Pakistan Veterinary Journal

ISSN: 0253-8318 (PRINT), 2074-7764 (ONLINE)
Accessible at: www.pvj.com.pk

RESEARCH ARTICLE

Loop-Mediated Isothermal Amplification Assay Targeting the *MOMP* Gene for Rapid Detection of *Chlamydia psittaci* Abortus Strain

Guo-Zhen Lin, Fu-Ying Zheng, Ji-Zhang Zhou, Guang-Hua Wang, Xiao-An Cao, Xiao-Wei Gong and Chang-Qing Qiu*

State Key Laboratory of Veterinary Etiological Biology, Key Laboratory of Veterinary Public Health of Ministry of Agriculture, Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences, No. 1 Xujiaping, Yanchangbao, Lanzhou 730046, China
*Corresponding author: cqqiu@126.com

ARTICLE HISTORY

Received: July 11, 2011
Revised: October 07, 2011
Accepted: November 19, 2011

Key words:
Chlamydia psittaci
LAMP
MOMP gene
PCR

ABSTRACT

For rapid detection of the *Chlamydia psittaci* abortus strain, a loop-mediated isothermal amplification (LAMP) assay was developed and evaluated in this study. The primers for the LAMP assay were designed on the basis of the main outer membrane protein (*MOMP*) gene sequence of *C. psittaci*. Analysis showed that the assay could detect the abortus strain of *C. psittaci* with adequate specificity. The sensitivity of the test was the same as that of the nested-conventional PCR and higher than that of chick embryo isolation. Testing of 153 samples indicated that the LAMP assay could detect the genome of the *C. psittaci* abortus strain effectively in clinical samples. This assay is a useful tool for rapid diagnosis of *C. psittaci* infection in sheep, swine and cattle.

To Cite This Article: Lin GZ, FY Zheng, JZ Zhou, GH Wang, XA Cao, XW Gong and CQ Qiu, 2012. Loop-mediated isothermal amplification assay targeting the *MOMP* gene for rapid detection of *Chlamydia psittaci* abortus strain. Pak Vet J, 32(2): 273-276.

INTRODUCTION

Chlamydia psittaci has a wide range of hosts, including avian and mammalian species (Yang *et al.*, 2011). In humans, the pathogen can cause psittacosis and Reiter syndrome (Ariane *et al.*, 2011). In swine, sheep and cattle it causes abortion, orchitis, enteritis and pneumonia. Generally, infected birds are the source of *C. psittaci* infection for other species because they carry persistent infection (Vanrompay *et al.*, 1995).

In China, *C. psittaci* has been reported to occur in many areas for the last 50 years. From 1978, *C. psittaci* abortus strains isolated from sheep, swine and cattle have been studied systematically in our laboratory. Inactivated vaccines for sheep, swine and cattle were developed successfully and applied to protect livestock against *C. psittaci* infection from 1981 (Qiu *et al.*, 2001 and 2006; Zhou *et al.*, 2007). Chlamydiosis has been controlled effectively in China by the widespread application of inactivated vaccines against *C. psittaci* (Caro *et al.*, 2005).

For the detection of anti-*C. psittaci* antibody, an indirect haemagglutination test (IHA), ELISA, complement fixation reaction and immunofluorescence test have been developed (Chen, 2006). For detection of the *C. psittaci* agent, conventional polymerase chain reaction (PCR), DNA probes and isolation of *C. psittaci* by inoculation of Specific Pathogen Free (SPF) chick embryos have been used (Chen, 2006). All the methods mentioned above contribute to the diagnosis of the disease.

A novel method, known as loop mediated isothermal amplification (LAMP), was developed by Notomi *et al.* (2000). In order to carry out this method, the only equipment required is a heating block or hot water bath. The method enables the amplification of specific DNA sequences, up to 10^9 copies, at 60–65°C in less than 1 h under isothermal conditions and the amplification products are observed visually (Mori *et al.*, 2001). With high specificity, sensitivity, rapidity and simplicity, this method has already been applied for the diagnosis of bacterial, viral, fungal and parasitic diseases in humans, animals and plants (Zhang *et al.*, 2009; Ai *et al.*, 2010; Chen *et al.*, 2011; Hanaki *et al.*, 2011; Lin *et al.*, 2011; Wang *et al.*, 2011; Zheng *et al.*, 2011b).

In this study, a LAMP assay was developed and assessed. Its viability as potential diagnostic tool was compared with the nested PCR method for the rapid detection of *C. psittaci* abortus strains from clinical samples obtained from swine, sheep and cattle.

MATERIALS AND METHODS

Vaccinal strains and clinical reference samples: The vaccine strains of *C. psittaci* strains D13, A and SX5 were isolated from swine, sheep and cattle respectively, in our laboratory. The livestock affected by the strains D13, A and SX5 showed the main clinical sign of abortion. The recombinant plasmid pMD-MOMP, which includes the *MOMP* gene of the *C. psittaci* SX5 strain, was constructed in our previous work (Song *et al*., 2010). The recombinant plasmid Cpn0695-pGEX-6p including *C. pneumoniae MOMP* gene was presented by Professor Guangming Zhong at University of Texas Health Science Center at San Antonio, USA (Chen *et al*., 2010). *C. trachomatis* T55 strain (CVCC1954), *Mycoplasma mycoides subsp. Caprinum* (CVCC 87001), *Brucella abortus* (CVCC 13), *Escherichia coli* (clinical isolate), *Vibrio cholera* (Vbo, non-O1) and *Clostridimu perfringens* (CMCC 50094) were stored at our laboratory. A total of 153 samples, consisting of 63 from cattle, 55 from swine and 35 from sheep were collected from a breeding farm in northwestern China, from domestic animals those were suspected of *C. psittaci* infection. The samples comprised abortus, placenta, lung, spleen, and gastric juice.

Design of primers for LAMP: According to the published *MOMP* gene sequence of *Chlamydia abortus* strains (Gen Bank accession numbers DQ471955.1, HQ622433.1, EU086705.1 and EF202609.1), four primers (F3, B3, FIP and BIP) were designed for the LAMP assay from the most conserved area of the *MOMP* gene. The names, positions and sequences of the primers are displayed in Fig. 1.

DNA extraction: DNA templates for *C. psittaci* were extracted from the vaccine strains (D13, A and SX5), *C. trachomatis* T55 strain, bacterial isolates (CVCC 87001, CVCC 13, *Escherichia coli*, Vbo and CMCC 50094) and clinical samples using a QIAamp DNA mini kit (Qiagen, Hilden, Germany), and the procedure was performed according to the manufacturer's instructions. The templates were stored at –70°C until used.

Reaction regime of LAMP and nested-PCR: The LAMP reaction was carried out as described previously (Zheng *et al*., 2011a). The mixture contained the primers F3, B3, FIP, BIP, betaine (Sigma-Aldrich, St. Louis, MO, USA), $MgSO_4$, dNTPs, ThermoPol reaction buffer (New England Biolabs, Ipswich, MA, USA) and template DNA. The final concentrations of the aforementioned ingredients were the same as reported previously (Zheng *et al*., 2011a). The mixture was heated at 95°C for 5 min and chilled immediately on ice, followed by the addition of 8 U Bst DNA polymerase (New England Biolabs, USA). The reaction mixture was incubated at 62°C for 60 min, and the reaction was terminated by heating at 80°C for 2 min. The LAMP amplification products were analyzed by 2% (w/v) agarose gel electrophoresis or visual inspection using the intercalating dye SYBR® green I (Invitrogen, Eugene, Oregon, USA). The plasmid pMD-MOMP was used as the positive control and a reaction sample without any DNA template was the negative control in this test.

The conventional nested-PCR was performed in a 50 µl volume using a method based on our previous work (Lin *et al.*, 2011). For the first amplification, the primers used were ompA1 (5' GAG GTG AGT ATG AAA AAA CTC TTG 3') and ompA2 (5' CAA GGT TGT AAT CTC TAG GTT TCA 3'), and the procedure comprised an initial denaturation step at 94°C for 5 min, 35 cycles (denaturation at 94°C for 30s, annealing at 52°C for 1 min and primer extension at 72°C for 2 min) and a final extension step at 72°C for 10 min. The PCR products (1 µl) were used as the templates in the second cycle. For the second amplification, the primers were ompA3 (5' TAT GAA AAA ACT CTT GAA ATC GGC 3') and ompA4 (5' GAT AGC GGG ACA AAA AGT TAG GAT 3'), and the procedure consisted of 20 cycles (denaturation at 94°C for 30s, annealing at 50°C for 1 min and primer extension at 72°C for 1.5 min). The expected PCR products exhibited bands of 1170bp in a 2% agarose gel. Positive and negative controls were included in this assay.

Specificity and sensitivity of LAMP detection: The specificity of the LAMP assay was determined by digestion of the amplified products with the *Taq*I enzyme, the restriction site was present between F1c and B1c. The digested samples and LAMP products were analyzed in a 2% agarose gel. In addition, the plasmid Cpn0695-pGEX-6p, *C. trachomatis* and other bacterial pathogens (*Mycoplasma mycoides subsp. Caprinum*, *Brucella abortus*, *Escherichia coli*, *Vibrio cholera* and *Clostridimu perfringens*), were investigated using the LAMP assay, because these bacterial agents can cause similar clinical signs to those of *C. psittaci*. At the same time, the D13, A and SX5 strains of *C. psittaci* were detected. All the aforementioned pathogens were stored at our laboratory. On the other hand, all the plasmid and pathogens above-mentioned were detected with the conventional nested-PCR.

The sensitivity of the LAMP assay was compared with that of the conventional nested-PCR using serial templates, which were extracted from the *C. psittaci* SX5 strain. The 10-fold diluted templates comprised 4×10^7, 4×10^6, 4×10^5, 4×10^4, 4×10^3, 400, 40, 4 and 0.4 copies/tube of total DNA. The copy number was calculated using the following formula (2 µl/tube indicates that 2 µl template was added to each reaction tube):

$$\text{Amount (copies/tube)} = \frac{6.02 \times 10^{23}\ (\text{copies/mol}) \times \text{conc (g/µl)}}{\text{MW (g/mol)}} \times 2\ \text{µl/tube}$$

Applicability of the LAMP assay: One hundred and fifty-three clinical samples were subjected to the LAMP assay and conventional nested-PCR analysis. At the same time, all the samples mentioned above were isolated in 7-day-old SPF chick embryos. Inoculated SPF chick embryos were incubated for 4-7 days, and five blind passages were performed. The yolk sac of each chick embryo that died within 3-5 days post-inoculation was collected. A smear was prepared using the yolk sac, stained with Giemsa and observed with an electron microscope. A positive result was determined where the characteristic elementary body (EB) of *C. psittaci* was observed.

RESULTS AND DISCUSSION

The LAMP primer sequences showed high homology with the corresponding DNA sequences of *C. psittaci* abortus strains, lower relative homology with those of *C. psittaci* isolates from poultry and other birds, and very low homology with those of other pathogens. This assured that the DNA from the abortus strains of *C. psittaci* could be detected specifically. Other assays, for detection of *C. psittaci* isolated from poultry and other birds, will be studied in the future.

To determine the specificity of the LAMP assay, LAMP products digested by the *Taq*I enzyme yielded two DNA bands which were consistent with the theoretical deduction of sizes 90 and 134bp (Fig. 2). The test gave positive results using the DNA templates of *C. psittaci* D13, A and SX5 strains. No DNA-ladder band was observed in 2% agarose gels using the plasmid Cpn0695-pGEX-6p and the genome templates of other pathogens (Fig. 2). The conventional nested-PCR gave the same results for detection of the plasmid and pathogens DNA templates above-mentioned. Although *C. pecorum* should be detected by the LAMP assay, unfortunately *C. pecorum* was isolated in Japan and has not been reported in China (Pudjiatmoko *et al.*, 1998), and an isolate was not available to facilitate study of the specificity of LAMP. The results revealed that the LAMP assay could detect abortus strains of *C. psittaci* specifically. The specificity of the LAMP assay was the same as that of the conventional nested-PCR method in this study.

With regard to the assessment of sensitivity, both LAMP and nested-PCR showed the same detection limit of four copies/tube of DNA templates and therefore had high sensitivity (Fig. 3). According to a previous report, the sensitivity of a multiplex real-time PCR assay for detection of *C. trachomatis* was 25 genome copies (Tanya *et al.*, 2006). Therefore, the detection sensitivity of LAMP was the same as that of the conventional nested-PCR method, and more sensitive than the multiplex real-time PCR.

For the 153 clinical specimens comprised abortus, placenta, lung, spleen, and gastric juice, the LAMP and nested-PCR tests gave the same results: 50 positive samples and 103 negative samples, which verified further that the sensitivity of the LAMP assay was the same as that of the nested-PCR. Using chick embryo isolation, *C. psittaci* was isolated from 35 of the 50 PCR-positive samples, and no pathogens were obtained from the other 132 samples. These results showed that the LAMP and nested-PCR assays were more sensitive and are therefore superior to chick embryo isolation for diagnosis of *C. psittaci* infection in the clinical setting.

In China, the epidemiology of *C. psittaci* has been conducted in goats and sheep, swine, cattle and poultry. The highest percentage of infection in goat and sheep, swine, cattle and poultry were 52, 53.66, 39.3 and 59.9%, respectively, which confirms that *C. psittaci* is of great concern for animal and public health (Qiu *et al.*, 2001 and 2006). Animals infected by *C. psittaci* must be detected and the infection controlled promptly.

In this report, a novel LAMP assay for rapid and accurate detection of abortion-related strains of *C. psittaci* from clinical specimens was developed. The assay could be performed in a local laboratory without any special equipment. It proved to be an effective tool for the diagnosis of *C. psittaci* infection in sheep, swine and cattle.

Fig. 1: LAMP primers specific for the *MOMP* gene of *C. psittaci* abortus strains. The displayed sequence is between 730bp and 966bp of the *MOMP* gene of the *C. psittaci* abortus strain. The arrowed letters indicate the sequences and location of the primers, and the underlined letters show the location of the *Taq*I restriction enzyme site.

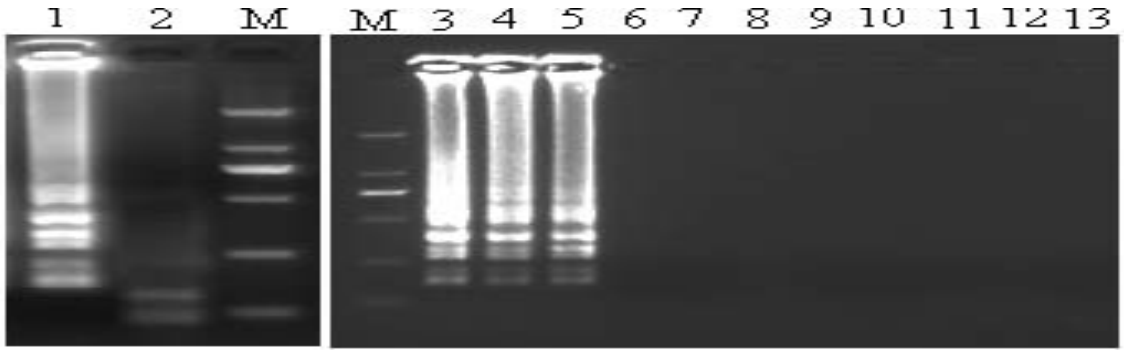

Fig. 2: Specificity analysis of the LAMP assay. Lane M, DL2000 DNA marker (2000bp, 1000bp, 750bp, 500bp, 250bp and 100bp); Lane 1, positive control for detection of the pMD-MOMP plasmid; Lane 2, LAMP products digested by *Taq*I enzyme yielded two DNA bands (90bp and 134bp); Lanes 3–5, LAMP products for detection of genomes of *C. psittaci* D13, A and SX5 strains respectively; Lanes 6–12, LAMP assay gave negative results using the DNA templates of *C. pneumoniae, C. trachomatis* and other bacterial pathogens (*Mycoplasma mycoides subsp. Caprinum, Brucella abortus, Escherichia coli, Vibrio cholera* and *Clostridimu perfringens*), respectively; Lane 13, negative control without any DNA template.

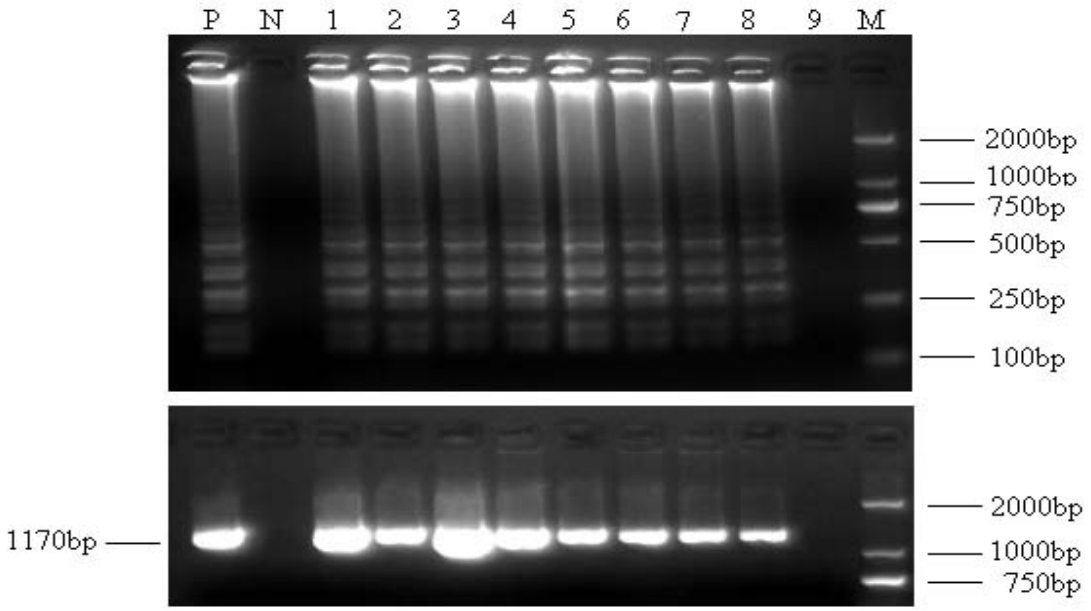

Fig. 3: The sensitivity of the LAMP assay (above fig.) compared with that of the conventional nested-PCR (below fig.); the tests exhibited the same sensitivity. Lane M, DL2000 DNA marker; Lane P, positive control for detection of pMD-MOMP plasmid; Lane N, negative control without any DNA template; Lanes 1–9, LAMP and nested-PCR products using 4×10^7, 4×10^6, 4×10^5, 4×10^4, 4×10^3, 400, 40, 4 and 0.4 copies/tube of *C. psittaci* DNA, respectively.

Acknowledgement: We appreciate the help of Professor Guangming Zhong of University of Texas Health Science Center at San Antonio. This work was supported by grants from the Special Basic Research Fund (2008FY210200).

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