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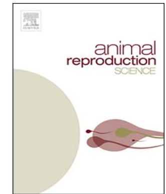

A nested-PCR strategy for molecular diagnosis of mollicutes in uncultured biological samples from cows with vulvovaginitis☆

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ABSTRACT

Bacteria classified in *Mycoplasma* (*M. bovis* and *M. bovigenitalium*) and *Ureaplasma* (*U. diversum*) genera are associated with granular vulvovaginitis that affect heifers and cows at reproductive age. The traditional means for detection and speciation of mollicutes from clinical samples have been culture and serology. However, challenges experienced with these laboratory methods have hampered assessment of their impact in pathogenesis and epidemiology in cattle worldwide. The aim of this study was to develop a PCR strategy to detect and primarily discriminate between the main species of mollicutes associated with reproductive disorders of cattle in uncultured clinical samples. In order to amplify the 16S-23S rRNA internal transcribed spacer region of the genome, a consensual and species-specific nested-PCR assay was developed to identify and discriminate between main species of mollicutes. In addition, 31 vaginal swab samples from dairy and beef affected cows were investigated. This nested-PCR strategy was successfully employed in the diagnosis of single and mixed mollicute infections of diseased cows from cattle herds from Brazil. The developed system enabled the rapid and unambiguous identification of the main mollicute species known to be associated with this cattle reproductive disorder through differential amplification of partial fragments of the ITS region of mollicute genomes. The development of rapid and sensitive tools for mollicute detection and discrimination without the need for previous cultures or sequencing of PCR products is a high priority for accurate diagnosis in animal health. Therefore, the PCR strategy described herein may be helpful for diagnosis of this class of bacteria in genital swabs submitted to veterinary diagnostic laboratories, not demanding expertise in mycoplasma culture and identification.

1. Introduction

Granular vulvovaginitis (GVV) is an infectious genital disease affecting heifers and cows at reproductive age. Clinically, GVV is characterized by the observation of vulvar discharge, hyperemia, and the presence of granulation and vesicles in genital mucosa soon after the service. Due to inflammatory reactions, infected animals can also present salpingitis, endometritis, dystocia, and abortion (Doig, 1981; Pfützner and Sachse, 1996; Givens and Marley, 2008; Ghanem et al., 2013).

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Bacteria classified in *Mycoplasma* (*M. bovis* and *M. bovigenitalium*) and *Ureaplasma* (*U. diversum*) genera are associated with cases of GVV in cattle (Hirth et al., 1966; Doig et al., 1980). Mycoplasmas and ureaplasmas belong to the class mollicutes, order *Mycoplasmatales*, and family *Mycoplasmataceae*. These microorganisms represent the smallest self-replicating and free-living form of life, being characterized by their lack of a cell wall, small genome size, and “fried egg” colony morphology (Trachtenberg, 2005; Razin and Hayflick, 2010).

Reproductive diseases related to infection by mollicutes have been described in beef and dairy cattle herds worldwide, including those from Brazil (Ghanem et al., 2013; Doig et al., 1979; Buzinhani et al., 2007; Petit et al., 2008; Lysnyansky et al., 2009; Argue et al., 2013; Gaeti et al., 2014).

Despite their ubiquitous character, the importance of these pathogens in the reproductive diseases of cattle is still unclear. This fact may be explained by the challenges experienced during laboratory identification and differentiation of mollicutes by culture and by serology methods that hamper assessment of their impact in pathogenesis and epidemiology of GVV in cattle worldwide (Buzinhani et al., 2007).

The traditional means for detection and speciation of mollicutes from clinical samples obtained from diseased animals have been culture and serology (Mcvey et al., 2013). However, as growth in culture requires specialized and enriched media as well as 7–10 days of incubation, identification of these microorganisms through culture can present several limitations (Miller et al., 1994; Quinn et al., 2011). Furthermore, contamination of biological samples with other bacteria, inadequate handling and storage conditions, and prolonged intervals between sampling and processing in the laboratory may result in increased false negative results (Maunsell et al., 2011). Additionally, due to the limited number of biochemical reactions for phenotypic characterization, the differentiation of mollicutes at the species level has been made through serology involving a complete serum panel. This method is labor-intensive, time-consuming, and currently available only in reference laboratories (Pettersson et al., 2000; Volokhov et al., 2006). To overcome difficulties regarding laboratory identification of mollicutes and to provide more accurate and rapid diagnostic results, diverse PCR assays have been developed in recent years, allowing the detection of these bacteria even in DNA purified directly from the collected samples (Buzinhani et al., 2007; Azevedo et al., 2017).

The aim of this study was to develop a PCR strategy to detect and primarily discriminate between the main species of mollicutes associated with vulvovaginitis and other reproductive disorders of affected cattle in uncultured clinical samples.

2. Materials and methods

2.1. Consensual and species-specific nested-PCR for mollicutes

Initially, conserved regions of 16S and 23S rRNA genes deposited in the GenBank database allowed the selection of a degenerate primer pair (MolliF/MolliR) for amplification of the 16S-23S rRNA internal transcribed spacer (ITS) region of the mollicute genome. This consensual PCR assay for mollicute detection amplifies PCR products with similar lengths, ranging from 847 to 866 bp. Table 1 shows the main characteristics for the primers MolliF and MolliR.

Based on the observation of significant interspecies polymorphism in the ITS region, three pairs of inner primers targeting *M. bovis* (MbF/MbR), *M. bovigenitalium* (MbgF/MbgR), and *U. diversum* (UdivF/UdivR) genomes were designed to amplify specific genomic fragments of 488, 296, and 404 bp lengths, respectively (Table 2 and Fig. 1).

DNA samples purified from vaginal swabs of animals with GVV, previously known to be infected by *M. bovis* (BRA/UEL2 strain; GenBank accession number KJ767191); *M. bovigenitalium* (BRA/UEL1; strain accession number KJ767190); and *U. diversum* (BRA/UEL3 strain; accession number KJ767192) were employed to aid in the optimization of the PCR system, both consensual and species-specific nested-PCR assays, and the evaluation of specificity of all developed PCR reactions.

2.2. Clinical samples

Vaginal swab samples from 31 cows were collected, immersed in PBS solution, pH 7.2, and stored at −80 °C until analysis. The cattle evaluated in this study were Nelore females and belonged to two beef herds from the Brazilian states of Minas Gerais ($n = 12$) and Mato Grosso do Sul ($n = 11$), as well as Holstein cows ($n = 8$) from Parana state. At the sampling, vulvovaginitis was observed in

Table 1
Sequences and characteristics of degenerate primers used on consensual PCR to amplify the ITS region of the mollicute genome.

Mollicute species	Accession number on GenBank	MolliF (5′-3′)	MolliR (5′-3′)	Amplicon length (bp)
		CCGTCAAACYATGGGAGC*	GTGYCCCGCCMTACTCAGG*	
M. bovis	AY780798	————————C————————	—C———C———————	864
M. bovigenitalium	AY780797	————————C————————	—C———C———————	863
M. agalactiae	NC_013948	————————C————————	—C———C———————	866
U. diversum	JN935894	————————T————————	—T———A———————	847
U. urealyticum	NC_011374	————————T————————	—T———C———————	852
U. parvum	NC_010503	————————T————————	—T———C———————	851

*Degenerate bases: Y = C, T; M = C, A.

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