

A novel LAMP field test for the diagnosis of contagious agalactia caused by *Mycoplasma agalactiae*

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Abstract

The aim of the work was to develop a new, robust, portable diagnostic system based on the latest development of loop-mediated isothermal amplification (LAMP) technology for the detection of the main causative agent of contagious agalactia, an important disease of small ruminants. The integrated diagnostic system consists of a portable instrument and a kit, specific for the pathogen. The kit is ready to use and provides both the reagents for a rapid nucleic acid extraction from milk, and the amplification reagents using LAMP technology. The test showed a high sensitivity identifying only *M. agalactiae* DNA with a detection limit of less than 100 CFU / ml in milk samples, exceeding the traditional laboratory diagnostics for speed and handling. The test also proved to be more tolerant to the inhibitory effect of milk components that affect amplification in the PCR. These preliminary results show that the LAMP system can be a practical and effective diagnostic tool for the diagnosis in the field of *M. agalactiae* pathogen.

Introduction

Contagious agalactia (CA) is caused by four different pathogenic mycoplasmas: *Mycoplasma agalactiae*, the pathogen with the highest prevalence in Mediterranean areas accounting for over 80% of diagnoses, followed by *M. mycoides* subsp. *capri*; *M. capricolum* subsp. *capricolum* and *M. putrefaciens* [1]. CA is today one of the top priorities for the sheep and goat livestock sector due to the large losses in milk production in ewes, the production and poor survival of weak lambs, low fertility, vaccine costs and indirect expenses such as treatment, time and labour needed for the management of the disease.

Rapid, low-cost, and user-friendly strategies are urgently needed for early diagnosis of disease and timely treatment, particularly for on-site screening of pathogens of animals often at great distances from diagnostic laboratories. The different diagnostic tests currently used for CA fail to provide sufficiently rapid responses. Serological tests like ELISA are not able to detect specific antibodies until at least 25-30 days by ELISA [2]. The excretion of the antigen itself in infected milk is only detectable by culture from the 9th day of infection onwards to which must be added the additional days, sometimes even weeks, for in vitro isolation [3,4]. More recently, the polymerase chain reaction (PCR) has come into common use due to its ability to increase the speed of response, sensitivity and specificity of the diagnosis [5,6]. However, it remains an established fact that the extraction of the pathogenic DNA from the milk is not always easy due to the natural inhibitors present in the sample that hinders their extraction. In practice, it is often observed that samples of milk with low levels of antigen, as occurs in bulk milk, may be falsely negative when tested. The PCR also has critical factors that exclude its use in the field including the need for a well-equipped laboratory and expensive reagents.

The aim of the work was to develop a new, robust, portable diagnostic system (ICGene[®]) based on the latest evolution of loop-mediated isothermal amplification (LAMP) technology which reduces all the classical phases of molecular biology, overcoming the use of expensive laboratory equipment needed for traditional or real time-PCR. The new method provides results directly in the field in about 1 hour with automatic interpretation of the results.

Material and method

Sensitivity

For the sensitivity test, freshly collected sheep milk was used, originating from a flock free of contagious agalactia, and distributed in 8 tubes of 10 ml, to the first of which was added 10% (1ml in 9ml of milk) of culture of *Mycoplasma agalactiae* (NCTC reference strain code 10123) at maximum growth (1X10⁸ colony forming units (CFU) / mL) in mycoplasma culture broth (Mycoplasma broth, Hayflick, Oxoid[®]). Then one ml of milk was taken from the first test tube and diluted in the subsequent one at a rate of 1/10 until it was estimated that 10 CFU of mycoplasma remained in the last tube. The exact amount of antigen was tested in parallel with viable colony counts on mycoplasma agar [7].

Specificity

In parallel, four sheep milk tubes each containing other pathogenic mycoplasmas *M. putrefaciens*, *M. m. capri*, *M. capricolum* and the

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closely related bovine pathogen *M. bovis* were prepared and tested in order to evaluate the specificity of the method.

The integrated diagnostic system consists of a portable instrument and a kit, specific for the pathogen (Figure 1). The kit is ready to use and provides both the reagents for a rapid nucleic acid extraction starting from 1 ml of milk, and the amplification reagents using LAMP technology.

Six primers were used for the p40 gene (Rekha V. et al., 2015): F3 (external forward primer) GGTTTATTAAGTGCATCA; B3 (complementary external primer) CAACAGTTGCATTCGTCTT; FIP (Forward inner primer) CCTTATCACCATTATCTTGTGGATCAGTGCCTTTATTAG; BIP (Backward Inner Primer)ATTAGGTGAAGTTGTCAAAAATATTGAGCTTGCTTCAGGAATTCTGCTA; LF (Forward Loop Primer) GTGAATTTTCGTTCTTATCATCAC; LB (Backward Loop Primer) ACAAATCTAGGTGAAATAGTATTACC.

Result

The test showed a detection limit of less than 100 CFU/ ml in artificially infected milk samples, comparable with the traditional



Figure 1. LAMP method in operation on farm

laboratory diagnostics for sensitivity but much better in speed and handling. It also proved to be more robust to the inhibitory effect of milk components that affect amplification of the PCR.

The system showed a high specificity identifying only the *M. agalactiae* DNA and not that from the other animal mycoplasmas tested or controls just containing milk.

Discussion

These preliminary results show that the ICGene® system can be a practical and effective diagnostic tool for the diagnosis in the field of *M. agalactiae* pathogen. It can easily be adapted for the detection of other animal and human pathogens particularly for mycoplasma diseases like contagious bovine and caprine pleuropneumonia which occur in areas of the world where diagnostic laboratories are many kilometers from diagnostic laboratories. The innovative test is a significant improvement on traditional laboratory tests in terms of speed and robustness. Work is now continuing to take this test into the field and preliminary results look promising.

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