Bulgarian Journal of Veterinary Medicine, 2023 ONLINE FIRST
ISSN 1311-1477; DOI: 10.15547/bjvm.2023-0014

Short communication

MOLECULAR DETECTION OF *MYCOPLASMA AGALACTIAE* BY qPCR IN SHEEP AND GOATS FROM BULGARIA

C. EVSTATIEV[1], V. URUMOVA[2], L. IVANOV[2] & V. PETROV[2]

[1]PhD student, Faculty of Veterinary Medicine, Trakia University, Stara Zagora, Bulgaria;
[2]Department of Veterinary Microbiology, Infectious and Parasitic Diseases, Faculty of Veterinary Medicine, Trakia University, Stara Zagora, Bulgaria

Summary

Evstatiev, C., V. Urumova, L. Ivanov & V. Petrov, 2023. Molecular detection of *Mycoplasma agalactiae* by qPCR in sheep and goats from Bulgaria. *Bulg. J. Vet. Med.* (online first).

Contagious agalactia is an infectious disease of small ruminants caused by several *Mycoplasma* species with leading role of *M. agalactiae*. Clinical signs are manifested by mastitis, arthritis, keratoconjunctivitis, abortion, pneumonia and septicaemia often in various combinations. The aim of the study was to tested milk samples from sheep and goats in herds with different health status by a rapid, sensitive, specific method for the detection and quantification of DNA of *M. agalactiae*. A total of 67 milk samples from sheep and goats were tested by real-time polymerase chain reaction (qPCR). Thirteen were positive for DNA and 54 were negative. The present study is the first report of detection of *M. agalactiae* DNA in Bulgaria.

Key words: *Mycoplasma agalactiae*, contagious agalactia, sheep, goats, qPCR, milk samples

Contagious agalactia is an infectious disease of small ruminants, endemic in Mediterranean countries and the Balkan Peninsula. It is caused by several *Mycoplasma* species with leading role of *Mycoplasma agalactiae* (*M. agalactiae*). It is responsible for severe losses in milk production in sheep (Kumar *et al.*, 2014; Tumino *et al.*, 2020). Clinical signs are manifested by mastitis, arthritis, keratoconjunctivitis, abortion, pneumonia and septicaemia often in various combinations. In lactating sheep and goats, the course of the disease usually starts as unilateral mastitis, with warm, swollen, painful parenchyma, followed by a change in milk quality with a drastic decline in milk production. This form ends with sclerosis of the udder (Nicholas *et al.*, 2008a; Tolone *et al.*, 2019). The spread of the infection in the affected farms is rapid and after a short period of time can affect up to 30–85% of the animals (Campos *et al.*, 2013).

Early diagnosis is necessary for effective disease management, control of the spread of infection within a herd and reduction of direct losses. Diagnostics of contagious agalactia is still challenging, time-consuming and requires well-equipped laboratories to confirm outbreaks. Therefore, rapid and accurate diagnostic tests are needed. Classical methods include isolation followed by differentiation and serological tests (Kumar *et al.*, 2014). The isolation of the mycoplasmae in selective enrichment media is a laborious and time-consuming process, as mycoplasmae grow very slowly. Primary isolation and subsequent biochemical identification procedure takes at least 2 weeks (WOAH, 2018). Culture and identification of the organism are recommended for individual animal testing in herds free from infection, control of efficacy after eradication programme implementation and confirmation of clinical cases.

At the same time these methods are recommended yet with limitations for the population free from infection and are not appropriate to assess the prevalence of infection (surveillance programmes) (WOAH, 2018). Serological tests can be ineffective in the first stage of the disease, which can lead to false negative results, since antibodies are detected only after 10–15 days after infection. Furthermore, serological tests cannot distinguish post-infection from post-vaccination antibodies, making them not applicable for diagnosis in vaccinated herds. (Buonavoglia *et al.*, 1999; Tumino *et al.*, 2020). In recent decades, a number of polymerase chain reaction (PCR) and real-time quantitative PCR-based assays have been developed for the rapid detection of *M. agalactiae.* Conventional and real-time PCR are recommended for individual animal testing in infection-free herds and for confirmation of clinical cases, but are recommended with limitations for assessment of infection prevalence (surveillance programmes) (WOAH, 2018). Real-time PCR for *M. agalactiae* is very sensitive, being able to detect as few as 10 copies of DNA. Compared to classical PCR protocols, the time required for sample processing is shorter, contamination risks are lower due to the absence of post-PCR steps, and specificity is increased by the use of probe hybridisation. In addition, real-time PCR avoids the need from mycoplasmae culturing. Therefore, real-time PCR analysis is a useful, practical and rapid technique for the diagnosis of *M. agalactiae* infections (Lorusso *et al.*, 2007; Lin *et al.*, 2019).

The aim of the study was to test milk samples from sheep and goats in herds with different health status by a rapid, sensitive, and specific method for detection and quantification of DNA of *M. agalactiae.*

Eight farms were included in the study. In farm 1, 65 mixed breed animals were housed, herd 2 was formed by 24 mixed breed sheep, farm 3 housed 190 sheep and 60 goats again from a mixed breed and farm 4 – 47 mixed-breed animals. The animals in all farms were housed in a barn with indoor and outdoor parts, they were fed hay, concentrate feed and on pasture. Prophylactic deworming was performed twice a year.

In these four farms, field studies for monitoring of the postvaccinal immune response in sheep and goats after vaccination against contagious agalactia were previously conducted (Evstatiev *et al.*, 2020; 2021). All animals were vaccinated every 6 months with Agalax S (Laboratorio SYVA). No clinical signs compatible with those of infectious agalactia were recorded.

From each herd, 6 milk samples were obtained from at least 4-year-old animals. They were tested for asymptomatic carriage of *M. agalactiae* in vaccinated small ruminants.

In farm 5 about 1,200 goats, divided into six herds and fed a total mix ration (TMR) were reared. The animals were milked automatically, in a milking parlour. In 2021, despite the vaccination with Agalax S (Laboratorio SYVA) against infectious agalactia, mastitis with the development of hypo- or agalactia, arthritis as well as keratoconjunctivitis were found in more than 100 animals. Blood, milk and synovial fluid samples were sent to a laboratory in the Netherlands and were tested by qPCR. The result obtained was positive for *Mycoplasma* spp. and negative for *M. capricolum* (personal communication). Subsequently, the vaccine used to prevent the disease was changed to Galazel (Intervet Schering Plow Animal Health). In the following months, clinical signs characteristic of infectious agalactia were observed only in single goats. Twenty milk samples from clinically healthy animals with data on previous mastitis were obtained from this farm.

In farm 6, 85 sheep were housed, in farm 7 – 200 goats and 150 sheep, and in farm 8 – 300 goats. In these three farms, the animals were housed in barns with indoor and outdoor parts, fed roughage (hay and silage), grain mixtures and on pasture. Regular prophylactic dewormings were carried out twice a year.

The clinical signs characteristic of contagious agalactia were observed at farms 2, 3 and 4. Animal owners reported worsening of the general condition (anorexia, lethargy) and fever. Mastitis with the development of hypo- or agalactia, arthritis and keratoconjunctivitis were found. During the milking period, the mammary glands of the affected animals were warm, swollen and painful. The milk was yellowish in colour and with reduced amount. From these farms, 7, 10 and 6 milk samples were obtained from untreated animals with clinical signs of disease, respectively.

A total of 67 milk samples were tested. Samples were obtained in sterile containers after aseptic preparation of the milk papilla and discarding of the first amounts of milk. The samples were transported at 4–8 °C to the Molecular Genetics Laboratory of the Department of Veterinary Microbiology, Infectious and Parasitic Diseases at the Faculty of Veterinary Medicine of Trakia University. The summary data on the number of samples, clinical signs in the animals from which they were obtained, milk quality are presented in Table 1.

For DNA extraction, the DNeasy Blood Tissue kit (Qiagen Germany) and the spin-column technology was used. DNA extraction was performed as described in the manufacturer's instructions with a modification similar to that used by Volk *et al.* (2014) for pretreatment of the samples. Modification consisted of the following: in the initial step, 1 mL of each milk sample was centrifuged for 10 minutes at 14,000 rpm. The supernatant was then decanted. The remaining pellet was used as the starting sample material.

qPCR assays were performed with a Stratagene Mx3000P (Agilent Technologies) using the company's pre-validated MycAga dtec-qPCR kit (Genetic PCR solutionsTM, Spain), following the manufacturer's recommendations. The thermal profile of the amplification reaction included denaturation at 95 °C for 10 minutes and the next step involved 3 stages repeated in 40 cycles (Table 2).

Table 1. Number of samples tested in each farm with clinical signs, milk quality, and number of qPCR positive and negative samples

	Samples number	Clinical signs of animals (number of animals)	Milk quality (number of animals)	qPCR positive	qPCR negative
Farm 1	6	Healthy	Normal	0	6
Farm 2	6	Healthy	Normal	0	6
Farm 3	6	Healthy	Normal	0	6
Farm 4	6	Healthy	Normal	0	6
Farm 5	20	Healthy	Normal	5	15
Farm 6	6	Mastitis (6) Hypogalactia (3) Keratoconjunctivitis (1) Arthritis (1)	Yellowish (3) Watery consistence (2) Bloody (1)	2	4
Farm 7	10	Mastitis (10) Hypogalactia (4) Keratoconjunctivitis (1) Arthritis (2)	Yellowish (6) Yellowish with clots (2) Watery consistence (1) Bloody (1)	4	6
Farm 8	7	Mastitis (6) Hypogalactia (4) Keratoconjunctivitis (1) Arthritis (1)	Yellowish (3) Yellowish with clots (3) Watery consistence (1)	2	5

Table 2. The qPCR programme

Steps		Time	Temperature
40 cycles	Activation	60 s	95 ºC
	Denaturation	10 s	95 ºC
	Annealing/ elongation	60 s	60 ºC

The Ct values of the samples were interpreted based on the Ct value of the positive control (≤ 29).

All 24 samples from the first 4 farms (monitoring of the postvaccinal immune response) were negative. In none of them qPCR has found Ct values.

Out of the 43 milk samples obtained from farms 5–8 (with history or clinical signs of contagious agalactia), 13 showed Ct values lower than those of the positive control and were defined as positive. In 23 samples, Ct values were not found. In the remaining 8 samples the Ct values were higher than those of the positive control and were defined as negative. The results for positive and negative milk samples with Ct values are presented on Fig. 1.

Contagious agalactia is in the World Organization for Animal Health (OIE/ WOAH) list of notifiable diseases (OIE, 2020). In recent years, the disease has been reported in several Balkan countries – Greece, Albania, North Macedonia. Other Southeastern European countries (Bulgaria, Croatia, Slovenia, Montenegro, Bosnia and Herzegovina, and Serbia) have not reported any spread of contagious agalactia (Jaÿ & Tardy, 2019). However,

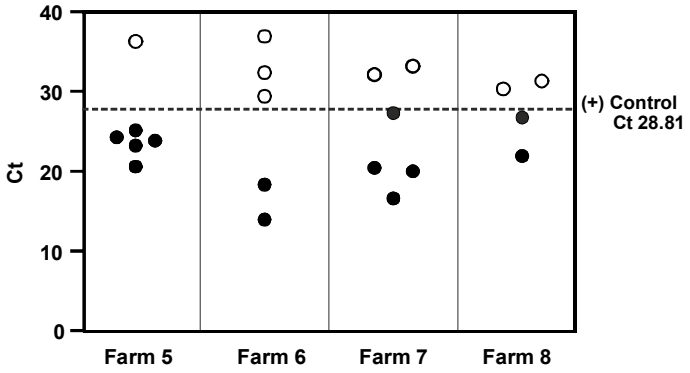

Fig. 1. Positive (black dots) and negative (white dots) qPCR amplifications for *M. agalactiae* DNA extract from milk samples from farms 5–8. The dotted line corresponds to positive control.

the clinical cases or studies of contagious agalactia for several of these countries are encountered in the scientific literature. In Bosnia and Herzegovina, Maksimović *et al.* (2016) examined samples from the respiratory and reproductive system, milk, ear and eye secretions. Nine samples positive for *M. mycoides* subsp. *capri*, 2 for *M. capricolum* subsp. capricolum and 1 for *M. putrefaciens* were established. The authors did not report *M. agalactiae*, although 58 samples were determined to be *Mycoplasma* spp. positive. For the purposes of the research, they used cultivation and identification of the isolates on the basis of colony morphology, sensitivity to digitonin, fermentation of glucose, hydrolysis of arginine, phosphatase activity, film and spot production, the growth inhibition test. PCR assays for *Mycoplasma* group were also used, but not for *M. agalactiae*, which may explain the absence of positive samples for this microorganism.

In the present study has found 13 qPCR positive DNA samples of *M. agalactiae*, which indicates the presence of the pathogen in the examined animals with clinical signs of the disease. In Bulgaria, targeted etiological studies on contagious agalactia have not been conducted since the end of the 1970s (Shabanov *et al.*, 1973). In recent years, only one report suggesting an infection with *M. agalactiae* in Bulgaria was published. In a study on the characterisation of microbial causative agents of subclinical mastitis in goats, Hristov *et al.* (2016) reported that during microscopic examination of milk samples, small cells with mycoplasma morphology were observed. Also, there are no official data on the presence of the disease. However, veterinarians and farmers across the country are reporting diseased sheep and goats with signs of contagious agalactia. As a prophylactic measure, vaccination against this disease is carried out in most farms.

Our qPCR data confirmed the presence of the *M. agalactiae* infection among small ruminant herds. This result was expected, considering that the two neighbouring countries – Turkey and Greece, belonging to the Mediterranean basin, have been endemic for decades (Kumar *et al.*, 2014; Tumino *et al.*, 2020). In Turkey, however, most registered outbreaks are in the Asian part of the country (Öztürkler & Otlu, 2020; Karatekeli & Kenar, 2022). However, Göçmen *et al.* (2016)

reported positive PCR results from a farm in the area of Edirne where clinical manifestations characteristic of contagious agalactia had been observed in the past. While Turkey just declares to the OIE that the disease is present, Greece also reports a number of outbreaks. Thus, for the period 2014–2018, they are 17 (Loria *et al.*, 2019). While in Bulgaria contagious agalactia still remains overlooked by national veterinary services, in 2006 Greece introduced legislation to reduce the frequency of the disease. Farmers have access to free laboratory testing. Owners whose flocks are affected receive compensation for the value of the slaughtered animals, the loss of milk production and the associated costs of disease control. After 2010, when the incidence of contagious agalactia in goats was found to be higher than that in sheep, the control plan was modified to eradicate the disease in goat-only and mixed livestock (Loria *et al.*, 2019).

REFERENCES

Buonavoglia, D., A. Fasanella, G. Greco & A. Pratelli, 1999. A study of an experimental infection of sheep with *Mycoplasma agalactiae*. *The New Microbiologica*, **22**, 27–30.

Campos, C., E. O. Azevedo, M. D. B. Alcântara, R. B. S. Silva, A. A. Cordeiro, A. G. Mamede, M. A. Melo, E. R. Nascimento & R. S. Castro, 2013. Efficiency of inactive vaccines against contagious agalactia in Brazil. *Arquivo Brasileiro de Medicina Veterinária e Zootecnia*, **65**, 1394–1402.

Göçmen, H., R. S. Rosales, R. D. Ayling & M. Ülgen, 2016. Comparison of PCR tests for the detection of *Mycoplasma agalactiae* in sheep and goats. *Turkish Journal of Veterinary & Animal Sciences*, **40**, 421–427.

Evstatiev, C., K. Gospodinova & V. Petrov, 2020. Comparative analysis of immune response in goats and sheep after vaccination against contagious agalactia – a field study. *Tradition and Modernity in Veterinary Medicine*, **5**, 3–9.

Evstatiev, C., K. Gospodinova & V. Petrov, 2021. Comparative analysis of the post-vaccinal immune response in sheeps in two farms after vaccination against infectious agalacia – a field study. *Trakia Journal of Sciences*, **19**, 29–34.

Hristov, K., T. Popova, R. Pepovich & B. Nikolov, 2016. Characterization of microbial causative agents of subclinical mastitis in goats in Bulgaria. *International Journal of Current Microbiology and Applied Sciences*, **5,** 316–323.

Jaÿ, M. & F. Tardy, 2019. Contagious agalactia in sheep and goats: current perspectives. *Veterinary Medicine: Research and Reports*, **10**, 229–247.

Karatekeli, U. & B. Kenar, 2022. An investigation of contagious agalactia disease of sheep and goats in Isparta and Afyonkarahisar in Turkey. *Indian Journal of Animal Research*, **56**, 358–361.

Kumar, A., A. Rahal, S. Chakraborty, A. K. Verma & K. Dhama, 2014. *Mycoplasma agalactiae*, an etiological agent of contagious agalactia in small ruminants: A review. *Veterinary Medicine International*, **2014**, 286752.

Lin, Y., J. Jiang, J. Zhang, W. You, & Q. Hu, 2019. Development of a TaqMan real-time PCR for detection of the *Mycoplasma mycoides* subsp. *capri*. *Small Ruminant Research*, **175**, 31–36.

Loria, G. R., R. Puleio, G. Filioussis, R. S. Rosales & R. A. J. Nicholas, 2019. Contagious agalactia: Costs and control revisited. *OIE Revue Scientifique et Technique*, **38**, 1–16.

Lorusso, A., N. Decaro, G. Greco, M. Corrente, A. Fasanella & D. Buonavoglia, 2007. A real-time PCR assay for detection and quantification of *Mycoplasma agalactiae* DNA. *Journal of Applied Microbiology*, **103**, 918–923.

Maksimović, Z., A. Bačić & M. Rifatbegović, 2016. Mycoplasmas isolated from ruminants in Bosnia and Herzegovina between 1995 and 2015. *Veterinaria*, **65**, 27–31.

Nicholas, R. A. J., R. D. Ayling & G. R. Loria, 2008a. Ovine mycoplasmal infections. *Small Ruminant Research*, **76**, 92–98.

Nicholas, R., R. Ayling & L. McAuliffe, 2008b. Detection of *Mycoplasma* species using polymerase chain reaction (PCR). In: *Mycoplasma Diseases of Ruminants,* CAB International, Wallingford, UK, pp. 28–34.

OIE, 2020. OIE Listed Diseases, http://www.animalhealthsurveillance.agriculture.gov.ie/oielisteddiseases (11 May 2023 date last accessed).

Öztürkler, O. & S. Otlu, 2020. A phylogenetic analysis of *Mycoplasma* strains circulating in sheep pneumonia in the Kars region of Turkey. T*urkish Journal of Veterinary & Animal Sciences*, **44**, 805–813.

Shabanov, M., A. Toshkov, L. Mihailova & L. Shirova, 1973. Experimental *Mycoplasma agalactiae* mastitis. *Veterinary Science (Sofia)*, **10**, 81–84.

Tolone, M., A. M. Sutera, S. Borrello, S. Tumino, M. L. Scatassa, B. Portolano, R. Nicholas & G. R. Loria, 2019. Effect of *Mycoplasma agalactiae* mastitis on milk production and composition in Valle dell Belice dairy sheep. *Italian Journal of Animal Science*, **18**, 1067–1072.

Tumino, S., M. Tolone, A. Parco, R. Puleio, G. Arcoleo, C. Manno, R. Nicholas & G. R. Loria, 2020. Validation of Loop-Mediated Isothermal Amplification (LAMP) field tool for rapid and sensitive diagnosis of contagious agalactia in small ruminants. *Animals*, **10**, 509.

Volk, H., S. Piskernik, M. Kurinčič, A. Klančnik, N. Toplak & B. Jeršek, 2014. Evaluation of different methods for DNA extraction from milk. *Journal of Food & Nutrition Research*, **53**, 97–104.

WOAH, 2018. World Organization for Animal Health (OIE). Chapter 3.7.3 Contagious agalactia. In: *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (Terrestrial Manual),* 8th edn, Volume 1, OIE Paris, France, pp. 1430–1440.

Paper received 03.02.2023; accepted for publication 05.05.2023

Correspondence:

Vladimir Petrov
Department of Veterinary Microbiology, Infectious and Parasitic Diseases,
Faculty of Veterinary Medicine,
Trakia University,
6000 Stara Zagora, Bulgaria,
phone: +359 42 699 610,
e-mail: vlado72@abv.bg