CONTAGIOUS AGALACTIA BY Mycoplasma agalactiae IN SMALL RUMINANTS IN BRAZIL: FIRST REPORT

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ABSTRACT

Two outbreaks of contagious agalactia by Mycoplasma agalactiae occurred in Paraíba State, Northeastern Region of Brazil are reported. The disease was characterized by mastitis, agalactia and polyarthritis in does and polyarthritis and conjunctivitis in kids and lambs. Fever and anorexia were also observed. Morbidity was from 26.1% to 100% in does, 36.5 to 100% in kids and 49% in lambs. In one farm 14.3% of the lactating goats and 6.4% of the kids died or were euthanized. In the other, 3.3% of the does, 36.5% of the kids and 22.9% of the lambs died and 84 affected goats were euthanized to control the disease. M. agalactiae was isolated from milk, joint exudates, nasal swabs and ear washings. The colonies were characteristic of Mycoplasma and the agent did not ferment both glucose and arginin. It was typed as Mycoplasma agalactiae by immunoperoxidase and PCR. This is the first report of M. agalactiae infection in Brazil, but the source of the infection remains unknown.

Key words: contagious agalactia, Mycoplasma agalactiae, small ruminants

INTRODUCTION

Mycoplasma agalactiae causing contagious agalactia (ACOC) was the first Mycoplasma isolated from sheep and goats (19). Other important Mycoplasma affecting goats are M. capricolum subsp capricolum which causes contagious caprine pleuropneumonia (CCPP), M. conjunctivae, M. arginini, M. mycoides subsp mycoides and M. mycoides subsp capri (14,20,28).

ACOC occurs in Europe, Asia and Africa, mainly in the countries around the Mediterranean (11,12,25,28) causing important losses due to decreased milk production, death of animals and cost of treatment and prevention. The disease is characterized by mastitis, agalactia, polyarthritis, keratoconjunctivitis and occasionally, abortion and pneumonia. The main agent of ACOC is Mycoplasma agalactiae, although M. capricolum subsp capricolum, M. mycoides subsp mycoides (large colony), M. putrfaciens and M. mycoides subsp capri can also cause the disease (13,19). Goats infected by M. mycoides subsp capri and M mycoides subsp. mycoides LC can have mastitis and pneumonia and arthritis (17,28).

The incubation period of infection by M. agalactiae in goats and sheep is one to eight weeks. Initially, affected animals are depressive, anorectics and pyrexia, followed by sudden drop of milk production, mastitis, agalactia, and polyarthritis mainly in the carpal and tarsal joints. Pneumonia and abortion can also occur in chronically infected animals (29). M. agalactiae and other mycoplasmas can be isolated from the external auditory duct and from Raillietia caprae or Psoroptes cunicul that parasites wich can be found in the the outer ear of sheep and goats and have been involved in the maintenance of the infection in carrier animals (6).

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In Brazil ACOC was reported by the first time in 1942 in the state of São Paulo, however the isolated Mycoplasma was not identified (22). Pneumonia, keratoconjunctivitis and arthritis caused by organisms related to M. mycoides subsp mycoides and M. mycoides subsp capri have been reported (21). Mycoplasma spp and M. arginini have been isolated from goats with polyarthritis and pneumonia, respectively (4,14). M. mycoides subsp mycoides SC (small colony) have been recovered from the external auditory meatus of clinically normal goats (23). M. conjunctivae have been identified from sheep and goats with and without clinical signs of keratoconjunctivitis (1,15).

The aim of this paper is to report contagious agalactia by Mycoplasma agalactiae in goats and sheep for the first time in Brazil.

**MATERIAL AND METHODS**

**Animals, clinical signs and pathlogy**

The two outbreaks occurred in the state of Paraíba, Northeastern of Brazil. The first one occurred from August to November 2001, and the second from December 2001 to January 2002. From 265 animals of the first affected herd (British Alpine, Anglo Nubian and Brown Alpine), 109 were lactating kids and 156 adult does, including 89 lactating does with a daily mean milk production of 2.1kg. In this farm the kids were separated from does immediately after parturition. The second affected herd had 172 adult does and 8 adult males that included Saanen, Toggenburg and Anglo Nubian, recently acquired in different commercial dairy goats herds from the states of São Paulo, Minas Gerais and Pernambuco to start milk production. This farm had also animals for meat production, including 52 Boer goats and 200 Santa Inês and Dorper sheeps kept in an intensive production system and 1500 sheep in a semi-intensive system.

Affected animals were clinically examined, mainly in relation to respiratory and locomotor systems, udder and eyes. Four kids and four goats severely affected were euthanized and necropsied. Samples of joint capsule, udder, lung, heart, spleen, lymphnodes, kidney and central nervous system were fixed in 10% buffered formalin, embedded in paraffin, cut at six µm, and stained by hematoxylin-eosin.

**Isolation and identification of Mycoplasma**

The clinical specimens used in this study are listed in Table 1. The nasal swabs were placed in tubes with the cotton part immersed in approximately 2 ml of a solution with 50% glycerol in PBS (PBS-G). Joint exudates, milk, and washings with mites from the external ear were homogenized (v/v) with PBS-G. Then in PBS-G the specimens were shipped in ice boxes to the laboratory and stored in freezer at -20ºC. Samples of joint capsule, udder, lung, heart, spleen, lymphnodes, kidney and central nervous system were fixed in 10% buffered formalin, embedded in paraffin, cut at six µm, and stained by hematoxylin-eosin.

<table>
<thead>
<tr>
<th>Specie</th>
<th>Milk</th>
<th>Joint exudate</th>
<th>Nasal swabs</th>
<th>External ear washings</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caprine</td>
<td>11</td>
<td>08</td>
<td>06</td>
<td>29</td>
<td>54</td>
</tr>
<tr>
<td>Ovine</td>
<td>-</td>
<td>-</td>
<td>16</td>
<td>37</td>
<td>53</td>
</tr>
</tbody>
</table>

Table 1. Clinical specimens used for *Mycoplasma* isolation.

a fried egg aspect. Fragments of agar with the colonies were transferred to tubes containing 3ml liquid Hayflick medium, which were incubated at 37ºC for 72-96h. Then, 300 µl of the diluted (10^3 to 10^6) culture were re-inculated in plates with solid medium to isolate colonies, observe films and spots production and perform Dienes stain (10).

**Indirect immunoperoxidase test**

Areas of typical colonies were used for typification by the indirect immunoperoxidase test reported by Imada et al. (16) with some modifications. The incubation period was prolonged from one to four hours during the phase of antiserum adsorption and from one to three hours in the phase of reaction with the conjugate. Immediately before using the serum was diluted (1:20) in TBS (50 mM Trizma and 100 mM NaCl at pH 7.4), and the conjugate diluted (1:50) in TBBS (TBS with 1% of bovine serum albumine). Initially, paper discs saturated with 25 µl of hyperimmune rabbit seraums against *M. agalactiae*, *M. capricolum*, *M. putrefaciens*, and *M. arginini* were placed over the colonies in solid media and incubated at 37ºC for four hours. Then, the discs were replaced by new discs previously embedded in 25 µl of anti-rabbit conjugate labeled with peroxidase and incubated for three hours at 37ºC. After incubation, each plate was washed in 10 ml washing buffer (100 ml TBS plus 2.0 ml equine serum plus 50 µl tween 20) and immediately revealed by the addition of a solution (10 ml of methanol plus 30 mg 4-cloro-1-naphtol plus 50 µl TBS plus 30 µl H2O2). This revelation was performed at room temperature and protected from light for 30 min. It was observed a stereoscopic microscope and considered positives the colonies with brown discoloration.

**Polymerase Chain Reaction**

DNA samples for PCR were prepared by phenol-chloroform method. Briefly, one ml culture were centrifuged at 20,000 x g for 20 min at 4ºC. The pellets were then re-suspended in 400 µl of lysis buffer [Dextrose-EDTA buffer, pH 8.4, plus 30 µl of proteinase K (240 µg/ml) plus 30 µl of SDS-Sodium Dodecyl Sulphate, 10%], and incubated for 30min at 50ºC. After fast cooling it was carried out double extraction with 500 µl of phenol (pH 7.8) followed by one extraction with 500 µl of chloroform and addition of 100 µl of ethanol to the final aqueous phase and stored overnight at -20ºC. Samples were centrifuged at 20,000 x g for 10min at 4ºC and the pellets were re-suspended
in 100 µl TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). For \textit{M. agalactiae}-specific amplification the primers set 5'-CCT TTT TTT AGATGGA TAG CGG ATG - 3' and 5'- CCG TCA AGG TAG CGT CAT TTC CTA C - 3' derived from the \textit{Mycoplasma agalactiae} PG2 strain were used (GenBank accession number U44763) as published by Chávez González et al. (5). The PCR reaction was conducted using thermocycler (mod. PTC-100, MJ Research). The reaction volume was 100 µl, with 59 µl of MilliQ water, 10 µl PCR buffer (10x), 5 µl MgCl$_2$, 5 µl of each dNTP mix (0.25 mM), 2 µl of each primer, 15 µl DNA extracts, 2U Taq Polymerase and 50 µl of mineral oil to overlay the mixture. The PCR was performed by using one cycle at 95ºC for 5min, the denature step followed by 40 cycles of denaturation at 94ºC for 1min, annealing at 57ºC for 1min, extension at 68ºC for 1min, and finally an extra extension at 70ºC for 10min. Bands were run and analyzed on 2% agarose stained with ethidium bromide (0.5pg/ml) and visualized under ultraviolet light after electrophoresis.

**RESULTS**

**Epidemiology, clinical signs and pathology**

In the first affected herd clinical signs included observed mastitis and agalactia in four goats, characterized by flaccid udder with aqueous secretion with presence of clots. Five days later, 21 other goats had mastitis with arthritis and fever. At the same time, 21 kids had fever lameness with enlarged and painful joints, mainly in the forelegs, and difficult gait with rigidity and dorsal flexion. After two weeks, all the 89 milking goats presented mastitis and agalactia. There was a fall of 90% of milk yield and milking was suspended. In a period of 120 days, 43 out of 89 goats (48.3%) had also arthritis or polyarthritis. Affected goats were treated with 20 mg/kg tylosin daily, during five days. Most animals recovered, although seven were euthanized and seven died spontaneously. At the same time all kids were affected and some of them were treated in the same way. Four of the 109 kids (3.7%) died and three (2.7%) were euthanized. No cases were observed in the group of non lactating does, which was separated from the affected goats at the start of the outbreak.

In the other herd, three weeks after the first parturition, two lactating goats had anorexia, mastitis, agalactia and arthritis, and six kids and eight lambs had arthritis, conjunctivitis, fever and anorexia. In 15 days 40/63 (63.5%) kids, 30/61 (49%) lambs, and 18/180 (10%) goats were affected. Twenty three (36.5%) kids, 14 (22.9%) lambs, and six (3.3%) goats died. In the same period 84 adult non lactating goats were separated from the flock as a control measure. These animals were not affected. Thirty days after the initiation of the outbreak 88 adult goats and eight bucks from the dairy goat herd were euthanized for control measure. Thirty seven of these had arthritis, mastitis and/or conjunctivitis, and 51 had no clinical signs. No clinical cases were observed in adult Boer goat. Among adult sheep, only two had arthritis and anorexia, but recovered after treatment with tylosin as described above.

At necropsies tumefaction of the joint capsule with presence within the joint of yellow viscous exudates with fibrin were observed in the animals with arthritis. On the histologic examination the joint capsule had infiltration of neutrophils, areas of necrosis, edema, and occasionally microabscesses. In one kid the spleen had diffuse necrosis of the white pulp, but the germinative centers were not affected. Another kid had an area of consolidation in the right apical lobe. Histologically, this lesion was characterized by interstitial pneumonia with mononuclear infiltration. The mammary gland contained yellow exudates with clots. Histologically, the gland had severe infiltration of mononuclear cells and neutrophils in the connective tissue, atrophy of the secretor ducts, and occasionally desquamation of the epithelial cells. Eosinophilic contents and cellular debris were observed within the ducts (Fig. 1).

**Isolation and identification of \textit{Mycoplasma agalactiae}**

\textit{Mycoplasma} positive to the Dienes test were isolated from joint exudates of two does, one kid and one lamb with polyarthritis, milk samples of four does with mastitis, nasal swabs from four kids without respiratory signs, and the ear washings of one pregnant does, one buck with arthritis, and two apparently healthy adult sheep. All isolates showed transparent colonies with fried egg appearance, and yielded films and spots on solid medium.

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**Figure 1.** Udder. Goat infected by \textit{M. agalactiae}. There is severe infiltration by mononuclear cells and neutrophils in the interstitial tissue. Eosinophilic exudates is observed within the ducts, and in some ducts the epithelium is desquamated (arrow). HE, x 100.
Hayflick medium supplemented with equine serum. The bacteria did not fermented glucose and arginin.

Agar blocks with typic *Mycoplasma* colonies from all culture species, when submitted to immunoperoxidase test, exhibited positive reactions only to *M. agalactiae*.

On the PCR test, all isolates showed specific amplicon at 360 bp to *M. agalactiae* (Fig. 2).

*Psoroptes cuniculi* was observed on ear washings of 68% (20/29) goats, and 37.8% (14/37) sheep.

**DISCUSSION**

The results reported in this paper confirmed for the first time in Brazil the occurrence of *Mycoplasma agalactiae* as an agent of AOCO. Agalactia and lameness were reported in São Paulo in a goat that died without significant lesions some months after been affected. Some kids from the same herd died with pulmonary congestion and at necropsy one of them had hyperemic foci in the lung. Microorganism with characteristics of *Mycoplasma* was isolated from the goat, and the bacterium was inoculated in kids causing lameness, enlargement of the prescapular lymph nodes, and death in five days. At necropsy, hyperemic foci were observed on the lung and gut (23). ACOC caused by *M. agalactiae* occurs in countries with intensive rearing of sheep and goats (3,7,11,12,18,25,30).

In these outbreaks the identification of the isolates was performed by their culture characteristics as well as by the immunoperoxidase test and PCR. PCR is faster than the traditional tests and it can be used as confirmatory test in the diagnosis of ACOC, even after storage of the samples at -20ºC for 24 months (30). The high morbidity of the disease (26.1% to 100% of the goats, 36.5 to 100% of kids, and 49% of lambs), suggests that the disease was introduced in farms where the *M. agalactiae* did not exist previously. After the occurrence of these outbreaks, the disease spread to Paraíba, Rio Grande do Norte, and Pernambuco States causing important economical losses. However, after the introduction of the agent in the farms the disease became enzootic with sporadic cases (unpublished data). These features strongly suggest that *M. agalactiae* was introduced in the Northeastern region and probably in Brazil, where the disease had not been reported. The source of the infection remains unknown, but until 1988 the farm where the first outbreak occurred imported goats and sheep from other countries, and the other farm bought many animals from the states of São Paulo, Minas Gerais and Pernambuco to start dairy production.

The infection was probably introduced by carrier animals, which are very important in epidemiology of the disease. In this study the role of carrier animals is suggested by the isolation of *M. agalactiae* after five days of treatment with tylosin, and also from the ear washings of sheep and goats infected by *Psoroptes cuniculi*. In other countries *P. cuniculi* is highly prevalent in goats and *M. agalactiae* has been isolated from this acarid (6,8). Other acarid, *Railetia caprae* that also can be a carrier of *Mycoplasma* spp is the most prevalent in other Brazilian regions (26).

The main clinical signs observed in the outbreaks reported in this paper were mastitis and agalactia in the does, and polyarthritis in kids and lambs, but numerous does had also polyarthritis. The high frequency of mastitis and agalactia in goats with some of them having arthritis is highly suggestive of infection by *M. agalactiae*. Other causes of mastitis are commonly observed in goats in Northeastern Brazil, but without the high frequency of agalactia and arthritis observed in these outbreaks. To EGWO *et al.* (11) the observation of agalactia is suggestive of *M. agalactiae* infection. In non milking goats other common causes of arthritis can be confused with infection by *M. agalactiae*, including other *Mycoplasma* and bacterial infections, virus infections and traumatic injuries, which are common in young animals, but the high frequency of polyarthritis is suggestive of *Mycoplasma* infection. In cases of arthritis by *M. agalactiae*, the thickening of the joint capsule, the liquid aspect of the exudates, the absence of umbilical lesions or abscesses in other organs, associated with the high prevalence of polyarthritis suggest the infection by *M. agalactiae* (2,9), but other *Mycoplasma*, including *M. mycoides* subsp. *capri* can also cause high frequency of arthritis (21,25). Despite the observation of a small lesion of interstitial pneumonia in one kid, respiratory signs were not observed in these outbreaks. Respiratory signs are frequently observed in goats infected by *M. mycoides* subsp *mycoides*, *M. capricolum* subsp *capricolum*, and *M. mycoides* subsp *capri* (27). *M. agalactiae* have been also isolated from goats with lung lesions (3,25).
The occurrence of ACOC by *M. agalactiae* in Brazil suggests that the diagnosis of *Mycoplasma* infections of the sheep and goats should be introduced in the routine diagnostic laboratories. In the country, other *Mycoplasma*, including *M. mycoides* subsp *mycoides*, *M. mycoides* subsp *capri*, *M. arginini*, and *Mycoplasma* spp, have been isolated in goats and sheep with pneumonia, mastitis, arthritis or keratoconjunctivitis (1,4,14,15,21,23,24,26), but their economic importance remains unknown.

**RESUMO**

**Agalaxia contagiosa por Mycoplasma agalactiae em pequenos ruminantes no Brasil: Primeiro relato**

Dois surtos de agalaxia contagiosa causada por *Mycoplasma agalactiae* são descritos no Estado da Paraíba, região Nordeste do Brasil. A doença caracterizou-se por mastite, agalaxia e poliartrite em cabras e poliartrite e ceratoconjuntivite em cabritos e cordeiros. Febre e anorexia também foram observadas. A morbidez variou de 26,1% a 100% nas cabras, 36,5% a 100% em cabritos e 49,0% em cordeiros. Na primeira fazenda, 14,3% das cabras em lactação e 6,4% dos cabritos morreram ou foram sacrificados. Na outra propriedade, 3,3% dos caprinos adultos, 36,5% dos cabritos e 22,9% dos cordeiros morreram e outros 84 caprinos foram sacrificados para controle da doença. *M. agalactiae* foi isolado a partir de leite, líquido articular, suabe nasal e lavado do conduto auditivo externo. Colônias características de *Mycoplasma* e que não fermentaram a glicose e arginina foram observadas. A identificação de *M. agalactiae* foi realizada por imunoperoxidase indireta e PCR. Sendo assim, *M. agalactiae* é descrito pela primeira vez no Brasil, mas a origem da infeção permanece desconhecida.

**Palavras-chave:** agalaxia contaminosa; *Mycoplasma agalactiae*; pequenos ruminantes

**REFERENCIAS**

Mycoplasma agalactiae in Brazil


