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ORIGINAL ARTICLE

A real-time PCR assay for detection and quantification of *Mycoplasma agalactiae* DNA

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Abstract

Aims: The aim of this study was to develop a rapid, sensitive, specific tool for detection and quantification of *Mycoplasma agalactiae* DNA in sheep milk samples.
Methods and Results: A real-time polymerase chain reaction (PCR) assay targeting the membrane-protein 81 gene of *M. agalactiae* was developed. The assay specifically detected *M. agalactiae* DNA without cross-amplification of other mycoplasmas and common pathogens of small ruminants. The method was reproducible and highly sensitive, providing precise quantification of *M. agalactiae* DNA over a range of nine orders of magnitude. Compared with an established PCR assay, the real-time PCR was one-log more sensitive, detecting as few as 10^1 DNA copies per 10 μl of plasmid template and $6{\cdot}5 \times 10^0$ colour changing units of reference strain Ba/2.
Conclusions: The real-time PCR assay is a reliable method for the detection and quantification of *M. agalactiae* DNA in sheep milk samples. The assay is more sensitive than gel-based PCR protocols and provides quantification of the *M. agalactiae* DNA contained in milk samples. The assay is also quicker than traditional culture methods (2–3 h compared with at least 1 week).
Significance and Impact of the Study: The established real-time PCR assay will help study the patterns of shedding of *M. agalactiae* in milk, aiding pathogenesis and vaccine efficacy studies.

Introduction

Contagious agalactia (CA) is one of the most serious diseases affecting small ruminants and is regarded by health authorities as being endemic in most Mediterranean countries (Bergonier *et al.* 1997). The syndrome is characterized by mastitis, arthritis, keratoconjunctivitis and, occasionally, abortion (De la Fe *et al.* 2005). The reduced milk production, the increased kids' mortality and the cost of veterinary assistance cause significant economic losses.

Mycoplasma agalactiae is the classical aetiological agent of CA of small ruminants (DaMassa *et al.* 1992), although other mycoplasma species included in the 'mycoides cluster', i.e. *Mycoplasma mycoides* subsp. *mycoides* large colony, *Mycoplasma capricolum* subsp. *capricolum* and *M. mycoides* subsp. *capri*, may induce a similar disease, often associated to clinical signs of respiratory distress (DaMassa *et al.* 1987). *Mycoplasma putrefaciens* causes a disease with identical clinical signs, consisting of mastitis, agalactia and arthritis (Nicholas 2002).

By means of biochemical and immunological methods and 16S rRNA sequence comparison, *M. agalactiae* has been shown to be closely related to *Mycoplasma bovis* that induces similar clinical signs in cattle, including mastitis, arthritis and respiratory diseases (Ross 1993). *Mycoplasma bovis* is therefore included in specificity studies, although in practice it is rarely found in sheep and goats. Because of the negative impact of CA in small ruminant flocks, it appears needful to develop and use diagnostic tools that allow rapid and specific detection of *M. agalactiae* in biological samples. Traditionally, identification of *M. agalactiae* is achieved using time-consuming techniques such as

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immunofluorescence and bacteriological investigations. Mycoplasma isolation and subsequent identification by cultural and biochemical methods is laborious, requiring up to 3 weeks and specific culture media such as Hayflick broth or agar (DaMassa 1995).

Moreover, diagnosis based on serological assays such as enzyme-linked immunosorbent assay (ELISA) or growth inhibition test may be affected by phenomena of cross-reactivity, as observed between *M. agalactiae* and *M. bovis* or among mycoplasmas of the 'mycoides cluster' (Leach *et al.* 1989; Mattsson *et al.* 1994), as well as by delayed seroconversion, with antibodies being detectable only 28–35 days postinfection (Buonavoglia *et al.* 1999). Polymerase chain reaction (PCR) is regarded as a reliable method for diagnosis of *M. agalactiae* infection (Dedieu *et al.* 1995; Tola *et al.* 1996). A multiplex PCR (m-PCR) assay has been optimized for simultaneous detection and characterization of several *Mycoplasma* species that affect small ruminants (Greco *et al.* 2001). In addition, several PCR assays have been developed to differentiate *M. agalactiae* from *M. bovis* (Chavez Gonzalez *et al.* 1995; Subramaniam *et al.* 1998; Pinnow *et al.* 2001; Foddai *et al.* 2005).

A number of real-time PCR assays have been developed for detection of mycoplasmas of animals, such as *M. mycoides* subsp. *mycoides* small colony (Gorton *et al.* 2005). Herewith, we describe a real-time PCR assay based on TaqMan® technology for specific and sensitive detection and quantification of *M. agalactiae* nucleic acid. The assay was applied for detection of *M. agalactiae* in milk samples.

Materials and methods

Samples collection and template DNA preparation

Reference strains for *M. mycoides* subsp. *mycoides* large colony, *M. capricolum* subsp. *capricolum* and *M. mycoides* subsp. *capri* were kindly supplied by the Istituto Zooprofilattico Sperimentale della Sardegna (Sassari, Italy). A reference strain for *M. bovis* was provided by the Istituto Zooprofilattico Sperimentale della Sicilia (Palermo, Italy) while *M. agalactiae*, *M. putrefaciens* and *M. capricolum* subsp. *capripneumoniae* were available in our collection. *Mycoplasma agalactiae*-positive milk samples ($n = 33$) collected from sheep with mastitis in Southern Italy were processed directly ($n = 18$) or after propagation in culture ($n = 15$) (Table 1).

All reference strains and 15/33 field strains were grown in modified Hayflick broth at 37°C with stirring until the mid-log phase was reached, according to Freundt (1983). Cells were pelleted by centrifugation at 10 000 ***g*** for 20 min, washed in phosphate-buffered saline (PBS, 0·1 mol l^{-1} phosphate, 0·33 mol l^{-1} NaCl, pH 7·4) and resuspended in Tris-EDTA buffer (10 mmol l^{-1} Tris-HCl, 1 mmol l^{-1} EDTA, pH 8·0). DNA extraction was carried out as described by Ausubel *et al.* (1993) and the final product (200 μl) was stored at −70°C. DNA extraction from the milk samples (200 μl) that were not cultured was carried out using the commercial kit DNeasy Tissue Kit (Qiagen S.p.A., Milan, Italy), according to the manufacturer's instructions. The DNA was eluted with 200 μl of the supplied AE buffer (elution buffer; Qiagen) and stored at −70°C until use. All field strains had been identified as *M. agalactiae* species by means of m-PCR (Greco *et al.* 2001).

Table 1 *Mycoplasma agalactiae* DNA titres detected by real-time polymerase chain reaction (PCR) in milk samples of infected sheep that were processed directly or after propagation in culture

Sample	Origin	Year	DNA titre*
Milk	Apulia	1999	$6·25 \times 10^{9}$
Milk	Apulia	1999	$8·82 \times 10^{6}$
Milk	Apulia	1999	$2·11 \times 10^{9}$
Milk	Apulia	2000	$1·33 \times 10^{8}$
Milk	Apulia	2000	$3·67 \times 10^{5}$
Milk	Apulia	2001	$6·98 \times 10^{8}$
Milk	Apulia	2001	$1·87 \times 10^{8}$
Milk	Apulia	2001	$6·57 \times 10^{8}$
Milk	Apulia	2001	$7·43 \times 10^{5}$
Milk	Apulia	2003	$8·55 \times 10^{4}$
Milk	Apulia	2003	$2·33 \times 10^{6}$
Milk	Apulia	2003	$4·84 \times 10^{8}$
Milk	Apulia	2004	$3·54 \times 10^{7}$
Milk	Basilicata	2000	$1·02 \times 10^{11}$
Milk	Basilicata	2000	$6·20 \times 10^{4}$
Milk	Basilicata	2001	$2·00 \times 10^{9}$
Milk	Basilicata	2002	$5·77 \times 10^{6}$
Milk	Basilicata	2002	$5·77 \times 10^{6}$
Culture	Apulia	1999	$6·14 \times 10^{9}$
Culture	Apulia	1999	$3·01 \times 10^{10}$
Culture	Apulia	2000	$9·97 \times 10^{9}$
Culture	Basilicata	2000	$3·45 \times 10^{10}$
Culture	Basilicata	2002	$4·10 \times 10^{10}$
Culture	Sardinia	2000	$3·22 \times 10^{10}$
Culture	Sardinia	2001	$5·15 \times 10^{10}$
Culture	Sardinia	2001	$4·04 \times 10^{10}$
Culture	Sardinia	2002	$5·55 \times 10^{10}$
Culture	Sardinia	2002	$6·00 \times 10^{10}$
Culture	Sardinia	2002	$2·56 \times 10^{10}$
Culture	Sardinia	2002	$4·60 \times 10^{10}$
Culture	Sardinia	2004	$4·12 \times 10^{10}$
Culture	Sardinia	2004	$7·70 \times 10^{11}$
Culture	Sardinia	2004	$1·62 \times 10^{5}$

*Titres are expressed as copy numbers of *M. agalactiae* DNA per millilitre of milk or Hayflick broth.

Design of primers and dual-labelled probe

In order to design a real-time PCR assay specific for *M. agalactiae*, the sequences of the 81-kDa surface mem-

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brane protein gene (*mp81*) of *M. agalactiae* (accession X95628) and *M. bovis* (accession AY627040) were retrieved from GenBank and aligned using the Bioedit software package (www.mbio.ncsu.edu/BioEdit/bioedit.html). Pairwise alignment using ClustalW displayed a 73% nucleotide identity between the two *Mycoplasma* species. Assay target region was identified by visual inspection of sequence alignment and then primer and probe sequences were chosen by using a primer design software (Beacon Designer; Bio-Rad Laboratories Srl, Milan, Italy), in order to amplify specifically a 117-bp fragment within the *M. agalactiae mp81* gene sequence. Primers and probe were synthesized by MWG Biotech AG (Ebersberg, Germany), with the probe labelled with the fluorescent reporter dye 6-carboxyfluoroscein (6-FAM) at the 5′-end and with blackhole quencher 1 (BHQ1) at the 3′-end.

DNA standard for quantification

A 650-nucleotide fragment of the *mp81* gene of *M. agalactiae* was amplified for the standard plasmid DNA construction by using primers Lp80for (5′-AATGATT GGACTTTCTTCTGCAA-3′) and Lp80rev (5′-TCACTTT CATCGCCCTTACC-3′). The resulting PCR product was cloned into pCR4-TOPO vector (TOPO TA cloning; Invitrogen, Milan, Italy) and propagated in competent *Escherichia coli* TOP10F' cells, following manufacturer's instructions. Plasmid DNA was purified using Wizard Plus Midiprep (Promega Italia, Milan, Italy) and then quantified by spectrophotometrical analysis at 260 nm. Input target copies were calculated based on the size of the plasmid and the corresponding DNA mass. Tenfold dilutions of the standard plasmid, representing 10^0–10^9 copies of plasmid DNA per 10 μl of the template, were made out in TE buffer. Aliquots of each dilution were frozen at −70°C and used only once.

Real-time PCR

Real-time PCR was conducted in a 7500 Real-time PCR System (Applied Biosystems, Foster City, CA, USA). Duplicates of *M. agalactiae* standards (plasmid DNA) and DNA templates were subjected simultaneously to real-time analysis with each run including one negative control (no template control). Various PCR reaction parameters were examined including primer and probe concentrations, cycling time and temperature in order to obtain the optimal working conditions. Conditions were chosen such that the cycle threshold (C_T) values were the lowest possible and the fluorescence acquisition curves were robust and parallel to each other at various template concentrations. Amplification was performed in a 25-μl reaction volume containing 12.5 μl of iTaq™ Supermix (Bio-Rad Laboratories Srl) added with ROX (reference dye used for normalization of intensity by background), 600 nmol l^{-1} of each primer Ma-For (5′-AAACTTTGA AGATAATGACAAA-3′) and Ma-Rev (5′-TGGAATTA TGAATGAACCATT-3′), 200 nmol l^{-1} of probe Ma-Pb (5′-6-FAM-TTAATCTTGATAATATAAGTGAACAGTTTA CTACT-BHQ1-3′) and 10 μl of template or standard DNA. The thermal cycle protocol used was the following: activation of iTaq DNA polymerase at 95°C for 10 min and 40 cycles consisting of denaturation at 95°C for 1 min, primer annealing at 50°C for 30 s and extension at 60°C for 1 min. The increase in fluorescent signal was registered during the extension step of the reaction and the data were analysed with the appropriate sequence detector software (7500 System Software v.1·3·1). The reporter dye (FAM) signal was measured against the reference dye (ROX) signal to normalize the signals for non-PCR-related fluorescence fluctuations that occur from well-to-well. The PCR cycle at which the increase in normalized fluorescence of a sample exceeds background noise is called the C_T values, which is inversely proportional to the initial copy number of the target template (Heid *et al.* 1996). The absolute *M. agalactiae* DNA copy numbers contained in the unknown samples were calculated using standard curves generated by analysing 10-fold dilutions of the plasmid DNA (from 10^9 to 10^1 DNA copies) made in a *M. agalactiae*-negative milk sample.

Internal control

In order to verify the absence of DNA losses during the extraction step and of PCR inhibitors in the DNA templates, an internal control (IC), consisting of canine parvovirus type 2 (CPV-2) DNA (Desario *et al.* 2005), was added to the AL buffer (lysis buffer; Qiagen S.p.A.) at the concentration of 10 000 DNA copies per millilitre of buffer prior to milk digestion. The fixed amount of the IC added to each sample had been calculated to give a mean C_T value in a real-time PCR assay (Decaro *et al.* 2005) of 36·26 with an SD of 0·81 as calculated by 50 separate runs. Real-time PCR for IC detection was carried out in a separate run, using primers CPV-For (5′-AAACAGG AATTAACTATACTAATATATTTA-3′) and CPV-Rev (5′-AAATTTGACCATTTGGATAAACT-3′) and probe CPV-Pb (5′-6-FAM-TGGTCCTTTAACTGCATTAAATAATGTA CC-TAMRA-3′).

Validation of the assay

The specificity of *M. agalactiae mp81* gene detection by real-time PCR was evaluated by testing DNA preparations from sterile water (no template controls), *M. agalactiae*-

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negative milk samples, different mycoplasma species (*M. mycoides* subsp. *mycoides* large colony, *M. capricolum* subsp. *capricolum*, *M. mycoides* subsp. *capri*, *M. bovis*, *M. putrefaciens* and *M. capricolum* subsp. *capripneumoniae*) and other pathogens of small ruminants, including Maedi-Visna virus, orf virus, ovine adenovirus, ovine herpesvirus type 2, caprine arthritis-encephalitis virus, *Clostridium perfringens*, *Brucella melitensis*, *Brucella abortus*, *Chlamydophila pecorum*, *Chlamydophila abortus*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Mannheimia haemolytica*, *E. coli*, *Streptococcus agalactiae*, *Streptococcus dysgalactiae*, *Streptococcus uberis*, *Bacillus cereus*, *Pseudomonas aeruginosa* and *Enterococcus durans*. The DNA concentrations of the extracts from ruminant pathogens used in the specificity test were comprised between 0·5 and 1·9 μg μl^{-1}. Nontarget viruses and bacteria were retrieved from our collection, with the exception of the mycoplasma reference strains provided by the Istituto Zooprofilattico Sperimentale della Sardegna or Istituto Zooprofilattico Sperimentale della Sicilia. To evaluate the detection limit of the real-time PCR assay, 10-fold dilutions of the plasmid DNA, ranging from 10^9 to 10^0 copies, were made in a milk sample collected from a *M. agalactiae*-negative sheep and subsequently tested. In addition, 10-fold dilutions of a *M. agalactiae*-positive milk sample containing $4·12 \times 10^8$ DNA copies per millilitre were processed. In order to determine the lowest number of bacteria detected by the established assay, the reference strain Ba/2 (Greco *et al.* 2002) was titrated according to the Taylor's method (Meynell and Meynell 1970) and 10-fold dilutions made in Hayflick broth were tested by real-time PCR. A *M. agalactiae*-negative milk sample was also spiked with serial 10-fold dilutions of the same reference strain and the spiked samples were subjected to culture and real-time PCR. Reproducibility of the assay was performed by testing repeatedly field samples containing several concentrations of *M. agalactiae* DNA, as previously described for other real-time PCR protocols (Decaro *et al.* 2005).

Gel-based PCR

To verify whether the real-time PCR assay had an acceptable sensitivity, its performances were compared with a classic PCR protocol for detection of *M. agalactiae* DNA, modified from Tola *et al.* (1996). A PCR assay targeting a specific *M. agalactiae* DNA fragment (pBT5) was carried out on 10-fold dilutions of the *M. agalactiae*-positive milk sample used for evaluation of the sensitivity of real-time PCR, using AccuPrime™ *Taq* DNA Polymerase System (Invitrogen), according to the manufacture's instructions. After a 2-min step at 94°C for template denaturation and enzyme activation, amplification was obtained with 40 cycles of denaturation at 94°C for 30 s, annealing at 57°C for 30 s and extension at 68°C for 1 min. The PCR product (8 μl) was detected by electrophoresis through a 1·5% agarose gel and visualization under ultraviolet (UV) light after ethidium-bromide staining.

Results

Performances of real-time PCR

Serial 10-fold dilutions of plasmid DNA and corresponding C_T values were used to plot a standard curve (Fig. 1). The generated standard curve covered a dynamic range of nine orders of magnitude and showed linearity over the entire quantification range (slope = 3·493), ensuring an accurate measurement over a very large variety of starting target amounts. The detection limit of the assay was assessed as 10^1 DNA copies per 10 μl of the plasmid template and $4·12 \times 10^1$ DNA copies per millilitre of milk (corresponding to dilution 10^{-7}) for standard DNA and *M. agalactiae*-positive sample, respectively. The titre of the reference strain Ba/2 determined by the most probable number method was $6·5 \times 10^9$ colour changing units (CCU) (average of five replicates), whereas real-time PCR detected the bacterial DNA until 10^{-9} dilutions, corresponding to $6·5 \times 10^0$ CCU. One millilitre of the reference strain Ba/2 was diluted 1 : 10 in milk until a 10^{-10} dilution. After 1-week culturing, the load of mycoplasma was evaluated in the various milk dilutions revealing growth of bacteria until the 10^{-6} dilution, while mycoplasma DNA was detected by real-time until the 10^{-7} dilution.

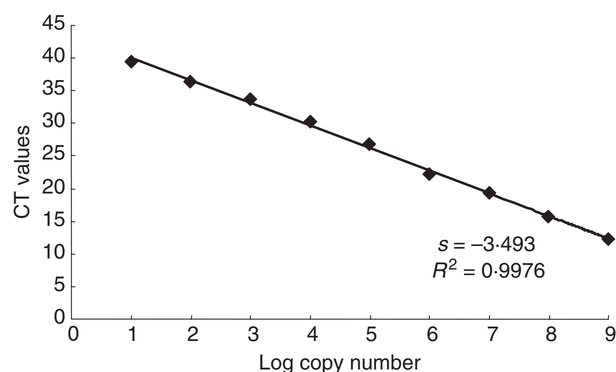

Figure 1 Standard curve of the *Mycoplasma agalactiae* real-time polymerase chain reaction (PCR) assay. Tenfold dilutions of standard DNA prior to amplification were used, as indicated on the *x*-axis, whereas the corresponding cycle threshold (C_T) values are presented on the *y*-axis. Each dot represents the result of duplicate amplifications of each dilution. The coefficient of determination (R^2) and the slope value (*s*) of the regression curve were calculated and are indicated.

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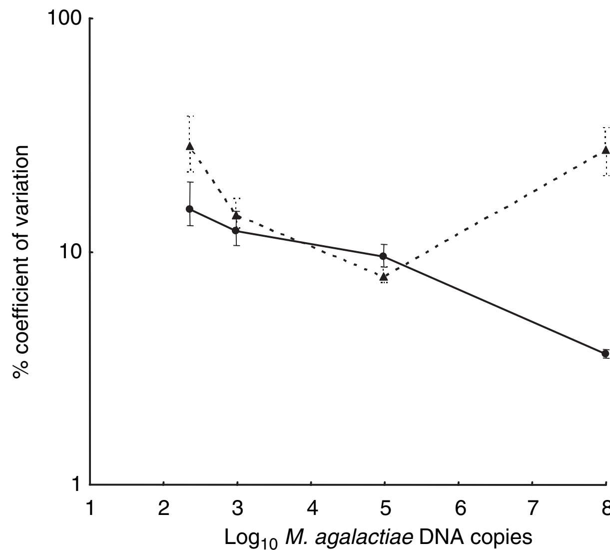

Figure 2 Coefficients of variation intra-assay (●) and interassay (▲) (means ± SE) over the dynamic range of the *Mycoplasma agalactiae* real-time polymerase chain reaction assay.

No detectable fluorescence signal was obtained from the template control, from negative milk samples and from other common pathogens of small ruminants, such as mycoplasmas of the 'mycoides cluster' and *M. bovis*, thus confirming that the assay is highly specific for detection of *M. agalactiae* DNA. In order to determine the reproducibility of the assay, intra-assay and interassay studies were undertaken (Fig. 2). Intraassay CVs ranged from 3·63% (samples containing 10^8 DNA copies) to 15·15% (10^2 DNA copies), while the interassay CVs were comprised between 7·82% (10^5 DNA copies) and 28·41% (10^2 DNA copies).

Comparison with gel-based PCR

By gel-based PCR, detectable amplicons of *M. agalactiae* in the field sample were obtained until the 10^{-6} dilution, which corresponds to $4{\cdot}12 \times 10^2$ copies of *M. agalactiae* DNA per millilitre of milk. Amplicons were obtained until the 10^{-8} dilution of the reference strain Ba/2, which corresponds to $6{\cdot}5 \times 10^1$ CCU. Accordingly, gel-based PCR proved to be one-log less sensitive than real-time PCR.

Analysis of milk samples

Milk samples collected from sheep with clinical signs typical of CA were found to contain varying quantities of DNA ranging between $6{\cdot}20 \times 10^4$ and $6{\cdot}25 \times 10^9$ DNA copies per millilitre of milk. Higher DNA loads were detected in the cultured samples, with the DNA load ranging between $1{\cdot}62 \times 10^5$ and $7{\cdot}70 \times 10^{11}$ DNA copies per millilitre of broth (Table 1). In all the field samples positive to *M. agalactiae* by real-time PCR, the presence of mycoplasma was confirmed using an m-PCR assay (Greco *et al.* 2001).

IC detection

The IC was detected in all the examined samples, with C_T values below the threshold value of 37·88 (average plus 2SD). Therefore, significant DNA losses did not occur during nucleic acid extraction and DNA polymerase inhibition was not observed during real-time PCR amplification.

Discussion

In this study, a real-time PCR assay was developed for detection and quantification of *M. agalactiae* DNA in milk samples. The assay displayed several advantages over conventional PCR, allowing increase in laboratory throughput. Real-time PCR for *M. agalactiae* was more sensitive than conventional PCR based on ethidium-bromide staining, being able to detect as few as 10^1 copies of plasmid DNA and $6{\cdot}5 \times 10^0$ CCU of the reference strain Ba/2. The assay was highly reproducible and linear over a range of nine orders of magnitude, from 10^1 to 10^9 copies of plasmid DNA, ensuring a precise calculation of *M. agalactiae* DNA load in milk samples, as demonstrated by the good reproducibility (intra-assay and interassay CVs). Compared with classical PCR protocols, the time required for processing samples by real-time PCR is shorter, contamination risks are lower because of the lack of post-PCR steps and specificity is increased by the use of probe hybridization.

Hitherto methods allowing rapid and specific quantification of *M. agalactiae* were not available. Titration in broth medium is not carried out routinely as it is time-consuming, requiring approximately 1 week for a definitive assessment of the mycoplasma counts. Recently, flow cytometry has been proposed to enumerate mycoplasmas in broth medium in order to replace traditional assays such as plate count, but this technique requires preliminary adaptation of mycoplasmas to growth *in vitro* (Assunção *et al.* 2006). In contrast, real-time PCR was carried out directly on the original samples avoiding the use of bacterial cultures. Accordingly, the real-time PCR assay proved to be a useful, practical and fast technique to enumerate mycoplasmas. Importantly, the novel real-time PCR assay may be applied profitably to the study of the pathogenesis of *M. agalactiae* to gain insights into the patterns (magnitude and duration) of shedding in naturally infected animals. Furthermore, the assay may be applied conveniently to the study and development of vaccines, to evaluate the patterns of shedding of *M. agalactiae* in large-scale trials for evaluation of vaccine efficacy.

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References

Assunção, P., Rosales, R.S., Rifatbegovic, M., Antunes, N.T., De la Fe, C., Ruiz de Galarreta, C.M. and Poveda, J.B. (2006) Quantification of mycoplasmas in broth medium with sybr green-I and flow cytometry. *Front Biosci* **11**, 492–497.

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A. and Struhl, K. (1993) *Current Protocols in Molecular Biology*, **vol. 1**. New York: Wiley Interscience.

Bergonier, D., Berthelot, X. and Poumarat, F. (1997) Contagious agalactia of small ruminants: current knowledge concerning epidemiology, diagnosis and control. *Rev Sci Tech* **16**, 848–873.

Buonavoglia, D., Fasanella, A., Greco, G. and Pratelli, A. (1999) A study on an experimental infection of sheep with *Mycoplasma agalactiae*. *New Microbiol* **22**, 27–30.

Chavez Gonzalez, Y.R., Ros Bascunana, C., Bolske, G., Mattsson, J.G., Fernandez Molina, C. and Johansson, K.E. (1995) *In vitro* amplification of the 16S rRNA genes from *Mycoplasma bovis* and *Mycoplasma agalactiae* by PCR. *Vet Microbiol* **47**, 183–190.

DaMassa, A.J. (1995) Mycoplasma infections of goats and sheep. In *Molecular and Diagnostic Procedures in Mycoplasmology*, **vol. II** ed.Tully, J.G. and Razin, S. pp. 265–273. San Diego: Academic Press.

DaMassa, A.J., Brooks, D.L., Holmberg, C.A. and Moe, A.I. (1987) Caprine mycoplasmosis: an outbreak of mastitis and arthritis requiring the destruction of 700 goats. *Vet Rec* **102**, 409–413.

DaMassa, A.J., Wakenell, S. and Brooks, D.L. (1992) Mycoplasmas of goat and sheep. *J Vet Diagn Invest* **4**, 101–113.

De la Fe, C., Assunção, P., Antunes, T., Rosales, R.S. and Poveda, J.B. (2005) Microbiological survey for *Mycoplasma* spp. in a contagious endemic area. *Vet J* **170**, 257–259.

Decaro, N., Elia, G., Martella, V., Desario, C., Campolo, M., Di Trani, L., Tarsitano, E., Tempesta, M. *et al.* (2005) A real-time PCR assay for rapid detection and quantitation of canine parvovirus type 2 DNA in the feces of dogs. *Vet Microbiol* **105**, 19–28.

Dedieu, L., Mady, V. and Lefevre, P.C. (1995) Development of two PCR assays for the identification of mycoplasmas causing contagious agalactia. *FEMS Microbiol Lett* **129**, 243–250.

Desario, C., Decaro, N., Campolo, M., Cavalli, A., Cirone, F., Elia, G., Martella, V., Lorusso, E. *et al.* (2005) Canine parvovirus infection: which diagnostic test for virus? *J Virol Methods* **121**, 179–185.

Foddai, A., Idini, G., Fusco, M., Rosa, N., De la Fe, C., Zinellu, S., Corona, L. and Tola, S. (2005) Rapid differential diagnosis of *Mycoplasma agalactiae* and *Mycoplasma bovis* based on a multiplex-PCR and a PCR-RFLP. *Mol Cell Probes* **19**, 207–212.

Freundt, E.A. (1983) Culture media for classic mycoplasmas. In *Methods in Mycoplasmology*, **vol. I** ed.Razin, S. and Tully, J.G. pp. 127–135. New York: Academic Press.

Gorton, T.S., Barnett, M.M., Gull, T., French, R.A., Lu, Z., Kutish, G.F., Adams, L.G. and Geary, S.J. (2005) Development of real-time diagnostic assays specific for *Mycoplasma mycoides* subspecies *mycoides* small colony. *Vet Microbiol* **111**, 51–58.

Greco, G., Corrente, M., Martella, V., Pratelli, A. and Buonavoglia, D. (2001) A multiplex-PCR for the diagnosis of contagious agalactia of sheep and goats. *Mol Cell Probes* **15**, 21–25.

Greco, G., Corrente, M., Buonavoglia, D., Aliberti, A. and Fasanella, A. (2002) Inactivated vaccine induces protection against *Mycoplasma agalactiae* infection in sheep. *New Microbiol* **25**, 17–20.

Heid, C.A., Stevens, J., Livak, K.J. and Williams, P.M. (1996) Real time quantitative PCR. *Genome Res* **6**, 986–994.

Leach, R., Costas, M. and Michelmore, D.L. (1989) Relationship between *Mycoplasma mycoides* subsp. *mycoides* (large-colony strain) and *M. mycoides* subsp. *capri*, as indicated by numerical analysis of one-dimensional SDS-PAGE protein patterns. *J Gen Microbiol* **135**, 2993–3000.

Mattsson, J.G., Guss, B. and Johansson, K.E. (1994) The phylogeny of *Mycoplasma bovis* as determined by sequence analysis of the 16S rRNA gene. *FEMS Microbiol Lett* **115**, 325–328.

Meynell, G.C. and Meynell, E. (1970) Quantitative aspects of microbiological experiments. In *Theory and Practice in Experimental Bacteriology*, 2nd edn. pp. 173–196. London: Cambridge University Press.

Nicholas, R. (2002) Improvements in the diagnosis and control of diseases of small ruminants caused by mycoplasmas. *Small Rumin Res* **45**, 145–149.

Pinnow, C.C., Butler, J.A., Sachse, K., Hotzel, H., Timms, L.L. and Rosenbusch, R.F. (2001) Detection of *Mycoplasma bovis* in preservative-treated field milk samples. *J Dairy Sci* **84**, 1640–1645.

Ross, R.F. (1993) Mycoplasma-animal pathogens. In *Rapid Diagnosis of Mycoplasmas* ed.Kahane, I. and Adoni, A. pp 69–109. New York: Plenum Press.

Subramaniam, S., Bergonier, D., Poumarat, F., Capaul, S., Schalatter, Y., Nicolet, J. and Frey, J. (1998) Species identification of *Mycoplasma bovis* and *Mycoplasma agalactiae* based on the *uvr*C genes by PCR. *Mol Cell Probes* **12**, 161–169.

Tola, S., Idini, G., Manunta, D., Galleri, G., Angioi, P.P., Rocchigiani, A.M. and Leori, G. (1996) Rapid and specific detection of *Mycoplasma agalactiae* by polymerase chain reaction. *Vet Microbiol* **51**, 77–84.

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