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Detection of genes encoding multidrug resistance and biofilm virulence factor in uterine pathogenic bacteria in postpartum dairy cows

V.R. Kasimanickam*, K. Owen, R.K. Kasimanickam

Department of Veterinary Clinical Sciences, College of Veterinary Medicine, Washington State University, Pullman, Washington, USA

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ABSTRACT

Reckless use of antibiotics and/or development of biofilm are the rationale for the development of multidrug resistance (MDR) of pathogenic bacteria. The objective of the present study was to detect MDR genes in *Trueperella pyogenes* and to detect biofilm virulence factor (VF) genes in *Escherichia coli* isolated from the uterus of postpartum dairy cows. Uterine secretions from different parity postpartum Holstein cows (n = 40) were collected using cytobrush technique after a sterile procedure from cows with varying degree of uterine inflammatory conditions. The cytobrush was stored in a specimen collector, placed in a cooler with ice, and transported to the laboratory within 2 hours. The pathogens were isolated and were identified initially by their colony morphology and biochemical characteristics. To further identify and classify the single species, and to determine the presence of MDR and VF genes, the genes fragments were amplified using the respective primers by either singleplex or multiplex polymerase chain reaction protocol, and amplicons were detected by electrophoresis method. *T pyogenes* was isolated in 17 of 40 (42.5%) cows in the study population as recognized by the *16S rRNA* gene. Of the positive *T pyogenes* samples, 8 of 17 (42.1%) were positive for integron type 1 (*intI I*), and none were positive for integron type 2 (*intI II*). Of those 8 positive for *intI I*, six of eight (66.7%) were positive for amplicons *aadA5* and *aadA24-ORF1* at 1048 and 1608 bp, respectively, associated with specific drug resistance. Presence of *addA5* indicated resistance to sulfadiazine, bacitracin, florfenicol, and ceftiofur. Presence of *addA24-ORF1* indicated resistant to sulfadiazine, bacitracin, penicillin, clindamycin, and erythromycin. *E coli* was isolated in 18 of 40 (45.0%) cows in the study population. The genes for VF, *Agn43a*, and *Agn43 b*, associated with biofilm production, were found in 6 of 18 (33.3%) of the positive isolates. Both *T pyogenes* MDR gene and *E coli* biofilm VF existed in more severe form of uterine diseases than subclinical endometritis. In conclusion, 35% of *T pyogenes* isolates found were positive for a gene cassette associated with antibiotic resistance, and 33% of the *E coli* isolates contained genes for the VF associated with biofilm production.

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1. Introduction

In postpartum dairy cows, reproductive tract inflammation is usually associated with intrauterine bacterial infection immediately after calving. Many species of bacteria can be found in the uterus during the early postpartum period [1–3]. Bacteria such as *Streptococcus* spp. and *Staphylococcus* spp. are commonly isolated in animals without signs of metritis during the first week after calving, whereas *Escherichia coli*, *Trueperella pyogenes*, *Fusobacterium necrophorum*, and *Prevotella melaninogenicus* are generally isolated from animals with metritis [1,3,4]. In the uterus, presence of *E coli* early in the postpartum affects the function of defense mechanism causing clinical

* Corresponding author. Tel.: +1 (509) 335 6060; fax: +1 (509) 335 0880.
E-mail address: vkasiman@vetmed.wsu.edu (V.R. Kasimanickam).

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endometritis [5,6]. In addition to infection with *E coli* in the first week postpartum, presence of infection with *T pyogenes* in week 3 resulted in clinical or subclinical endometritis, and impaired reproductive performance [7].

Treatment of uterine infection has historically involved systemic, intrauterine infusion of antibiotics [8–10], or injection with PGF2a [9–11]. A large body of conflicting reports exists regarding the benefit of treatment improving uterine health and reproductive performance. Efficacy of treatment is dependent on time postpartum at treatment, severity of inflammation in the uterus, and presence of a CL [12].

Drug regulations and milk withdrawal after antibiotic treatment resulted in selection of specific class of antibiotics to treat uterine disease. The extensive use of such antibacterial has resulted in the increasing resistance of pathogenic bacteria [13,14]. The establishment of bacterial drug resistance can eventually jeopardize the treatment success. The occurrence of multidrug resistance (MDR) in bacteria could be caused by genetic modification or biofilm formation. The MDR is often caused by the accumulation of genes, each coding for resistance to a single drug, on resistance transfer factor plasmids [15]. The assembly of resistance genes on a single resistance transfer factor is achieved by mechanisms provided by transposons, integrons, and insertion sequence common region elements [15,16]. Integrons, for example, are especially powerful in producing MDR because they assemble several resistance genes in a correct orientation and supply a strong promoter for their expression [15,16]. Furthermore, the resistance gene once incorporated into an integron becomes tagged, so that it could easily become a part of another integron. The antibiotic susceptibility of bacterial cells is affected by their physiological states. Another important consequence of the drug resistance phenomenon is the occurrence of "persister" cells and biofilm formation [17–19]. Biofilm is produced when planktonic, or free floating, bacteria attach to a surface and start secreting extracellular polymeric substances [19]. When bacteria are in a community, different genes are activated, allowing expression of polysaccharides and other molecules that the biofilm is created from. Up to 800 genes can be activated when a bacteria joins a biofilm community. The biofilm serves to sequester the bacteria from antibiotics. Biofilm exists naturally throughout the body, and often, it is normal or opportunistic flora that colonizes it and serves to protect the body from other potential threats [19]. If biofilm is disturbed or unbalanced, there will be opportunities for pathologic bacteria to take residence within it. The antibiotic resistance that biofilms create is a challenge that requires new kinds of novel therapies.

The objectives were to determine the prevalence of: (1) MDR genes in *T pyogenes* (*16S rRNA*, *intI 1*, *intI 2*, and Cassette genes) and (2) Biofilm virulence factor (VF) genes (*Agn43aCFT073* and *Agn43bCFT073*) in *E coli* isolated from the uterus of postpartum dairy cows.

2. Materials and methods

2.1. Animals and endometrial sample collection

Lactating Holstein cows of different parity (n = 40), from a dairy farm located in Washington state with no history of peripartum metabolic diseases were enrolled in this study. Cows were fed, twice daily a total mixed ration to meet or exceed dietary requirements for lactating Holstein cows weighing 545 to 770 kg and producing 27 to 36 kg of 3.5% fat-corrected milk and were evaluated during weekly visits from calving to 7 weeks postpartum for the presence of metritis, clinical endometritis, or subclinical endometritis, using diagnostic techniques and criteria described previously [20–23]. All cows were reevaluated 2 weeks after the initial diagnosis to determine proportion of cows that experienced either spontaneous recovery or persistent infection. Cows that were diagnosed with uterine disease at both initial and follow-up examinations were considered to have persistent inflammation. The prevalence of persistent inflammation was calculated as number of cows with uterine disease at both the initial and follow-up examinations, divided by cows that had uterine inflammatory conditions at initial examination.

No treatments were administered during the study period except that all cows received one to two uterine lavages in the first week after calving. After the study period, diseased cows either received antibiotic treatment or PGF2a via breeding protocol based on the decision of farm management. Briefly, metritis was defined as cows with fetid vaginal discharge associated with fever (rectal temperature >39.5 °C) during first 14 DIM. Clinical endometritis was defined as presence of mucopurulent or purulent uterine discharge externally or in the anterior vagina on vaginoscopic examination between 28 and 35 DIM. Subclinical endometritis was defined as presence of polymorphonuclear neutrophils (PMN) exceeding 18% on endometrial cytology collected by cytobrush between 28 and 35 DIM. In the follow-up evaluation, metritis is defined as purulent uterine discharge or greater than 18% PMN in endometrial cytology between 14 and 28 DIM, clinical endometritis is defined as mucopurulent uterine discharge or greater than 18% PMN in endometrial cytology between 35 and 49 DIM, and subclinical endometritis is defined as greater than 10% PMN in endometrial cytology with no uterine discharge between 35 and 49 DIM. The study was conducted in accordance with institutional animal care and use policies (04342-001 and 04391-001). It should be noted that the samples for this study were collected from same animals at the same time when samples were collected for use in another study [24].

Endometrial cytology samples from all cows were collected through a sterile procedure using a Cytobrush Plus GT (Medscand Inc., Hollywood, FL, USA), modified for use in large animals as previously described [20,24]. One clinician collected all endometrial samples. Between uses, the stainless steel devices were thoroughly cleaned with water to remove biological material, rinsed with alcohol and sterile PBS and cold-sterilized, and washed with sterile PBS before use. It should be noted that this method of sterilization is not considered adequate for any major surgical procedures but can be used for minor procedures [25].

2.2. Isolation of Trueperella pyogenes

The pathogens were isolated after performing the methods described previously [26,27]. Briefly, the cytobrush with uterine secretions was stored in an Anaerobic

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