Isolation and identification of *Mycoplasma agalactiae* associated with respiratory tract infection in goats: A case study

RM Reji, M Mini, BK Mani, S Sankar, V Thirupathy, PS Reshma, N Alswarya and TS Akkara

Abstract

Respiratory tract infections are the major cause of economic loss in goats. *Mycoplasma agalactiae* acts as an important etiological agent in mastitis, arthritis, keratoconjunctivitis, pleuropneumonia and septicaemia syndrome (MAKePS). This paper reports a case study regarding the outbreak of respiratory disease associated with *M. agalactiae* in a herd of goats in Thrissur district, Kerala. The goats were presented with symptoms of respiratory distress, conjunctivitis, lethargy and reduced production. Nasal swabs were collected and dispensed in modified Hayflick’s broth without crystal violet. After incubation at 37°C for about 72 h, DNA was isolated from the broth and subjected to Mycoplasma genus specific PCR, which revealed amplicons of 280 bp confirming the genus. Also, the solid medium which was inoculated with filtered drops of incubated broth presented typical fried egg like colonies after five days of incubation and the isolates were identified as *Mycoplasma* by PCR. The species was confirmed as *M. agalactiae* by nucleotide sequencing and this is the first report documenting the detection and isolation of *M. agalactiae* from goats of Kerala.

Keywords: Mycoplasma agalactiae, MAKePS, Modified Hayflick’s broth

1. Introduction

Goats are highly susceptible to a number of *Mycoplasma* spp., particularly *Mycoplasma capricolum* subsp. *capripneumoniae* and other mycoplasmas of the *Mycoplasma mycoides* cluster (Nicholas, 2002) [1]. Among the various diseases, MAKePS syndrome is a significant disease and is characterised by mastitis, arthritis, keratoconjunctivitis, pleuropneumonia and septicaemia (Thiaucourt and Bolske, 1996) [2]. The members of *Mycoplasma* implicated in MAKePS syndrome are *M. mycoides* subsp. *mycoides* Large Colony (MmmLC), *M. mycoides* subsp. *capri* (Mmc), *M. capricolum* subsp. *capricolum* (Mcc), *M. putrefaciens* (Mp) and *M. agalactiae* (Kumar et al., 2011) [3]. The infection due to *M. agalactiae* is also known as contagious agalactia (Bergonier and Poumarat, 1996) [4]. The incubation period of infection caused by *M. agalactiae* in goats and sheep is one to eight weeks (Azevedo et al., 2006) [5]. Since *M. agalactiae* has been isolated from nasal secretions, faecal samples, milk, and aborted foetus, it is believed that the primary site of predilection is the mucosa of respiratory tract, small intestine, and alveoli of mammary glands, respectively, depending upon the respiratory, oral, and mammary routes of infection. Common clinical symptoms associated with the infection include fever, anorexia, lethargy, and unwillingness to follow the herd, followed by clinical symptoms depending upon the involvement of various organs such as mammary glands, lungs, genitalia, joints, and conjunctiva (Kumar et al., 2014) [6]. This study documents the investigation of an outbreak of respiratory disease associated with *M. agalactiae* in a herd of goats.

2. Materials and methods

Respiratory disease was reported in a herd of goats in Thrissur district. The goats were presented with symptoms of depression, lethargy, fever, serous to mucopurulent nasal discharge, conjunctivitis, coughing and recumbency. Among 30 goats in the farm, 23 were affected. Nasal swabs were collected aseptically from four infected goats and dispensed in modified Hayflick’s broth. The medium contains PPLO (pleuropneumonia like organisms) infusion broth without crystal violet (17.5 g/L) as basal component and is supplemented with 25 per cent de complemented horse serum,
10 per cent fresh yeast extract, 0.4 per cent glucose, 0.8 per cent sodium pyruvate, 0.4 per cent of phenol red solution, 0.4 mL of five per cent thallium acetate solution and 250 mg ampicillin (Miles and Nicholas, 2010) [7]. The samples were incubated at 37°C in five per cent CO₂ for about five days. The broth was examined daily for growth, which is indicated by a colour change in the medium. The broth which showed colour change was filtered and transferred to solid medium. Also, DNA was extracted from the incubated broth after three days of enrichment using Hi Pura multi sample DNA extraction kit (HiMedia, India). The DNA samples were tested using *Mycoplasma* 16S rRNA genus specific Polymerase Chain Reaction (PCR). Primer pairs specific to *Mycoplasma* genus (GPO3F 5’-TGGGGAGCAAAACGAGATTAGATACC-3’ and MGSO 5’-TGCACCATCTGTCACTCTGTTAAC CTC-3’) as described by Botes et al. (2005) [8] was employed for the study. A 25 μL reaction mixture was set up for the single PCR reaction consisting of

<table>
<thead>
<tr>
<th>10X PCR master mix</th>
<th>12.5 μL</th>
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<tbody>
<tr>
<td>Forward Primer</td>
<td>2 μL</td>
</tr>
<tr>
<td>Reverse Primer</td>
<td>2 μL</td>
</tr>
<tr>
<td>Template DNA</td>
<td>6 μL</td>
</tr>
<tr>
<td>Nuclease Free Water</td>
<td>2.5 μL</td>
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</tbody>
</table>

The PCR tubes were placed in a thermal cycler (Eppendorf) and reaction was run as per the following protocol (Cetinkaya et al., 2009) [9].

**Table 1: Protocol for 16S rRNA Genus Specific PCR**

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Time</th>
<th>Steps</th>
</tr>
</thead>
<tbody>
<tr>
<td>94°C</td>
<td>2 min</td>
<td>Initial denaturation</td>
</tr>
<tr>
<td>94°C</td>
<td>15 sec</td>
<td>Denaturation</td>
</tr>
<tr>
<td>59.3°C</td>
<td>15 sec</td>
<td>Denaturation</td>
</tr>
<tr>
<td>72°C</td>
<td>15 sec</td>
<td>Denaturation</td>
</tr>
<tr>
<td>72°C</td>
<td>5 min</td>
<td>Final extension</td>
</tr>
<tr>
<td>4°C</td>
<td>10 min</td>
<td>Hold</td>
</tr>
</tbody>
</table>

Identification of the amplified products were done in a submerged agarose gel electrophoresis system using one per cent agarose gel stained with ethidium bromide, and Tris Borate EDTA buffer was used as the matrix at a voltage of 50V. The gel was visualised under a UV transilluminator and results were documented in a Gel Doc system (Biorad). The isolates obtained on solid plate were analysed under the low power objective of microscope and the colonies were subjected to DNA extraction and 16S rRNA genus specific PCR. Nasal swabs were also inoculated onto Blood agar (BA) and Sabouraud’s Dextrose Agar (SDA) for isolation of bacteria and fungi, respectively. Blood agar plates were incubated at 37°C and the duplicate samples on SDA plates were incubated at 37°C and at room temperature.

**3. Results and discussion**

In the present study, owner reported respiratory distress, lethargy and reduced production in a herd comprising of 30 goats. On BA, no bacterial organisms of pathogenic significance could be isolated even after 48h. of incubation. No growth could be obtained on SDA even after 7 days of incubation at 37°C and at room temperature. Diagnosis of caprine mycoplasmiosis can be done by various methods, but the gold standard test for confirmatory diagnosis is isolation and identification of the organism. It takes at least two to three weeks for cultural isolation, and the procedure is laborious (Aluotto et al., 1970) [10]. Polymerase chain reaction offers a rapid and a sensitive method for the detection and identification of *M. agalactiae* directly from clinical samples (Azvedo et al., 2006) [5]. The DNA extracted from the incubated broth was subjected to genus specific PCR for *Mycoplasma* species. A positive PCR result was obtained in case of all the four samples, which was indicated by the presence of a 280 bp fragment in electrophoresed gel under UV Tran illumination (Fig. 1). But, only two samples inoculated with filtered drops of incubated broth showed minute colonies on solid medium. On microscopy, typical fried egg like colonies could be appreciated in these samples (Fig. 2) and is in accordance with the observation of Kibor (1983) [11]. On Diene’s staining, dark blue central zone and light blue peripheral zone could be recognised clearly (Fig. 3) as described by Quinn et al. (2011) [12]. The isolates were identified as *Mycoplasma* by 16S rRNA genus specific PCR using the primer pair GPO3F and MGSO, which was found to be accurate in detecting *Mycoplasma* by Botes et al. (2005) [8]. The species identification of *Mycoplasma* isolates by biochemical and serological methods is complicated, since the members share many antigenic and genomic features (Thiaucourt and Bolske, 1996) [2]. Hence, in the present study, the amplicons were confirmed as *M. agalactiae* by nucleotide sequencing (Table 2). Even though, all the four samples produced amplicons on16S rRNA genus specific PCR, only two samples showed *Mycoplasma* colonies on solid medium. This lower recovery rate of *Mycoplasma* from clinical samples could be attributed to its fastidious nature (Francis et al., 2015) [13]. Also, isolation of *Mycoplasma* from nasal swabs was found to be challenging by Litamoi et al. (1990) [14] due to contamination with normal flora. This is the first report documenting the detection and isolation of *M. agalactiae* from goats in Kerala.

**Fig 1: Amplicons of 16S rRNA gene specific PCR**
### 4. Conclusion

The present study was an investigation in a herd of goats exhibiting respiratory distress, lethargy and significant production loss suggesting the involvement of *Mycoplasma* group of organisms. All the four samples tested showed positive results in PCR while isolates could be obtained from two samples. Even though, conventional isolation and identification is the gold standard technique for the detection of mycoplasmas, because of the laborious and time-consuming procedure, it is now replaced by nucleic acid-based techniques like PCR. The assay offers a rapid and sensitive alternative for accurate diagnosis of the disease, so that appropriate and timely strategies could be employed to control the disease.

### 5. Acknowledgement

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### 6. References