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Presence of *Mycoplasma agalactiae* in semen of naturally infected asymptomatic rams

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ABSTRACT

The purpose of the present study was to assess the presence of *Mycoplasma agalactiae* (Ma), the main causative agent of ovine contagious agalactia (CA), in semen of naturally infected rams. Therefore, semen samples from 167 rams residing in three different artificial insemination (AI) centers of a CA–endemic area were studied by microbiological and molecular techniques. In addition, serial ejaculates from the same rams were evaluated to determine the excretion dynamics of Ma. Of the 384 samples studied, Ma was detected in 56 (14.58%) which belonged to 44 different rams (26.35%). These findings confirm the ability of Ma to be excreted in semen of asymptomatic rams. Furthermore, these results also evidence the presence of these asymptomatic carriers of Ma in ovine AI centers, representing a serious health risk regarding the spread and maintenance of CA, especially in endemic areas. Moreover, the excretion of Ma in semen also points to the risk of venereal transmission of this disease. The current results highlight the need to implement control measures to prevent the admission of infected rams in AI centers and the necessity to continuously monitor semen samples to effectively detect infected individuals.

1. Introduction

Mycoplasma agalactiae (Ma) is the main causative agent of ovine contagious agalactia (CA), one of the most significant diseases affecting dairy small ruminants, which is endemic in nearly all countries of the Mediterranean basin. Therefore, most of the herds in these areas are chronically infected and usually do not show the characteristic triad of symptoms caused by this disease in its acute form. Only mastitis, which is often subclinical, is habitually shown by affected animals [1].

Contagious agalactia–causing mycoplasmas are commonly transmitted through the mammary route although systemic infection enables these bacteria to be present in other anatomic locations, explaining the existence of different transmission routes [2]. In this sense, in the last years there have been advances in the understanding of the epidemiological role of goat bucks, which include studies which have assessed the anatomic location and associated excretion routes of CA–causing mycoplasmas in asymptomatic carriers [3]. Moreover, other works have assessed the presence of these inapparent carriers in artificial insemination (AI) centers. These studies revealed the occurrence of subclinically infected male goats, which carried Ma and *Mycoplasma mycoides* subspecies *capri* (Mmc) mainly in their external auricular canal and also described the spread of this infection within the studied breeding centers [4]. In addition, the intermittent excretion of Ma and Mmc in semen has been reported in naturally infected goat bucks, pointing to the risk of venereal transmission of CA. Hence,

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these findings confirm the role of males as a permanent reservoir of CA–causing mycoplasmas, which implies that these individuals represent a health risk regarding the spread and maintenance of this disease in endemic areas [5–7].

However, contrary to the situation in caprine, the information available about the different excretion routes of Ma in rams has scarcely been reported, and the information available in the literature describes findings acquired after experimental inoculations of the agent. Thus, Or et al. [8] isolated Ma from semen of experimentally infected rams between the 7th and 15th day after inoculation, reporting the ability of this mycoplasma to colonize the ovine reproductive tract in experimental conditions without causing any specific CA symptoms. Still, these findings have not been reported in natural conditions. Hence, in view of the outcomes of the experimental inoculation of Ma, our hypothesis considers the ability of this pathogen to colonize the reproductive tract and to be present in semen of naturally infected asymptomatic rams.

Consequently, the aim of the present study was to assess the presence and excretion dynamics of Ma in semen of naturally infected asymptomatic rams. Therefore, semen samples from rams residing in three different ovine AI centers were analyzed by microbiological and molecular techniques.

2. Materials and methods

2.1. Study population and design

Semen samples (n = 384) were collected over 9 months from 167 rams admitted to three AI centers. Of the total 167 rams, 130 were sampled at least two times throughout this period, obtaining ejaculates at different time points (quarterly) to assess the excretion dynamics of Ma. These animals met the conditions set out in the Commission Regulation (EU) No 176/2010 on ovine semen donors and none reported clinical signs associated with CA at the moment of sampling [9].

2.2. Semen collection

Semen samples were collected in sterile vials (15 mL) using the artificial vagina method after inducing the erection of the penis with a teaser sheep. These samples were immediately refrigerated (4 °C) and transported to the laboratory, where they were processed before 24 hours after collection. All ejaculates were analyzed to detect Ma by culture and polymerase chain reaction (PCR).

2.3. Mycoplasma cultures

After homogenizing each ejaculate, 200 μL of semen were inoculated in solid and liquid PH media [3] 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 PCR.

2.4. DNA extraction and PCR

DNA was extracted from 200 μL of semen and *Mycoplasma* spp. positive cultures using a High Pure PCR Template Preparation Kit (Roche Diagnostics, Spain), following the manufacturer's instructions. Subsequently, Ma was detected using a specific PCR procedure [10], applying previously described conditions [5].

2.5. Detection limit

To assess the detection limit for Ma after applying the DNA extraction and specific PCR protocol used in this study, duplicate semen samples were inoculated with serial fold dilutions in the range 10 to 10^8 of a PG2 inoculum (reference strain of Ma NCTC 10123), and negative controls were included.

2.6. Statistical analysis

To determine the infection status of the studied rams regarding the number of samplings realized, the sensitivity and negative predictive values for each sampling strategy were estimated using Win Episcope 2.0 software [11]. In this sense, a ram was considered as positive when Ma was detected by PCR or culture in at least one semen sample.

3. Results

Of the 384 semen samples analyzed, 56 (14.58%) tested positive for Ma. Fifty-one of these positive samples were detected through PCR, whereas the microbiological analysis merely allowed the detection of Ma in three of the studied samples (Table 1). Besides, 21 (5.47%) of the 384 samples which tested positive for *Mycoplasma* spp. by culture were subsequently identified by PCR as *Mycoplasma arginini*. The 56 semen samples in which Ma was detected belonged to 44 of the 167 rams examined, which corresponds to a prevalence of 26.35%. Moreover, the prevalence data obtained in each AI center range from 16.00% to 32.20% (Table 1). In 33 (75%) of the positive rams, the presence of Ma in semen was detected only once, whereas in 11 rams (25%) Ma was identified repeatedly. Regarding the validity parameters for the number of serial samplings carried out, the sensitivity values obtained increased from 6.8% to 81.8% between one and three sampling points, as is shown in Table 2.

As for the detection limit of Ma in semen, the specific DNA extraction and PCR procedures used allowed the detection of samples which yielded between 10^5 and 10^6 ufc/mL.

4. Discussion

Our results provide the first evidence of the presence of Ma in semen of naturally infected asymptomatic rams. This had only been reported after the experimental inoculation of Ma, in which it was also reported that this pathogen is able not only to colonize the reproductive tract of rams but also to cause degenerative lesions at the testes and seminal vesicles without showing any apparent symptoms.