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Detecting asymptomatic rams infected with *Mycoplasma agalactiae* in ovine artificial insemination centers

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ABSTRACT

Mycoplasma agalactiae (Ma) is the main causative agent of ovine contagious agalactia, which is a serious disease of small ruminants. In endemic areas, its most common clinical situation consists of chronically infected herds, and asymptomatic infected individuals represent an epidemiological risk regarding the transmission of this disease. The aim of this work was to detect the presence of asymptomatic rams infected with Ma in different artificial insemination centers, and to determine the most effective way to identify these individuals so as to implement adequate surveillance protocols. For this purpose, 215 rams and 14 teaser sheep were sampled taking auricular, nasal, and vaginal swabs and serum samples. In addition, ejaculates from 147 rams were analyzed. These samples were subjected to specific culture and molecular techniques to isolate and identify mycoplasmas, and to a serological test to detect antibodies against Ma. *Mycoplasma agalactiae* was detected in 47 (4.4%) of the 1077 samples analyzed, and also one individual resulted seropositive. Thus, 37 (17.2%) of the 215 studied rams were infected with Ma. The specimens which proportionally yielded the greatest number of positive results for this pathogen were semen samples (13.6%), followed by nasal swabs (5.8%). In contrast, the sampling of the external auricular canal and the serological analyses resulted insufficient to effectively detect infected individuals. Asymptomatic rams infected with Ma were detected in all the analyzed artificial insemination centers, highlighting the need to implement adequate surveillance protocols to prevent the presence of these individuals in these centers, reducing the risk of transmitting contagious agalactia.

1. Introduction

Mycoplasma agalactiae (Ma) is the main causative agent of ovine contagious agalactia (CA). This disease is one of the most important affecting small ruminants because of its economic impact, especially in dairy herds. Notwithstanding, CA is endemic in most countries of the Mediterranean basin, and thus, its most common clinical situation consists of chronically infected herds with no signs of disease. Moreover, it has been demonstrated that both diseased and asymptomatic animals continue to shed Ma for long periods of time. These nonapparent infected individuals are responsible for maintaining and spreading this infection, not only within but also between different herds and thus, imply an epidemiological risk regarding the transmission of CA [1–3].

Previous works have assessed the presence of asymptomatic infected bucks in artificial insemination (AI) centers of CA-endemic areas, revealing that one of the most effective tools to detect these individuals was through

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the sampling of their external auricular canal (EAC) [4,5]. These findings motivated the implementation of control measures to prevent the admission and ulterior presence of unapparent infected males in these centers. Furthermore, the anatomic location and associated excretion routes of Ma in naturally infected goat bucks have also been studied, demonstrating that, apart from the mammary route, which is the main transmission route of CA, systemic infection enables mycoplasmas to be present at other anatomic locations such as the respiratory and reproductive tracts and the central nervous system, even without showing apparent symptoms [6]. However, few studies are available about the excretion routes of Ma in sheep and most of the available descriptions have been made after experimental inoculations of this agent. So, Ma has been retrieved from several distant internal organs, including lymph nodes, lungs, uterus, and brain, after being inoculated through the mammary canal of lactating sheep [7]. Otherwise, Ma was detected in milk samples, blood samples, and nasal and auricular swabs taken from nasally inoculated sheep [8], and also from the conjunctiva, nasal mucosa, spleen and lymph nodes of lambs after being inoculated conjunctivally [9]. In addition, Ma was also retrieved from the mammary gland and milk of conjunctivally inoculated lactating ewes [10]. Concerning experimental studies carried out on rams, Ma has been isolated from the reproductive tract and semen of inoculated individuals, which did not show apparent symptoms associated with CA [11]. Thus, these works demonstrate the capability of Ma to disseminate to distant body sites and its associated excretion routes, yet most of these findings have still not been demonstrated in natural conditions.

Therefore, the aim of the present work was to analyze different samples to detect the presence of asymptomatic rams infected with Ma in AI centers of a CA-endemic area and to determine the most effective way to diagnose these individuals with regard to the implementation of adequate control measures against this infection.

2. Materials and methods

2.1. Study population and design

In this study, a total of 215 rams housed in three AI centers of a CA-endemic area were sampled, taking two auricular swabs (left and right), two nasal swabs (left and right), and a serum sample from each individual (n = 5). From 147 of these rams, a semen sample was also collected. All the studied animals met the conditions set out in the Commission Regulation (EU) No 176/2010 on ovine semen donors, and none showed clinical signs associated with CA at the moment of sampling [12]. In addition, 14 teaser sheep from two of these AI centers were also sampled taking auricular, nasal, and vaginal swabs, and serum (n = 6). To detect Ma, specific culture, molecular, and serological techniques were performed.

2.2. Mycoplasma cultures

All the specimens except serum were immediately refrigerated (4 °C) and processed in the laboratory before 24 hours after collection. Nasal, auricular, and vaginal swabs and semen samples were cultured in liquid and solid PH media [6], and incubated at 37 °C in a 5% CO_2 humid atmosphere for 15 days before being considered as negative. With positive cultures, isolates from previously cloned single colonies were used for final identification, performed by polymerase chain reaction (PCR).

2.3. DNA extraction and PCR

DNA was extracted from 200 μL of culture aliquots from nasal, auricular, and vaginal swabs and from 200 μL of ejaculates using a High Pure PCR Template Preparation Kit (Roche Diagnostics, Spain), following the manufacturer's instructions. Subsequently, Ma was detected using a specific PCR protocol [13]. Other mycoplasma species were identified through the amplification and posterior-purified PCR product sequencing of a 1492 bp fragment of their 16S-ribosomal RNA gene [14]. The obtained sequences were then compared with the nucleotide database basic local alignment search tool [15] (http://blast.ncbi.nlm.nih.gov/Blast.cgi). In addition, isolates identified as *M arginini* and *M ovipneumoniae* were confirmed by PCR [16,17]. Primers and PCR protocols used are available at Supplementary Material S1.

2.4. Serological analysis

Serum samples (n = 215) were subjected to ELISA (IDEXX *M agalactiae* Verification Ab, P00400-10, France) to detect specific antibodies against Ma, following the manufacturer's instructions.

2.5. Statistical analysis

Individuals were considered as infected with Ma when this agent was detected in at least one of the analyzed samples and/or when a positive result for serology was obtained. The frequencies of Ma positive results for each sample type were compared by Chi-square tests.

3. Results

A total of 96 of the 1077 analyzed samples tested positive for *Mycoplasma* spp. (Table 1). Regarding the different anatomic locations studied, the specimens which yielded the highest number positive results were the nasal swabs (66 of 458), in which different mycoplasma species such as *M arginini* (Marg), *M ovipneumoniae,* and *M bovigenitalium* were identified. In addition, Ma and Marg were isolated from semen samples, vaginal swabs, and auricular swabs, as shown in Table 2.

In this study, 37 (17.2%) of the 215 analyzed rams, which were housed in the three studied AI centers, were infected with Ma. However, this pathogen was not isolated from any of the 14 teaser sheep. Concerning the identification of asymptomatic rams infected with Ma, 47 of the 1077 samples analyzed resulted positive. Hence, in some of these positive rams, Ma was detected in more than one sample. Specifically, nine individuals yielded two positive results, as in eight individuals Ma was detected in their nasal mucosae