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ORIGINAL ARTICLE

Development of a specific immunomagnetic capture-PCR for rapid detection of viable *Mycoplasma agalactiae* in sheep milk samples

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Abstract

Aims: To develop an immunomagnetic capture (IMC) to detect viable *Mycoplasma agalactiae* in routine ovine milk samples.
Methods and Results: Polyclonal antibodies against two *M. agalactiae* membrane surface proteins (P80 and P55) were covalently conjugated to magnetic beads (MBs) to form MB-Ab80 and MB-Ab55. *Mycoplasma agalactiae* cells were captured by a specific antigen–antibody reaction and magnetic separation. Immunomagnetic capture (IMC) was used to isolate and concentrate *M. agalactiae* in serial decimal dilutions and in artificially contaminated milk to facilitate subsequent detection by PCR. A 375-bp fragment of *M. agalactiae* was amplified using a pair of *M. agalactiae*-specific primers in PCR. The limit of detection of IMC-PCR method ranged from 10 to 10^2 CCU ml^{-1} when mycoplasmas were resuspended in PBS and from 10^2 to 10^3 CCU ml^{-1} when mycoplasmas were resuspended in uncontaminated ovine milk. This study also describes the application of IMC-PCR method to test for *M. agalactiae* in 516 milk samples collected from sheep with suspected contagious agalactia. Its performance was evaluated relative to culture.
Conclusions: This report has demonstrated for the first time, the effective use of rapid and reliable IMC combined with PCR assay for the detection of viable *M. agalactiae*.
Significance and Impact of the Study: The method IMC-PCR provides an alternative to conventional microbiological detection, method and it could be applied to quick detection of *M. agalactiae* in routine sheep milk samples.

Introduction

Mycoplasma agalactiae is a cell wall-less bacterium belonging to the class *Mollicutes* that has been identified as the main aetiologic agent of contagious agalactia (CA) in small ruminants. The disease has a worldwide distribution but is prominent in the Mediterranean basin (Corrales *et al.* 2007). It is characterized primarily by mastitis, arthritis, keratoconjunctivitis and, occasionally, abortion and pneumonia. Because of its importance in veterinary medicine, CA is listed as a notifiable disease by the World Organization for Animal Health (OIE). Disease notification requirements for OIE member nations are detailed in the OIE Terrestrial Animal Health Code (http://www.oie.int/international-standard-setting/terrestrial-manual/access-online/, chapter 2.7.5).

Conventional laboratory procedures currently used to identify *M. agalactiae* from infected animals are very time-consuming requiring over 1–2 weeks to produce results. The standard method (Gold standard method) includes culture of samples from living animals (milk, ear/nasal/conjunctival/swab and joint fluid) onto Hayflick-agar plates for 7 days at 37°C and subsequent identification of typical fried-egg colonies yielded in agar plates by biochemical, serological and, as increasingly, molecular tests such as PCR (Dedieu *et al.* 1995; Tola

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et al. 1996; Subramaniam *et al.* 1998; Foddai *et al.* 2005) and real-time PCR (Lorusso *et al.* 2007; Fitzmaurice *et al.* 2008; Oravcova *et al.* 2009).

To speed up acquisition of results, we developed a simple and rapid method for DNA extraction from sheep milk samples to be used for PCR diagnosis of *M. agalactiae*. The method enables to reduce the time required for diagnosis from several days to 5 h (Tola *et al.* 1997). However, PCR detection of *M. agalactiae* in milk samples does not prove the viability, thus infectivity of detected bacterial cells because the method cannot discriminate between DNA molecules from live and dead cells. The distinction between viable and dead bacterial cells represents a major challenge in PCR diagnostics, due to the persistence of DNA in the environmental sample after the cells have lost viability (Josephson *et al.* 1993).

Recently, the use of magnetic nanobeads coated with specific antibodies to extract and concentrate the target organism from the samples has become a low-cost routine procedure in food and veterinary microbiology laboratories. Immunomagnetic capture (IMC) is commonly used in combination with culture or molecular methods for the detection and isolation of pathogenic bacteria such as *Salmonella* spp., *Listeria monocytogenes* and *Escherichia coli* (Spanova *et al.* 2000; Uyttendaele *et al.* 2000; Favrin *et al.* 2003). In contrast, no IMC method has been developed for *M. agalactiae* detection.

The objectives of this study were to (i) develop an immunomagnetic capture (IMC) method using polyclonal antibodies against P55 and P80, two *M. agalactiae* immunodominant antigens (Fusco *et al.* 2007), (ii) evaluate the performance of the method, (iii) detect viable *M. agalactiae* from artificially contaminated milk samples by IMC-PCR technique, in which we combine the ability of IMC to differentiate viable from nonviable mycoplasmas with the specificity and sensitivity of PCR and (iv) detect viable *M. agalactiae* in clinical ovine milk samples.

Materials and methods

Mycoplasma agalactiae isolates and growth conditions

Mycoplasma agalactiae isolates used in this study are given in Table 1. All isolates included in this study were chosen among a bank of *M. agalactiae* isolates from contagious agalactia outbreaks in Italy. Each isolate was grown in a shaker incubator at 37°C in 100 ml of modified Hayflick medium containing 8% horse serum until logarithmic phase.

Table 1 *Mycoplasma agalactiae* isolates used in this study

Isolate	Year of isolation	Geographic origin (Region/Province)
PA119	1993	Sicily/Palermo
TE290	1993	Abruzzo/Teramo
338	1994	Sardinia/Cagliari
1852	2012	Sardinia/Nuoro
70976	2013	Sardinia/Sassari

Preparation of *Mycoplasma agalactiae* inoculums

To estimate the number of bacterial cells inoculated in each spiked sample included in this study, serial dilutions from original *Mycoplasma* culture were prepared as described below. One ml of each log-phase culture was dispersed into the first of a set of 10 glass tubes containing 9 ml of Hayflick medium. After 3 min of vortex, 1 ml of the first dilution was transferred onto the second tube. The procedure was repeated 10 times, to obtain a serial decimal dilution. Number of inoculated cells was estimated based on the last serial dilution showing sign of bacterial growth and expressed as color changing units (CCU ml^{-1}).

Preparation of spiked phosphate-buffered saline and milk samples

One milliliter of each serial decimal dilution was used for both (i) determining the sensitivity of immunocapture method using phosphate-buffered saline (PBS) pH 7·4 and (ii) determining the sensitivity of immunocapture method using milk sample from a sheep belonging to a flock without a clinical history of mycoplasma infection. In the first case, mycoplasmas from each serial decimal dilution were harvested by centrifugation at 9000 ***g*** for 30 min and resuspended in 1 ml of PBS pH 7·4. In the second case, mycoplasmas were resuspended in 1 ml of milk sample. The remaining sample from each serial dilution was incubated for 5 days at 37°C to estimate the number of viable mycoplasmas inoculated in each samples (CCU ml^{-1}) as described above.

Production of immune sera to *Mycoplasma agalactiae* P80 and P55

Two polyclonal sera against *M. agalactiae* P80 and P55 were used in this study. Briefly, protein bands corresponding to P80 and P55 were electroeluted from Coomassie blue-stained gels, concentrated and inoculated into two different lambs, as described by Tola *et al.* (2001).

In-house coating of commercially available superparamagnetic beads

Dynabeads MyOne carboxylic acid (Invitrogen, Dynal AS, Oslo, Norway) was used in this study. Beads were activated by the use of the hydrophobic N-cyclohexyl-N-(2-morpholinoethyl) carbodiimide methyl-p-toluenesulfonate (CMC, Sigma, St. Louis, Mo) that improves yield and orientation of the immobilized antibodies. Briefly, 300 μl of DynabeadsMyOne, corresponding to 3 mg, was washed twice with an equal volume of 0·01 mol l^{-1} NaOH and three times with deionized water. Beads, captured by the use of a magnetic stand (DynaMag™-2, Dynal), were incubated for 30 min at 4°C with 200 μl of cold 20 mg ml^{-1} CMC in slow rotation. After incubation, beads were washed once with 300 μl of 25 mmol l^{-1} 2-(n-morpholino)ethanesulfonic acid (MES, Sigma), pH 6·0. Beads, with activated carboxylic acid groups, were used for coating with a ligand containing primary amino groups.

Coating of anti-P80 and anti-P55 antibodies to activated magnetic beads

Polyclonal sera against *M. agalactiae* P80 and P55 were diluted 1/100, 1/500, 1/1000, 1/1500 and 1/2000 with 25 mmol l^{-1} MES pH 6·0 and measured spectrophotometrically by reading of absorbance at 280 nm. One ml of each serum dilution was thoroughly mixed and incubated with the activated Dynabeads at room temperature (r.t.) for 1 h under slow shaking. The mixtures of Dynabeads and P80/P55 serum dilutions were placed on the magnet for 1 min. After spectrophotometric reading of each supernatant at 280 nm, we selected the serum dilution with highest difference between first and second reading. Anti-P80/P55-coated magnetic particles were incubated at r.t. for 15 min with 1 ml of 0·05 mol l^{-1} Tris-HCl pH 7·4 in slow rotation. Beads were washed four times with PBS containing 0·1% bovine serum albumin (BSA, Sigma) and stored at 4°C for $<$6 months.

Immunomagnetic capture (IMC) of *Mycoplasma agalactiae* resuspended in PBS

One-ml PBS samples inoculated with increasing numbers of mycoplasmas were processed through IMC involving the use of ten μl of P80-coated particles and 10 μl of P55-coated beads per reaction. After incubation at 37°C for 1 h in slow rotation, the mycoplasma-magnetic particle complex was separated from the suspension, washed three times with PBS containing 0·05% Tween-20 (PBST) and resuspended in 100 μl of TE buffer (10 mmol l^{-1} Tris-HCl, 1 mmol l^{-1} EDTA, pH 8·0). Samples were then boiled for 15 min and centrifuged at 4500 ***g*** for 5 min. Five microliter of supernatant was used for each PCR.

Immunomagnetic capture (IMC) of *Mycoplasma agalactiae* resuspended in sheep milk

To maximize capture efficiency from milk of the new IMC method, different combinations of milk (95–5%) and PBST (5–95%) were considered. Mycoplasmas from serial decimal dilutions were resuspended in 1 ml of milk containing increasing amount of PBST and processed through immunomagnetic separation involving the use of both P80-coated and P55-coated beads, as described above.

Routine PCR

A 375-bp fragment based on the sequence reported by Tola *et al.* (1996) was amplified using the sense primer FS1 (5′-AAAGGTGCTTGAGAAATGGC-3′) and the antisense primer FS2 (5′-GTTGCAGAAGAAAGTCCAATCA-3′). PCR products were analysed by electrophoresis on an 1·2% agarose gel, stained with Sybr®Safe DNA gel stain (Invitrogen) and visualized under a UV transilluminator.

Immunocapture of viable *Mycoplasma agalactiae* in milk field samples

For this study, a total of 516 sheep milk samples, from 61 flocks with suspected contagious agalactia, were examined: 473 samples were analysed after 6–48 h of collection, while 43 were analysed after 3 or more days. An 850 μl aliquot of each milk sample was diluted with 150 μl of PBST and mixed thoroughly with 10 μl of P80-coated and P55-coated beads. After incubation at 37°C for 1 h in slow rotation, beads were processed as described above.

As comparative tests, all 516 sheep milk samples were cultured on Hayflick-agar plates, incubated for 5 days at 37°C in a humidified chamber and observed with a Zeiss Axiovert 35 inverted-light microscope.

SDS-PAGE and Immunoblotting

Twenty micrograms of total proteins from pooled isolates was electrophoresed on 12% (w/v) polyacrylamide gels containing 0·1% SDS. The apparent molecular weight of mycoplasma proteins was determined using appropriate molecular weight markers (kaleidoscope prestained standards, Bio-Rad, CA, USA). Electrophoresed proteins were transferred to nitrocellulose membranes in a Trans-Blot-semidry apparatus (Bio-Rad) as described by the manufacturer. Blots were incubated for 1 h at 37°C with

lamb sera diluted 1 : 1500 in PBS-2% skim milk. After several washings with PBS-2% skim milk, blots were incubated for a further hour at 37°C with alkaline phosphatase-conjugated anti-sheep antibodies (Sigma). After five more washes, blots were developed with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT, Promega, Madison, WI) in alkaline phosphatase buffer (100 mmol l^{-1} NaCl, 5 mmol l^{-1} $MgCl_2$, 100 mmol l^{-1} Tris, pH 9·5).

Results

Polyclonal sera against P80 and P55

The bands corresponding to P80 and P55 were electroeluted from the Coomassie-stained gels, concentrated and inoculated in two different lambs, as described by Tola *et al.* (2001). As shown by the immunoblotting in Fig. 1, the IgG antibodies produced against P80 recognized only this antigen; on the contrary, the lamb monospecific hyperimmune serum directed against the electroeluted P55 also recognized other antigens of middle and low molecular weight, suggesting that all these proteins might share common antigenic determinants.

Antibodies against P80/P55-conjugated magnetic beads (MB-Ab80 and MB-Ab55) for *Mycoplasma agalactiae* capture

Optimal working dilution for the two lamb polyclonal antibodies to be covalently conjugated to carboxylated MBs to form MB-Ab80 and MB-Ab55 was 1 : 1500. Use of 10 μl of MB-Ab80 and 10 μl of MB-Ab55 achieved capture from serial decimal dilutions of *M. agalactiae* broth cultures.

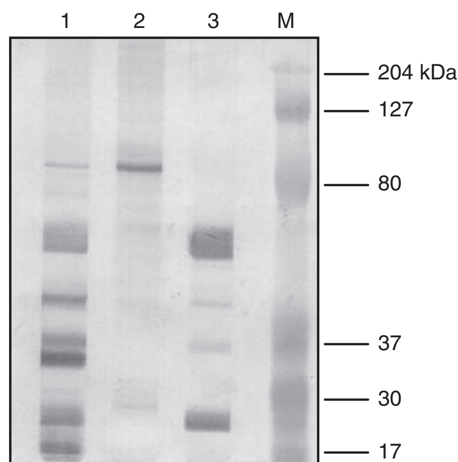

Figure 1 Immunoblotting analysis of reactivity of anti-P80 (lane 2), anti-P55 (lane 3) and pooled serum from naturally infected sheep with clinical symptoms (lane 1) with whole-cell *Mycoplasma agalactiae* antigens. M, kaleidoscope prestained standards. Molecular masses are indicated on the right.

Analytical sensitivity of the IMC-PCR

To find the lowest detectable concentration of *M. agalactiae*, IMC-PCR assay was carried on PBS and milk samples artificially contaminated at different level (10–10^{10} CCU ml^{-1}) with 5 *M. agalactiae* isolates (Table 1). The minimum number of mycoplasmas that can be detected in one round of PCR amplification with 30 cycles was determined by visualizing of amplification band. The lowest dilution that produced a 375-bp amplicon was taken as the limit of detection (Fig. 2). Analytical sensitivity of the IMC-PCR method ranged from 10 to 10^2 CCU ml^{-1} for spiked PBS samples and from 10^2 to 10^3 CCU ml^{-1} for artificially contaminated milk samples. The best result was obtained when mycoplasmas were resuspended in a mixture containing 850 μl of milk and 150 μl PBST.

Application of the IMC-PCR to milk field samples

A total of 516 sheep milk samples were subjected to culture and IMC-PCR. Overall, 296 milk samples were resulted *M. agalactiae* positive by culture method. One hundred and seventy-seven samples were negative, whereas the remaining 43 were culture doubtful samples. The sample was considered a doubtful sample when the growth of other bacteria did not allow visualizing mycoplasma colonies. For this reason, the 43 samples were excluded from the IMC-PCR analysis. Using the

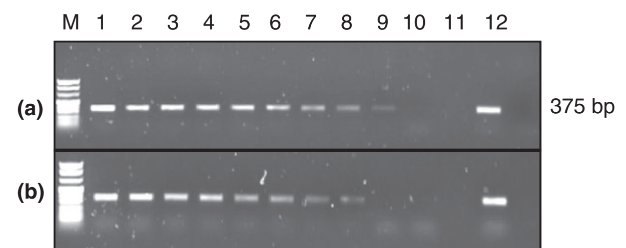

Figure 2 Analytical sensitivity of immunomagnetic capture (IMC)-PCR assay using different concentrations of *Mycoplasma agalactiae* 1852 isolate resuspended in PBS pH 7·4 (a) and uncontaminated sheep milk (b). The amount of mycoplasmas used in the PCR was 10^{10} CCU (lane 1), 10^9 CCU (lane 2), 10^8 CCU (lane 3), 10^7 CCU (lane 4), 10^6 CCU (lane 5), 10^5 CCU (lane 6), 10^4 CCU (lane 7), 10^3 CCU (lane 8), 10^2 CCU (lane 9) and 10 CCU (lane 10) ml^{-1}. Negative control (lane 11) and positive control (lane 12) of PCR. Lane M: marker VIII (Roche).

IMC-PCR method, 287 samples were positive and 186 negative.

Results of the IMC-PCR detection method are compared with culture results: IMC-PCR detected the presence of *M. agalactiae* in 287 samples (287/473 corresponding to 60·6%), whereas 296 samples (296/473, 62·5%) gave positive results by culture.

Discussion

Contagious agalactia (CA) is a notifiable disease pursuant to Decree n. 54/2009 of Sardinia Region in agreement with Italian Presidential Decree n. 380/1954 and European Directive n. 82/894/EEC and their amendments. The disease notification must be made as soon as possible so that all health measures are used to limit or prevent its spread. The time interval between clinical symptoms onset and disease notification depends on laboratory techniques used for the diagnosis of *M. agalactiae*. Culture-based method is the technique widely used in the isolation of mycoplasmas (Nicholas and Baker 1998). Isolation and subsequent identification of mycoplasma colonies by biochemical and/or serological tests have limitations such as length of time (two or more weeks) and labour intensity (Bradbury 1998; Poumarat 1998; Poveda 1998). As a consequence, several attempts have been made to develop PCR-based detection systems (Dedieu *et al.* 1995; Tola *et al.* 1996; Subramaniam *et al.* 1998; Foddai *et al.* 2005). These PCR-based detection systems have been used directly on conjunctival, nasal, synovial and tissue samples and on milk samples (Tola *et al.* 1997). Direct detection of *M. agalactiae* in milk samples by PCR has been reported more sensitive than the traditional method and much faster than culture reducing the time for diagnosis to about 5 h (Tola *et al.* 1997).

In this study, we have developed an immunomagnetic separation approach to be used in combination with the previously reported PCR assay (Tola *et al.* 1996), so as to provide a novel IMC-PCR assay for the detection of viable *M. agalactiae* in spiked and clinical milk samples. Specific polyclonal antibodies (pAbs) against two *M. agalactiae* membrane surface proteins (P80 and P55) were conjugated with carboxylated MBs. Unlike the anti-P80 serum, the anti-P55-serum reacts strongly not only with the protein band corresponding to P55, but also with a protein of about 26 kDa (Fig. 1). This might suggest that P55 shares antigenic similarity with P26. In a previous study, Santona *et al.* (2002) showed by N-terminal sequencing that P55 was identical to the AvgC protein encoded by the *avg*C gene. The *M. agalactiae* variable genes (*avg*) system is a cluster of four genes that encodes a family of surface lipoproteins characterized by high-frequency phase and size variations (Flitman-Tene *et al.* 2000). Another family of variable surface lipoproteins was identified in *M. agalactiae* and designated as Vpmas. Four *vpma* genes, *vpma*X, *vpma*Y, *vpma*U and *vpma*Z, were found to be identical to four *avg* genes, *avg*A, *avg*B, *avg*C and *avg*D (Glew *et al.* 2000). In particular, *avg*C corresponds to the *vpma*U gene. The *avg*C (*vmpa*U) gene is 717 bp in size and would encode a protein with an expected molecular mass of approximately 26 kDa. Therefore, the combination of the two polyclonal sera allows binding of antibodies with several *M. agalactiae* membrane surface proteins. The resulting MBs-pAbs were used to capture 5 *M. agalactiae* isolates and reference strain PG2 in serial decimal dilutions and in artificially contaminated milk samples. As expected, the better capture efficiency was obtained from cultures resuspended in PBS, with a limit of detection (LOD) ranging from 10 to 10^2 CCU ml^{-1}. When the IMC-PCR method is applied to spiked milk, the LOD ranged from 10^2 to 10^3 CCU ml^{-1}, with a 'matrix effect' 10-fold higher than PBS. The lower sensitivity of IMC-PCR method than the silica-based PCR, described by Tola *et al.* (1997), could be attributed to the method used for DNA extraction from mycoplasma-bead complex. However, the boiling method was chosen because of its simplicity and rapidity of execution. DNA extraction from complex matrix like milk is a critical step because milk samples contain high percentage of PCR inhibitors, fats, proteins, eukaryotic cells, and the proportions of which can vary considerable between bulk tank milk and mastitis milk (Bickley *et al.* 1996). Oravcova *et al.* (2009), using a sophisticated extraction protocol, have detected 250 *M. agalactiae* cells per 25 ml of milk, corresponding to 10^2 cells ml^{-1}, whereas Becker *et al.* (2012), using a commercially DNA extraction kit, have obtained a detection of 350 CFU ml^{-1}, corresponding to $3{\cdot}5 \times 10^2$ CFU ml^{-1}. However, the aim of these authors was the absolute quantification of DNA by real-time PCR and not to differentiate viable from nonviable mycoplasmas, main object of our study.

In the second part of our study, we applied the optimized IMC-PCR method to clinical milk samples from 61 flocks with suspected CA. Of a total of 516 milk samples, analysed during the 2012–2013 period, 473 were transferred from farms to our laboratories within 48 h while 43 from 3 to 10 days. However, we excluded the 43 samples because of remarkable growth of other bacteria that did not permit the observation of fried-egg colonies through an inverted-light microscope. The comparison between cultural and IMC-PCR methods has shown that only nine milk samples from individual sheep did not yield a positive result with the IMC-PCR. However, these false-negative samples, belonging to nine different flocks, did not modify the final diagnosis of CA because, from each suspected flock, usually milk samples ranging from 5 to 10% of all animals are received.

In conclusion, this report has demonstrated, for the first time, the effective use of a rapid and reliable IMC method that combines with PCR assay for detection of viable *M. agalactiae*. The method IMC-PCR provides an alternative to conventional microbiological detection method, and it could be applied to quick detection of *M. agalactiae* in routine sheep milk samples because it permits the diagnosis of contagious agalactia in about 6 h.

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Conflict of interest

No conflict of interest declared.

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