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Short Communication

Helcococcus kunzii and *Helcococcus ovis* isolated in dairy cows with puerperal metritis

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Puerperal metritis is a common disease in the first 3 weeks after calving in dairy cattle. Complicated parturitions and retained placenta are factors facilitating contamination of the uterine lumen by environmental and opportunistic pathogens. Post-partum uterine infections are considered factors able to reduce animal welfare and fertility, causing economic losses and early animal elimination from the herd (Williams et al., 2007). The most common pathogens associated with metritis are *Escherichia coli, Trueperella pyogenes* and anaerobes such as *Fusobacterium necrophorum, Prevotella* spp., alone or often in association (Sheldon et al., 2008; Williams et al., 2007). After parturition, the uterine microbial population can be very complex and follows an evolution in which a synergic effect can be exerted among bacteria conditioning the onset of a disease and future reproductive performance. Hence, as pathogenic mechanisms governing metritis are largely unknown, it is important to understand the diversity and prevalence of microorganisms involved. α-hemoltyic streptococci are listed in generic terms as opportunistic uterine contaminants (Williams et al., 2007) among bacteria isolated culturing uterine specimens but particular attention to their species identification has not been paid and little evidence has been documented.

New genera and species have been created to give a taxonomic position to slow-growing, catalase-negative, facultatively anaerobic, α-hemolytic Gram-positive cocci such as *Helcococcus kunzii* and *Helcococcus ovis* identified in human and animal clinical specimens respectively (Collins et al., 1993, 1999). Since *H. ovis*' first description in sheep organs after a *post-mortem* examination and in milk from a sheep suffering from subclinical mastitis (Collins et al., 1999), several papers have reported its isolation in horse and sheep broncopneumonia (Garcìa et al., 2012; Rothschild et al., 2004; Zhang et al., 2009) and bovine endocarditis (Kutzer et al., 2008; Post et al., 2003). Recently *H. ovis* DNA has been detected in milk samples from bovine

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mastitis, but without effective isolation (Schwaiger et al., 2012). *H. kunzii* has been found in human specimens (Lemaitre et al., 2008; Peel et al., 1997; Woo et al., 2005) and only recently in a sow with purulent cystitis as the first case of veterinary concern (Grattarola et al., 2010). With regards to diseases in the reproductive sphere, a study investigated antimicrobial susceptibility of seven *H. ovis* strains isolated from bovine metritis and/or abortion, but didn't mention *H. kunzii* (Bilk et al., 2011). This paper describes the isolation of 1 *H. ovis* and of 5 *H. kunzii* during bovine puerperal metritis. To the authors' knowledge, *H. kunzii* has never been identified in cattle infections. The report indicates the need of investigating the possible clinical significance of *Helcococcus* species' presence and their role in bovine metritis pathogenesis.

During the periods December 2009−April 2010 and June 2010−January 2011, 13 dairy farms were enrolled in a survey focused on puerperal metritis. All the dairy cows calving within the defined period underwent clinical evaluation within 3 weeks after parturition. They were examined through rectal palpation in order to determine uterine conditions. The practitioners collected data about cow parity, days in milk(DIM), general clinical conditions and degree of metritis. Sheldon's criteria (Sheldon et al., 2008) were used to identify cows with puerperal metritis and the degree of disease severity. Uterine content was sampled in all affected cows with open cervix, which allowed the insertion of a double protected swab device (Equivet, Marslev, Denmark). The swabs were stored at 4°C in Amies transport medium (Laboratorios Conda, Madrid, Spain) and carried to the laboratory within 24 h from collection. The swabs were transferred to sterile saline solution and 100 μl of opportune 10-fold dilutions were plated onto 5% sheep blood agar (Oxoid, Basingstoke, UK), MacConkey agar (Oxoid), and Schaedler blood agar (Carlo Erba, Milan, Italy). Culture plates were examined after 24- and 48-h incubation at 37°C (Schaedler blood agar in anaerobic conditions). Bacterial growth was recorded and colonies representative for specific pathogens were selected, picked up and cultured onto blood agar for further identification through evaluation of colony shape, hemolysis, Gram-stain, catalase, and oxidase reaction and eventually API Systems (bioMérieux, Marcy l'Etoile, France). In the case of failure in identification by commercial systems, sequencing of 16S rRNA gene was performed using the universal primers 16S27F (5′-AGA GTT TGA TCC TGG CTC AG-3′) and R1492 (5′-TAC GGY TAC CTT GTT ACG ACT T-3′), as described by Cremonesi (2009). Briefly, DNA samples were obtained using a commercial extraction kit (RBC Bioscience, New Taipei City, Taiwan) according to manufacturer's instructions. Amplification was performed using a commercial thermal cycler (C 1000^{TM} Thermal Cycler, Bio-Rad Laboratories, Inc., Hercules, CA) with the following cycling protocol: 30 cycles at 94°C for 45 s, 61°C for 45 s, 72°C for 45 s and a final extension step of 10 s at 72°C. The PCR products were sent to BMR Genomics (Padova, Italy) for purification and sequencing.

A total of 112 dairy cows were sampled as they met the required criteria to be considered affected by puerperal metritis. The pathogens most frequently isolated were *E. coli* (n=61; 54.5%) and *T. pyogenes* (n=55; 49.1%), which were recovered together in co-infection in 17 cases (15.2%). Overall, 37 *Streptococcus* spp. were recovered in 33.0% of samples, 13 isolated in pure culture, 13 in association with *E. coli* and 11 with *T. pyogenes*. Six (5.4%) out of all the 112 uterine samples revealed a diffuse growth of pinpoint, non-hemolytic colonies both on sheep blood agar and Schaedler blood agar at 24 h of incubation. Bacteria were definable as facultative anaerobes, and appeared slightly α-hemolytic after 48 h of incubation. Gram-stain revealed Gram-positive cocci tending to arrange in clusters, couples or tetrads. The isolates were catalase and oxidase negative and API 20 Strep (bioMérieux) yielded profiles corresponding to *Abiotrophia adiacens* and *Aerococcus viridans* (Table 1). Since findings of *Abiotrophia* spp. in animal specimens is unusual and the API system database is developed mainly for human isolates, sequence analysis of the 16S rRNA gene was performed to confirm this initial and doubtful identification. NCBI BLAST analysis of sequencing results showed that the six isolates obtained from bovine puerperal metritis belonged to the genus *Helcococcus*. The nucleotide sequences were compared with the reference sequences deposited in the GenBank database (X69837 for *H. kunzii* and NR_027228 for *H. ovis*). Five isolates (1106, 1095, 1094, 1257 and 1175) had high identity score matches to *H. kunzii*, and one isolate (1105) had a high identity score match to *H. ovis* (Table 1). *H. kunzii* has never been previously reported in a bovine specimen but recently in a sow purulent cystitis (Grattarola et al., 2010). All 16S rRNA gene sequences of the *H. ovis* and *H. kunzii* isolates have been lodged in the Gen-

Table 1. Compared identification of six strains with phenotypic and molecular methods.

Strain	Accession number	API 20Strep identification	Sequencing identification	Co-isolate	Max identity %	Parity	DIM
1095	JN861736	*Aerococcus viridans*	*H. kunzii*	*T. pyogenes*	98	2	16
1105	JN861733	*Abiotrophia adiacens*	*H. ovis*	*E. coli*	99	2	9
1106	JN861734	*Aerococcus viridans*	*H. kunzii*	*E. coli*	99	1	8
1094	JN861737	*Aerococcus viridans*	*H. kunzii*	—	98	n.a.	n.a.
1257	JN861738	*Aerococcus viridans*	*H. kunzii*	*T. pyogenes*	98	2	10
1175	JN861735	*Aerococcus viridans*	*H. kunzii*	*T. pyogenes*	98	1	n.a.

Abbreviations: DIM, Days in Milk; n.a., data not available.

Bank sequence database under accession numbers reported in Table 1.

These six isolates came from only three herds out of the 13 visited. Isolates 1095, 1257 and 1175 were obtained from the same farm, which provided the greatest number of total sample swabs ($n=22$); 1105 and 1106 were recovered from two swabs from another farm and 1094 from a farm with a total of 11 samples. Eighteen *H. ovis* strains from bovine valvular endocarditis were found in animals from 17 different dairy herds (Kutzer et al., 2008), which implies a uniform spread of the pathogen. The present study focused on completely different specimens and recovered *Helcococcus* spp. in samples from a limited group of the recruited herds. Differences in uterine bacterial flora between dairy herds have been described (Elkjær et al., 2013) and are of concern for antimicrobial treatment selection.

A discussed issue is the true pathogenic role of *Helcococcus* spp. in pure and mixed infections (Zhang et al., 2009). Mixed infections are very common in the context of bovine metritis (Williams et al., 2007). *Helcococcus* spp. belong to the genera detected in cows beyond 35 days after parturition by pyrosequencing of 16S rRNA gene applied to uterine lavage samples (Machado et al., 2012). *Helcococcus* spp., detected by such a culture-independent technique, were associated with uterine infections and significantly related to cows that were not pregnant at 200 days after parturition, suggesting that it may have a damaging impact on reproductive performances. Moreover, its detrimental effect seems supported by the fact that a systemic supplementation with trace minerals during gestation, probably enhancing immune response, was able to decrease the prevalence of bacteria associated with uterine infections, *Helcococcus* spp. included (Machado et al., 2012). In the present study, *H. kunzii* 1094 was isolated in pure culture in a single case, suggesting that it can sporadically be a primary pathogen. In the other cases, the concomitant isolation of common primary uterine pathogens such as *E. coli* or *T. pyogenes* (Table 1) could suggest a synergism with *Helcococcus* spp. and on the other hand could contribute to exclude mere sample contamination. Otherwise a close link between *Helcococcus* spp. and *T. pyogenes* has been already remarked by co-isolation (Collins et al., 1999). Moreover, detection of DNAs belonging to both pathogens in milk samples needs a reassessment of their reciprocal role in bovine mastitis pathogenesis too (Schwaiger et al., 2012).

Bovine metritis reported in the present study represents the second disease of veterinary interest in which *H. kunzii* has been isolated and it is likely it could be charged with a role. *H. ovis* has been isolated in cases of metritis and abortion and antimicrobial susceptibility of isolates from such specimens has recently been determined (Bilk et al., 2011). *H. ovis* is also considered an emerging pathogen in valvular endocarditis, a severe and life-threatening disease (Kutzer et al., 2008). It is arguable that metritis caused or even supported by *Helcococcus* spp. could represent a risk factor in this regard as endocarditis-causing bacteria could originate from peripheral infections through a hematogenous pathway (Post et al., 2003).

The findings of this work suggest that both *H. ovis* and *H. kunzii* are microbiota potentially involved to some extent in uterine infections of dairy cows. A sure charge cannot be ascertained as we did not survey healthy cows and a comparison of uterine flora is not possible in this work. Unlikely bacteriological diagnosis is routinely requested for bovine metritis and treatment is usually empirically decided according to farm protocol. Moreover, *Helcococcus* spp. grows slowly and has an appearance and biochemical profile that

make it easily misidentified. For these reasons, it is probably overlooked and consequently underreported. Nevertheless, *Helcococcus* spp. presence, specificities and potential role should not be ignored as part of bovine metritis pathogenesis. For further studies aimed to investigate *Helcococcus* spp. significance, the use of molecular tools such as sequencing should be considered the most reliable method for correct identification (Zhang et al., 2009).

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