus is contaminated with microorganisms from the vagina and vice versa. In our research, vaginal/vulvar swabs were sampled and tested negative for the presence of mycoplasmas. Thus, we can freely say that the mycoplasmas detected in the uterus of examined cows were from the uterus. Four *Mycoplasna* spp. positive and SCE positive cows observed in this study, tested positive for *Trueperella pyogenes* using conventional bacteriological methods. Some reports suggest that over 90 % of cows in the first two weeks after parturition have bacterial contamination of the uterus that decreases as the puerperium progresses [37] and by 6-8 weeks PP pathogens get eliminated [18]. The most commonly isolated pathogens from the uterus of cows with clinical endometritis are *Fusobacterium*, *Trueperella*, and *Peptoniphilus* [17]. Some studies [17,38] showed that *T. pyogenes* have no influence on the etiology of SCE. Bacterial cultures isolated from the uterus of cows with SCE do not differ from those isolated from the uterus of healthy cows [39]. The results of these studies suggest that common pathogens isolated from the uterus of cows with clinical endometritis do not play a significant role in the pathogenesis of SCE. Previously cited studies agree with the assumption that clinical endometritis and SCE represent two different risk factors that have a negative impact on reproductive performance [40]. Our Metricheck® score 3 can be associated with *T. pyogenes*. It is known that *T. pyogenes* leads to an increased incidence of PVD and purulent uterine lavage fluid [41]. In this case, although there was no spontaneous vaginal discharge, a PVD was obtained using a Metricheck®.

CONCLUSIONS

Prevalence of detected *Mycoplasma* spp. obtained in our research was 2.4% in cows with SCE. After sequencing PCR products, we established that 3 cows (1.8%) were *M. bovigenitalium* positive and 1 cow (0.6%) was *M. tauri* positive. After analyzing the data obtained in our study we concluded that *M. bovigenitalium* and *M. tauri* may have a certain role in the etiology of subclinical endometritis. Further studies are needed to elucidate their exact role as potential causes of subclinical endometritis.

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Authors' contributions

MM, BK and BM carried out the experimental work, made substantial contributions to the acquisition, analysis and interpretation of data and participated in manuscript writing. DM have been involved in drafting and manuscript writing. MM and BK supervised the entire work. BM, VM, NZ and JK carried out laboratory diagnostics. All authors provided critical feedback and helped to shape the research and final paper.

Declaration of interest statement

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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DETEKCIJA GENOMA *MYCOPLASMA BOVIGENITALIUM* I *MYCOPLASMA TAURI* KOD MLEČNIH KRAVA HOLŠTAJN-FRIZIJSKE RASE SA SUPKLINIČKIM ENDOMETRITISOM

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Mycoplasma spp. prouzrokuju oboljenja goveda koja za posledicu imaju smanjenje reproduktivne efikasnosti muznih krava. *Mycoplasma* spp. kod goveda dovode do hroničnog seminalnog vezikulitisa, vulvovaginitisa, distokije i neplodnosti. Cilj ove studije je bio da se ispita učestalost mikoplazmi u materici kod krava nakon teljenja i utvrdi da li postoji potencijalna veza sa nastankom subkliničkog endometritisa. Studija je obuhvatila 102 muzne krave Holštajn Frizijske rase od 22-32. dana nakon teljenja. Brisevi endometrijuma za citološku, bakteriološku i molekularnu analizu su dobijeni korišćenjem Cytoprint AI®. Metricheck® je korišćen za procenu kvaliteta cervikovaginalne sluzi. Nakon detekcije genoma *Mycoplasma* spp. u brisevima endometrijuma, uzorkovani su brisevi vulve, vagine, nosa i uzorci mleka kako bi se isključila moguća kontaminacija drugim mikroorganizmima kao potencijalnim prouzrokovačima subkliničkog endometritisa. Genom *Mycoplasma* spp. u brisu endometrijuma je utvrđen kod 4 krave (2,40%). Sekvenciranjem 16S RNK utvrđeno je da detektovane mikoplazme pripadaju *Mycoplasma bovigenitalium* i *Mycoplasma tauri* vrstama, sa prevalencijom od 1,80%, odnosno 0,6%. Citološkom analizom je utvrđen procenat polimorfonuklearnih ćelija od 69,90%, 54,87% i 48,33% kod krava pozitivnih na *M. bovigenitalium* i 61,64% kod krave pozitivne na *M. tauri*. Bakteriološkom analizom je utvrđena *Trueperella pyogenes* u uzorcima endometrijuma kod sve 4 ispitane krave. Ovo je prva studija u kojoj je *M. tauri* otkrivena u brisu iz materice žive krave sa subkliničkim endometritisom. U ovom radu, pretpostavljamo da *M. bovigenitalium* i *M. tauri* mogu imati izvesnu ulogu u etiologiji subkliničkog endometritisa.