

Short Communication

Detection of *Ureaplasma* spp. in semen samples from sheep in Brazil

Sandra Batista dos Santos¹, Orestes Luiz de Souza Neto¹, Pedro Paulo Feitosa de Albuquerque¹, André da Rocha Mota¹, Pomy de Cássia Peixoto Kim¹, Érica Paes Barreto Xavier de Moraes¹, Elmiro Rosendo do Nascimento², Rinaldo Aparecido Mota¹

¹Department of Veterinary Medicine, Federal Rural University of Pernambuco, Pernambuco, Recife, Brazil.

²Department of Veterinary Medicine and Public Health, Veterinary Medicine School, Federal Fluminense University, Rio de Janeiro, Niterói, Brazil.

Submitted: February 10, 2012; Approved: November 13, 2012.

Abstract

A study was conducted to verify the presence of mycoplasmas and ureaplasmas DNA in sheep semen samples from the State of Pernambuco. The PCR assay was conducted according with standard protocols with generic primers. *Mollicutes* DNA was detected in 26.0% and *Ureaplasma* spp. in 12.0% of semen samples.

Key words: mycoplasmas, reproduction diseases, semen, sheep.

Mycoplasmas are members of the class of *Mollicutes*, which are the smallest known prokaryotes with the capacity to self-replicate (Razin and Tully, 1996; Blanchard and Browning, 2005). These mollicutes are found in nature as parasites, endosymbionts, commensals and saprophytes in animals, plants and insects (Whitcomb and Bové, 1983; Garnier *et al.*, 2001). In ruminants, mycoplasmas can colonize different sites, including the urogenital tract, the respiratory system, the mammary glands, the joints, the ocular mucous, the placenta and the ear canals (Rodríguez *et al.*, 1996; Razin *et al.*, 1998). The main mycoplasma species commonly found in goats and sheep are: *Mycoplasma agalactiae*, *M. conjunctivae*, *M. putrefaciens* and several taxa from the *Mycoplasma mycoides* cluster (MMC), such as *Mycoplasma mycoides* subspecies *capri* (*Mmc*), *Mycoplasma capricolum* subspecies *capripneumoniae* (*Mccp*), *Mycoplasma capricolum* subspecies *capricolum* (*Mcc*) (Manso-Silvan *et al.*, 2009). In small ruminants mycoplasmosis is associated with various symptoms including mastitis, arthritis, keratoconjunctivitis, pneumonia and septicemia (Thiaucourt and Bolske, 1996). In Brazil, *M. agalactiae* and *Mmc* have been reported from contagious agalactia episodes (Nascimento *et al.*, 1986; Nascimento *et al.*, 1990; Azevedo *et al.*, 2006) and *M. conjunctivae* from outbreaks of keratoconjunctivitis in sheep

or in asymptomatic animals (Neto *et al.*, 2004). Mycoplasmas and ureaplasmas are recognized as some of the most important bacterial agents causing reproductive disorders (Junqueira and Alfieri, 2006). In cattle, goats and sheep, the main related reproductive problems are granular vulvovaginitis, endometritis, being repeatedly on heat, premature birth and miscarriage. In males, the main problems are infertility, a reduction in the frequency of intercourse, balanoposthitis, epididymitis, seminal vesiculitis, orchitis, and pathologies responsible for morphological and functional changes in spermatozooids (Singh *et al.*, 1974; Jones 1983; Kirkbride 1987; DaMassa *et al.*, 1992; Trichard *et al.*, 1993; Cardoso *et al.*, 2000; Gil *et al.*, 2003; Junqueira and Alfieri, 2006; Oliveira 2008). The mycoplasmas species involved in these cases in sheep and goats are *M. agalactiae*, *M. putrefaciens* and *Ureaplasma* spp. (Jones 1983; DaMassa *et al.*, 1992; Azevedo *et al.*, 2006; Corrales *et al.*, 2007; Oliveira 2008; De La Fe *et al.*, 2009), in addition to some species belonging to the *Mycoplasma mycoides* cluster (MMC) (Nascimento *et al.*, 1990; Neto *et al.*, 2004). *Mycoplasma* spp. and *Ureaplasma* spp. are also frequently found in animals showing no reproductive disorders (McCaughy and Ball, 1981; De La Fe *et al.*, 2009; Rizzo *et al.*, 2011), which may facilitate the dissemination of these agents among free herds, especially through the use

of contaminated semen and the transfer of embryos (De La Fe *et al.*, 2009; Rizzo *et al.*, 2011). Studies of mycoplasmas that affect the reproductive system of sheep and goats in Brazil are rare and the true epidemiological situation of this disease and the risk factors associated with these reproductive disorders are unknown, especially in the Northeast region of Brazil, which is the main sheep and goat producing area. The aim of this study is thus to detect the presence of mycoplasmas and ureaplasmas DNA in samples of semen from male sheep using PCR.

This study used 50 male sheep of differing breeds and ages from properties located in the State of Pernambuco, in the Northeast region of Brazil. The animals underwent a clinical examination and semen samples were collected using the artificial vagina method. The sperm was tested for macro and microscopic characteristics, in accordance with the guidelines of the Brazilian College of Animal Reproduction (CBRA) (1998). A quantity of 1.5 mL of semen was used for DNA extraction using a commercially available kit^a, according to the manufacturer's instructions. PCR for mollicutes was carried out in a mix with a final volume of 25 μ L containing: 5 μ L of buffer [10 mM of Tris-HCl, pH 8.3], 30 pmol of each "primer", 25 mM of MgCl₂, 50 μ M of each dinucleotide, 2.5 units (U) of Taq DNA polymerase^b, Mili-Q ultrapure water and 5 μ L of DNA mold. Amplification reactions were carried out in a thermocycler^c and the thermal profile was based on the protocol described in the literature, with primers based on conserved regions of V6 and V7 of the 16SrRNA gene (Van Kuppeveld *et al.*, 1992; Van Kuppeveld *et al.*, 1994). The PCR for *Ureaplasma* spp. was carried out on a final volume of 25 μ L containing: 5 μ L of DNA; the already described primers by Lauerman (1998) (UGP-F' and UGP-R') at 30 pmol; Mili-Q ultrapure water and 6.25 μ L of TopTaq Mastermix^d in accordance with the manufacturer's instructions. The thermal profile of the reactions was obtained using the standard protocol (Lauerman 1998). *Mycoplasma mycoides mycoides* (strain GM12) and *Ureaplasma diversum* (strain GMU132) were used as positive controls during PCR reactions for *Mycoplasma* and *Ureaplasma* detection, respectively. The amplified DNA was visualized by electrophoresis in 1.5% agarose gel with 100 bp molecular weight marker^e, colored with Bluegreen^f, viewed under ultraviolet light and photodocumented. The semen samples that were positive by PCR were cultured in medium specific to *Mycoplasma* and *Ureaplasma* (modified Hayflicks and the "U" medium), respectively (Razin and Tully, 1996; Whitford *et al.*, 1994). The plates and broth were incubated at 37 °C under microaerophilic conditions for up to 21 days and examined daily for colonies under a stereoscopic microscope (40X). In this study, none of the animals examined showed any clinical signs of reproductive disease and 100% exhibited no alteration in sperm and were considered suitable for reproduction. Of the 50 semen samples submitted to PCR, 26.0% (13/50) tested positive for *Mollicutes*

and 12.0% (6/50) for *Ureaplasma* spp. The amplicons obtained were 280 and 640 base pair, respectively.

It was not possible to identify the two agents in isolation, because, despite the selectivity of the media used for *Mycoplasma* and *Ureaplasma*, the samples were contaminated by fungi, making it impossible to confirm the viability of the agents. However, the presence of *Ureaplasma* DNA, as detected by PCR in the semen samples from the sheep under investigation shows that these agents can be carried among animals from infected and disease-free flocks. It should be noted that the sheep used showed no symptoms of reproductive disease, which could contribute to the dissemination of the two agents, since, in these cases, the rams passed the andrological examination and were used for artificial insemination. The presence of asymptomatic animals has been reported as a risk factor for outbreaks mycoplasmosis, such as those caused by *Mycoplasma agalactiae* in the Southeast and Northeast of Brazil (Nascimento *et al.*, 1986; Nascimento *et al.*, 1990; Gregory *et al.*, 2004; Neto *et al.*, 2004; Azevedo *et al.*, 2006; Oliveira 2008) and in some regions in Spain (Corrales *et al.*, 2007; De La Fe *et al.*, 2009). In these cases *M. agalactiae* was found in goats without reproductive disorders. The frequency of *Mollicutes* and *Ureaplasma* detected in this study is similar to that reported in other regions of Brazil in the semen of sheep and goats (Oliveira 2008; Rizzo *et al.*, 2011). In the present study, even though the frequency of the detection of *Ureaplasma* was low, this agent should be considered a pathogen that is important for the reproduction of sheep in the Northeast region of Brazil, since it is known that both *Mycoplasma* and *Ureaplasma* are sexually transmitted and it has been shown that this is through the semen (Mccaughey and Ball, 1981; Livingston Jr and Gauer, 1982; Corrales *et al.*, 2007; De La Fe *et al.*, 2009; Rizzo *et al.*, 2011). Mycoplasmosis in sheep and goats have still not been studied extensively in Brazil and, like reproductive disease, are often overlooked. The elimination of *Ureaplasma* from the semen of animals without symptoms should draw attention to the epidemiological role of these animals, which can act as a reservoir of the agents, which may also hinder programs to control diseases, such as Contagious Agalactia of Sheep and Goats, which is endemic in the region. Furthermore, the constant use of biotechnology in reproduction to improve flocks of sheep and goats genetically may contribute to the dissemination of these agents. In order to identify the *Mycoplasma* and *Ureaplasma* species present in the semen samples, and document their clinical significance, specific PCR assays are being currently performed in the laboratory.

Sources and Manufacturers

a. DNA Easy Blood and Tissues Kit[®] (catalog no. 69506), Qiagen Biotechnology Brazil Ltda., São Paulo, Brazil.

- b. TopTaq DNA Polymerase (50)[®] (catalog number 200201), Qiagen Biotechnology Brazil Ltda., São Paulo, Brazil.
- c. Bioer XP cycler[®], Bioer Technology, Hangzhou, China.
- d. TopTaq Master Mix Kit[®] (catalog no.200403), Qiagen Biotechnology Brazil Ltda., São Paulo, Brazil.
- e. Amresco[®], Ready Ladder 100 bp DNA Marker (no.550), Life Science Research Products & Biochemicals OH, USA.
- f. LGC Biotechnology Ltda. (LGC[®]), Cotia, São Paulo, Brazil.

Declaration of Conflicting Interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Research Ethics Committee

This project was approved by the ethics committee of the UFRPE/DMV.

Acknowledgments

This work was supported by the Conselho Nacional de Desenvolvimento Científico e Tecnológico - CNPq (MCT/CNPq) and Fundação de Amparo à Ciência e Tecnologia do Estado de Pernambuco - FACEPE (Pronem no APQ-1226-5.05/10; no APQ 1512-5.05/10).

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