Sensitivity of two methods to detect *Mycoplasma agalactiae* in goat milk

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**Abstract**

**Background:** Laboratory diagnostic techniques able to detect *Mycoplasma agalactiae* are essential in contagious agalactia in dairy goats. This study was designed: 1) to determine the detection limits of PCR and culture in goat milk samples, 2) to examine the effects of experimental conditions including the DNA extraction method, PCR technique and storage conditions (fresh versus frozen stored milk samples) on these methods and 3) to establish agreement between PCR and culture techniques using milk samples from goats with mastitis in commercial dairy herds. The study was conducted both on artificially inoculated and field samples.

**Results:** Our findings indicate that culture is able to detect *M. agalactiae* in goat milk at lower concentrations than PCR. Qualitative detection of *M. agalactiae* by culture and PCR was not affected by sample freezing, though the DNA extraction method used significantly affected the results of the different PCR protocols. When clinical samples were used, both techniques showed good agreement.

**Conclusions:** The results from this study indicate that both culture and PCR are able to detect *M. agalactiae* in clinical goat mastitis samples. However, in bulk tank milk samples with presumably lower *M. agalactiae* concentrations, culture is recommended within the first 24 h of sample collection due to its lower limit of detection. To improve the diagnostic sensitivity of PCR in milk samples, there is a need to increase the efficiency of extracting DNA from milk samples using protocols including a previous step of enzymatic digestion.

**Keywords:** Goat, Contagious agalactia, PCR, Culture, Milk, *Mycoplasma agalactiae*
a second experiment, agreement between PCR and culture techniques was assessed using milk samples from mastitic goats from dairy herds of a CA endemic area.

**Methods**

For the experimental study, five goat milk samples were obtained: one bulk tank milk sample and four from randomly selected goats. All samples tested negative for *M. agalactiae* by conventional culture [9] and PCR [5].

Each of these five samples was divided in seven 4 ml aliquots which were inoculated with serial fold dilutions in the range 10 to 10^7 (except one of the samples, which yielded a concentration of 10^6 CFU/ml of a PG2 inoculum (reference strain of *M. agalactiae* NCTC 10123). In addition, a negative control (non inoculated aliquot) was included for each sample. Then, each aliquot was divided into two 2 ml aliquots: one was examined after 24 h of refrigerated storage and the other after freezing (−21 °C) for 14 days and thawing at room temperature. A total of 80 aliquots were analyzed. 200 µl of the samples were cultured in liquid PH medium [10]. After 24 h of incubation at 37 °C, the samples were filtered (0.45 µm), and afterwards cultured in agar PH medium so as to detect the presence of typical mycoplasma colonies. Two further 200 µl-aliquots were obtained to use two different DNA extraction methods, one as described by Tola et al. [8] and the other using a commercial kit (High Pure PCR Template Preparation Kit, Roche Diagnostics). Subsequently, all samples were analyzed using the three PCR protocols previously described [4–6]. PCR conditions are detailed in Table 1. PCR results were compared with a negative control and a positive control (DNA of the PG2 strain).

For the field study, 255 clinical mastitis samples were obtained from 80 dairy goats herds reared in an endemic CA area. All samples collected were kept refrigerated at 4 °C and arrived at our laboratory within the following 24 h. Immediately after their arrival, these samples were cultured following the same protocol described in our experimental study. Then, 200 µl of each milk sample were processed with DNA extraction prior to PCR diagnosis [8, 5].

Agreement between PCR and culture techniques was determined using Win Episcope 2.0 software [11]. Thrusfield criteria [12] were used to interpret kappa values. The Chi squared test implemented in Epi Info 3.5.2 software [13] was used to assess the factors that affect the sensitivity of the techniques, which was calculated as the proportion of inoculated samples detected by the different methods used.

**Results and discussion**

The PCR protocols described by Tola et al. [4], Marenda et al. [5] and De la Fe et al. [6] did not differ significantly from each other. Moreover, no significant differences were detected between fresh and frozen samples. When comparing the DNA extraction methods used together with the PCR protocols, the commercial kit allowed the detection of 44.6 % positive samples (95 % CI; 37.8 %, 51.4 %), while this rate was significantly lower, 33.8 %, after using the extraction method described by Tola et al. [8] (95 % CI; 27.3 %, 40.3 %) (Table 2). Negative controls showed negative results in all cases. These differences observed between the DNA extraction methods could be explained by the additional step of enzymatic protein denaturation carried out by proteinase-K when using the commercial kit. Furthermore, it has been demonstrated that the use of this enzyme could also inactivate some inhibitors which are present in milk samples [14]. In this sense, Becker et al. [7] obtained a lower limit of detection of mycoplasmas when dilutions were prepared in water rather than milk.

The detection limit yielded was lower for the culture technique than for the PCR protocols (10 CFU/ml with a sensitivity of 33.3 %) (Table 3), and there were no significant differences between fresh and frozen samples. Thereby, the culture method was able to detect more positive samples than the PCR protocols used and its detection limit was also lower, suggesting a lower sensitivity of PCR when used directly in milk samples.

In previous studies, it has been observed that *M. agalactiae* can be found in milk at concentrations of 10^6 up to 10^8 CFU/ml [15]. Moreover, Castro-Alonso et al., [16] showed by experimental inoculation that this concentration could even reach values of 10^10 to 10^12 CFU/ml. Therefore, considering our limits of detection (above 10^6 with culture and PCR), both PCR and culture methods should be able to detect the presence of

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**Table 1 PCR conditions for each protocol**

| Reference                | Primers                                             | PCR conditions                                                                 |
|--------------------------|-----------------------------------------------------|--------------------------------------------------------------------------------|
| Tola et al. (1996) [8]   | 5'-AAAAGGTGCTTGAAGAAATGGC-3' 5'-GTTGGCAGAAGAAAGTCCAAATCA-3' | 94 °C, 5 min.; (94 °C, 1 min. 64 °C, 1 min.; 72 °C,1 min.) x 35 cycles; 72 °C, 10 min. |
| Marenda et al. (2005) [5]| 5'-CATGACCTACCTGATTCAATTTCG-3' 5'-CTATGTGATCATGCTTGTGGTTGA-3' | 94 °C, 5 min.; (94 °C, 1 min.; 58 °C, 1 min.; 72 °C, 1 min.) x 30 cycles; 72 °C, 10 min. |
| De la Fe et al. (2012) [6]| 5'-GCAGCTTTTGTAGTGAAGA-3' 5'-CCTAAAGCAACCTTTATAACTG-3' | 94 °C, 2 min.; (94 °C, 30 sec; 49 °C, 30 sec; 72 °C, 30 sec.) x 30 cycles; 72 °C, 5 min. |
mycoplasmas from the initial days postinfection when the concentrations of *M. agalactiae* are above 10^7 CFU/ml, because both techniques show high values of sensitivity [15, 16]. However, when the concentration of mycoplasmas is unknown, as it happens with the bulk tank milk samples, where obviously these values are lower than in individual samples from infected animals, the use of PCR protocols for bulk tank samples with a limit of detection of 10^7–10^8 could be inadequate. Therefore, for the diagnosis of CA from bulk tank milk, samples should be previously cultivated and the DNA extraction should be carried out on the culture rather than on milk sample, in order to improve its sensitivity [17].

Of the 255 clinical samples examined, 58 were culture positive and 47 were PCR positive for *M. agalactiae*, while 41 samples were culture and PCR positive and 191 scored negative using both techniques. Our agreement study revealed a kappa coefficient of 0.725, indicating a good agreement between the two methods [12]. This is probably due to the concentration range of *M. agalactiae* which is usually found in mastitic goat milk samples (10^7 to 10^12 CFU/ml), that has been previously described [15, 16].

### Table 2 PCR results obtained according to the DNA extraction method and the *Mycoplasma agalactiae* concentration used

| *M. agalactiae* concentration (CFU/ml) | Method of extraction | Negative | Positive | Se (%) | 95 % CI | Negative | Positive | Se (%) | 95 % CI |
|---------------------------------------|----------------------|----------|----------|--------|---------|----------|----------|--------|---------|
| 10^7                                  | Tola et al. (1997) [8] | 30       | 0        | 0      |         | 30       | 0        | 0      |         |
| 10^7                                  | Commercial kit       | 29       | 1        | 3.3    | (−3.1 %, 9.8 %) | 29       | 1        | 3.3    | (−3.1 %, 9.8 %) |
| 10^6                                  | Tola et al. (1997) [8] | 24       | 6        | 20     | (5.7 %, 34.3 %) | 24       | 6        | 20     | (5.7 %, 34.3 %) |
| 10^6                                  | Commercial kit       | 22       | 8        | 26.7   | (10.8 %, 42.5 %) | 22       | 8        | 26.7   | (10.8 %, 42.5 %) |
| 10^5                                  | Tola et al. (1997) [8] | 8        | 22       | 73.3   | (57.5 %, 89.2 %) | 24       | 6        | 20     | (5.7 %, 34.3 %) |
| 10^5                                  | Commercial kit       | 0        | 30       | 100    |         | 0        | 30       | 100    |         |
| 10^4                                  | Tola et al. (1997) [8] | 0        | 24       | 100    |         | 0        | 24       | 100    |         |
| 10^4                                  | Commercial kit       | 113      | 91       | 44.6   | (27.3 %, 40.3 %) | 113      | 91       | 44.6   | (27.3 %, 40.3 %) |

Se: sensitivity at each concentration

a, b: Values with different superscripts differ significantly (*p* < 0.05)

c: High Pure PCR Template Preparation Kit, Roche Diagnostics

### Table 3 Culture results according to the concentration of *Mycoplasma agalactiae* used to inoculate the goat milk samples

| *M. agalactiae* concentration (CFU/ml) | Culture results | Negative | Positive | Sensitivity (%) |
|---------------------------------------|----------------|----------|----------|-----------------|
| 10^7                                  |                | 38       | 18       | 33.3            |
| 10^7                                  |                | 12       | 42       | 77.8            |
| 10^8                                  |                | 12       | 42       | 77.8            |
| 10^8                                  |                | 6        | 48       | 88.9            |
| 10^9                                  |                | 0        | 54       | 100             |
| 10^9                                  |                | 0        | 54       | 100             |
| 10^10                                 |                | 0        | 42       | 100             |

### Conclusion

In conclusion, our findings show that both culture and PCR are able to detect *M. agalactiae* in clinical goat mastitis samples. However, in bulk tank milk samples with presumably lower *M. agalactiae* concentrations, culture within the first 24 h of sample collection is recommended. In order to improve the diagnostic sensitivity of PCR in milk samples, it is also necessary to increase the efficiency of the DNA extracting methods from milk samples, for example by using protocols with a previous enzymatic digestion so as to reduce the presence of inhibitors in our sample.

### Competing interests

The authors declare that they have no competing interests.

### Authors’ contributions

AS, JA, AC, JJC and CDF participated in study design and coordination, and drafted the manuscript. JTD drafted the manuscript. JTD, JA, MPH, AGM and AP participated in microbiological analysis. All authors read and approved the manuscript.

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### References

1. Corrales JC, Esnal A, De La Fe C, Sánchez A, Assunção P, Poveda JB, et al. Contagious agalactia in small ruminants. Small Ruminant Res. 2007;68:154–66.

2. Amores J, Sánchez A, Gómez-Martin A, Corrales JC, Contreras A, De La Fe C. Surveillance of *Mycoplasma agalactiae* and *Mycoplasma mycoides* subsp. capri in dairy goat herds. Small Ruminant Res. 2012;102:89–93.

3. Nicholas, R. Contagious agalactia. In. Office International of Epizootics, editors. Manual of Diagnostic Tests and Vaccines for Terrestrial Animals. Paris: Office International of Epizootics; 2008. p. 992–999.

4. Tola S, Idini G, Manunta D, Galleni G, Angioi A, Rochigiani AM, et al. Rapid and specific detection of *Mycoplasma agalactiae* by polymerase chain reaction. Vet Microbiol. 1996;67–84.

5. Marenda MS, Sagne E, Poumarat F, Citti C. Suppression subtractive hybridization as a basis to assess *Mycoplasma agalactiae* and
Mycoplasma bovis genomic diversity and species-specific sequences. Microbiology. 2005;151:475–89.

6. De La Fe C, Amores J, Tardy F, Sagne E, Nouvel L-X, Citti C. Unexpected genetic diversity of Mycoplasma agalactiae caprine isolates from an endemic geographically restricted area of Spain. BMC Vet Res. 2012;8:146–54.

7. Becker CAM, Ramos F, Sellal E, Moore S, Pournar M, Tardy F. Development of a multiplex real-time PCR for contagious agalactia diagnosis in small ruminants. J Microbiol Methods. 2012;90:73–9.

8. Tola S, Angioi A, Rocchigiani AM, Idini G, Manunta D, Galleri G, et al. Detection of Mycoplasma agalactiae in sheep milk samples by polymerase chain reaction. Vet Microbiol. 1997;54:17–22.

9. Nicholas R, Baker S. Recovery of Mycoplasmas from Animals. In: Miles R, Nicholas R, editors. Methods in Molecular Biology. Volume 104. Totowa, New Jersey: Humana Press Inc; 1998. p. 37–44. Mycoplasma protocols.

10. Gómez-Martin A, De la Fe C, Amores J, Sánchez A, Contreras A, Paterna A, et al. Anatomic location of Mycoplasma mycoides subsp. capri and Mycoplasma agalactiae in naturally infected goat male auricular carriers. Vet Microbiol. 2012;157:355–62.

11. Thrustfield M, Ortega C, Di Bias I, Noordhuizen JP, Frankena K. Win Episcope 2.0: improved epidemiological software for veterinary medicine. Vet Rec. 2001;148:567–72.

12. Thrustfield M. Diagnostic testing. In: Thrustfield, editor. Veterinary Epidemiology, Chapter 17. Oxford M, Blackwell Science Ltd; 2005. p. 305–29.

13. Dean AG, Dean JA, Coulombier D, Brederel KA, Smith DC, Burton AH, et al. Epi Info, Version 6.04a, a word processing, database, and statistics program for public health on IBM-compatible microcomputers. Atlanta: Centers for Disease Control and Prevention; 1996.

14. Bickley J, Short JK, Mcdowell DG. Polymerase chain reaction (PCR) detection of Listeria monocytogenes in diluted milk and reversal of PCR inhibition caused by calico ions. Lett Appl Microbiol. 1996;22:153–8.

15. Kinde H, DaMassa A, Wakenell P, Petty R. Mycoplasma infection in a commercial goat dairy caused by Mycoplasma agalactiae and Mycoplasma mycoides subsp. mycoides (caprine biotype). J Vet Diagn Invest. 1994;6:423–7.

16. Castro-Alonso A, De la Fe C, Espinosa de los Monteros A, Rodríguez F, Andrada M, Poveda JB, et al. Chronological and immunohistochemical characterization of the mammary immunoinflammatory response in experimental caprine contagious agalactiae. Vet Immunol Immunopathol. 2010;136:43–54.

17. Oravcová K, López-Enríquez L, Rodríguez-Lázaro D, Hernández M. Mycoplasma agalactiae p40 Gene, a Novel Marker for Diagnosis of Contagious Agalactia in Sheep by Real-Time PCR: Assessment of Analytical Performance and In-House Validation Using Naturally Contaminated Milk Samples. J Clin Microbiol. 2009;47:1445–50.

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