

Development of TaqMan MGB Fluorescent Real-time PCR Assay for the Rapid Detection of *Chlamydia psittaci* in Cattle

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Chlamydia psittaci (*C. psittaci*) is the causative agents of avian chlamydiosis and bovine enzootic abortion respectively. Here we describe a fluorescent quantitative real-time PCR (FQ-PCR) method developed for sensitive and specific measurement of *C. psittaci* infections in cattle based on TaqMan MGB primers and probes targeting the *ompA* gene of *C. psittaci*. The specificity and sensitivity of the established FQ-PCR assay were assessed. Besides, the established FQ-PCR assay was applied to detect *C. psittaci* in 376 specimens which collected from three large-scale dairy farms. The experimental results indicated that the detection limit for this FQ-PCR assay was 10 fg of total DNA, with a sensitivity of 100 times higher than that of the conventional gel-based semi-quantitative PCR assay targeting the *ompA* gene, and there was no cross-reactivity with *Chlamydia trachomatis*, *Chlamydia pneumoniae*, etc *Mycobacterium bovis*, *Brucella*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus*, *Streptococcus lactis*, *Klebsiella pneumoniae* and *Salmonella*, indicating that the established FQ-PCR assay had and high specificity. Furthermore, examination of field samples by the hemagglutination test kit, semi-quantitative PCR and established FQ-PCR revealed that the sensitivity and specificity of this FQ-PCR assay was significantly higher than IHA assay and PCR assay. This FQ-PCR assay for sensitive and specific detection of *C. psittaci* is suited for routine diagnosis, which renders it a useful tool for the recognition of outbreaks of psittacosis and bovine enzootic abortion.

Key words: *Chlamydia psittaci*, minor groove binder probe, real-time fluorescence quantitative PCR.

Chlamydia psittaci (*C. psittaci*) is the causative agent of psittacosis, a widespread infection in psittacine birds and domestic poultry. *C. psittaci* can only replicate when inside a cell, which is characteristic of a virus. *Chlamydial* infections in cattle have been described worldwide and cause disease syndromes such as pneumonia,

enteritis, conjunctivitis, polyarthritis, encephalitis, mastitis, abortion and other urogenital tract infections as well as subclinical infections (Harkinezhad *et al.* 2009; Longbottom and Coulter 2003). Because prevention and early detection are presently the most logical strategies for *C. psittaci* control, various diagnostic methods for *C. psittaci* detection including iodine staining, giemsa staining, immunofluorescence (IF), enzyme-linked immunosorbent assay (ELISA) and polymerase chain reaction (PCR) have been developed (Perez-Martinez *et al.* 1986; Schmeer *et al.* 1987; DeGraves

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et al. 2003; Yin et al. 2012). Although rapid antigen tests, bacterium isolation, and serological tests can be used to diagnose *C. psittaci* infection, these methods have limited use for routine diagnostics because of the inability to the low sensitivity and specificity, and the requirement of biosafety level 3 laboratory facilities. PCR is a powerful tool with exquisite sensitivity for detection of minute amounts of nucleic acids, even against a high background of unrelated nucleic acids. Fluorescent quantitative real-time PCR (FQ-PCR) technique has eliminated the need of sample post-amplification handling required by the conventional PCR assay and has paved the way towards fully automated detection systems now that they usually display very high sensitivity and broad dynamic capacity after optimization. TaqMan Minor Groove Binding (MGB) probes as an upgrade of the ordinary TaqMan probe has been widely used during the recent years for high specificity (Guo et al. 2009; Bass et al. 2007). Therefore, a TaqMan MGB-based real-time PCR method for detection and quantitation of *C. psittaci* is developed to serve as an alternative and improvement of the previously developed ordinary TaqMan real-time PCR method.

MATERIALS AND METHODS

Chlamydial strains and cells

For sensitivity and specificity analyses, Several Chlamydial strains were used. *Chlamydia psittaci* SX5 was separated and preserved by Chinese Academy of Agricultural Sciences Lanzhou Veterinary Institute of Animal Collection. *Chlamydia trachomatis* 55Y120 and *Chlamydia pneumoniae* AR39 were provided by Chinese Medical Culture Collection Center. Besides, Hela cells were purchased from Cell Bank of Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences. *C. psittaci* was cultured with Hela cells. The frozen-preserved samples were defrosted under the room temperature, which were then seeded into Hela cells with good condition. Meanwhile, positive *C. psittaci* SX5, and *Chlamydia trachomatis* 55Y120 or *Chlamydia pneumoniae* AR39 were also seeded, acting as positive and negative control each. The cells were incubated at 37 °C under 5% CO₂ for 48 to 72 hours. After that, the cells were collected for DNA abstraction. DNA abstraction of *C. psittaci* was

conducted according to the protocol of the Bacterial Genomic DNA Isolation Kit (Sangon Biotech, Shanghai).

Field samples preparation

Extraction of bacteria DNA

All DNA extractions from *Chlamydia trachomatis*, *Chlamydia pneumoniae*, *Mycobacterium bovis*, *Brucella*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus*, *Streptococcus lactis*, *Klebsiella pneumoniae*, *Salmonella* and cattle field specimens were performed using the Bacterial Genomic DNA Isolation Kit (Sangon Biotech Co., Ltd), following the manufacturers' instructions. DNA extracts were stored at -70°C until evaluation via PCR analysis.

Primers and probes design

Using software Primer 5.0, primers were designed according to the ompA gene. The forward primer was 5'-GGCACCATGTGGGAAGGTGCTTG-3' and reverse primer was 5'-GTCATTTGGAGAGGATCCTGTG-3'. Due to the gene fragments within primers, TaqMAN probe was also designed under the help of Primer Express with the sequence of 5'-GCGCAGGATACTACGGAGATTATG-3'. FAM was labeled at the 5' terminal while NFQ-MGB was labeled at the 3' terminal. The amplification length of the target fragment was 180 bp. Primers and probes were synthesis and labeled by ABI Company in US.

PCR and Real-Time PCR

The PCR analyses were performed on an ABI 2720 PCR System (Applied Biosystems). The 50 µl PCR included 2 µl PCR forward primer (10 µM), 2 µl PCR reverse primer (10 µM), 5 µl 10 × buffer (Mg²⁺), 4 µl dNTP (2.5 mM), 2 µl DNA, 1 µl Taq and 34µl ddH₂O. The reaction was conducted at 94 °C for 5 min, followed by 50 cycles of 94 °C for 40 s 62 °C for 40 s and 72 °C for 40 s. The FQ-PCR analyses were performed on an LightCycler (Roche) 4.8 and IQ5 (RD) Real-time PCR System (Applied Biosystems) under the conditions according to TaKaRa kit: the 20 µl PCR included 10 µl Premix ExTaq™ (Probe qPCR), 0.8 µl PCR Forward Primer (10 µM), 0.8 µl PCR Reverse Primer (10 µM), 1.0 µl Probe, 2.0 µl total DNA and 5.4 µl ddH₂O. The reaction was conducted at 95 °C for 30s, followed by 40 cycles of 95 °C for 5 s 62 °C for 20s 72 °C for 20s and cooling for 20 min at 37 °C till the end of the reaction.

Assessment on sensitivity of FQ-PCR

The sensitivity of the newly developed FQ-PCR assays for *C. psittaci* were tested with genomic DNA extractions from cultures of *C. psittaci* SX5. Tenfold serial dilutions of DNA in the range of 1 fg to 1 µg were prepared firstly. Then the semi-quantitative PCR and the FQ-PCR analysis were performed under the conditions as previously described. The semi-quantitative PCR products were electrophoresed on 3% agarose gels in 1 × TAE buffer. The standard curve was calculated by the FQ-PCR analyses.

Assessment on specificity of FQ-PCR

The specificity of the newly developed FQ-PCR assays for *C. psittaci* were tested with genomic DNA extractions of several bacterial species, including *Chlamydia psittaci* SX5, *Chlamydia trachomatis* 55Y120, *Chlamydia pneumoniae* AR39, *Mycobacterium bovis*, *Brucella*, *E. coli*, *Pseudomonas aeruginosa*, *Staphylococcus*, *Streptococcus lactis*, *Klebsiella pneumoniae*, *Salmonella*. The amplification curve of FQ-PCR was analyzed.

Test of established FQ-PCR assay using specimens for practical applications

All field specimens were provided as direct DNA extracts from blood or tissue samples. Case samples with clinical signs or evidence of *C. psittaci* infection (n = 116) and normal samples (n = 260) were obtained from three cattle farms in Yinchuan, Ningxia province. The *C. psittaci* assays were performed using IHA diagnostic reagent kit (Lanzhou Veterinary Research Institute, Lanzhou, China), the semi-quantitative PCR and the established FQ-PCR assay performed with the total DNA of 1 ng, 100 fg and 10 fg as PCR templates. All statistical analysis was performed by using SPSS18.0 for Windows software package (Chicago, IL, USA).

RESULTS

Analytical sensitivity

Ten-fold dilution series of DNA extractions from cultures of *C. psittaci* SX5 were tested by the semi-quantitative PCR and established FQ-PCR assay to evaluate the sensitivity of the system. The semi-quantitative PCR were performed on an ABI 2720 PCR system. Gel electrophoresis of the semi-quantitative PCR

products with the PCR templates from 1 µg to 1 ng revealed positive bands. But no band was detected with the PCR templates of 100 fg. The established FQ-PCR analysis was performed on LightCycler (Roche) 4.8 and IQ5 (RD) Real-time PCR System, and the detection limit was found to be 10 fg. As shown as Fig. 1, Fig. 2, and Fig. 3 LightCycler (Roche) 4.8 and IQ5(RD) Real-time PCR System revealed both good standard curves, and the results showed a single peak in the melting curve. The melting temperature (T_m) was 74.3 °C in the IQ5 and was 74.1 °C in the LightCycler. Comparisons were made between the semi-quantitative PCR and established FQ-PCR using dilution series to calculate the end point sensitivity

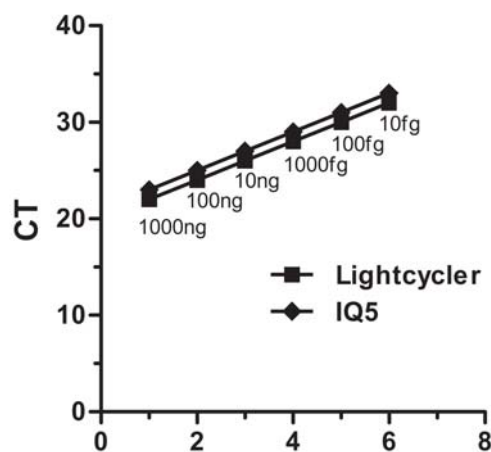


Fig. 1. Establishment of the fluorescent quantitative real-time PCR standard curve. The sensitivity of the newly developed FQ-PCR assays for *C. psittaci* were tested with genomic DNA extractions from cultures of *C. psittaci* SX5. Tenfold serial dilutions of DNA in the range of 1 fg to 1 µg were prepared. Each dot represents the result of triplicate amplification of each dilution

Table 1. The analytical sensitivity and specificity of IHA, PCR & FQ-PCR assays identified by field samples

Testing methods	Sensitivity	Specificity
IHA	81.82% (90/116)	92.69% (241/260)
PCR	88.79% (103/116)	94.61% (246/260)
FQ-PCR	100% (116/116)	96.15% (250/260)

of each assay. The results indicate that the established FQ-PCR is around 100 times more sensitive than the semi-quantitative PCR method, detecting *C. psittaci* down to dilutions of 10 fg, compared to dilutions of only 1 ng for the semi-quantitative PCR.

Specificity

The test using DNA from several other bacteria was used as template to examine the specificity of established FQ-PCR. The DNA samples including *Chlamydia psittaci* SX5, *Chlamydia trachomatis* 55Y120 and *Chlamydia pneumoniae* AR39 were analyzed by the established FQ-PCR. There were any amplification signal with the DNA samples from *Chlamydia trachomatis* 55Y120, *Chlamydia pneumoniae*

AR39, *Mycobacterium bovis*, *Brucella*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus*, *Streptococcus lactis*, *Klebsiella pneumoniae*, *Salmonella*. The results demonstrated that the established FQ-PCR assay is of highly specific.

Detection of *C. psittaci* in field samples

The blood samples of domestic milk cows were collected from three farms. The *C. psittaci* assays were performed using IHA diagnostic reagent kit, the semi-quantitative PCR and the established FQ-PCR assays with the total DNA from the blood and the tissues. As shown as Table 1, there were 116 samples with *C. psittaci* infection. IHA assays detected 90 samples with *C. psittaci* infection and its sensitivity was 81.82%. The semi-

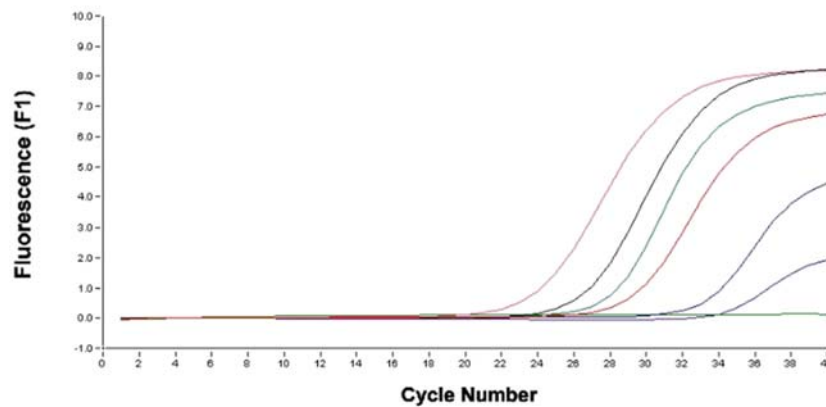


Fig. 2. The amplification curves of DNA extractions from cultures of *Chlamydia psittaci* SX5 by LightCycler. The sensitivity of the newly developed FQ-PCR assays for *C. psittaci* were tested with genomic DNA extractions from cultures of *C. psittaci* SX5. Tenfold serial dilutions of DNA in the range of 1 fg to 1 μ g were prepared

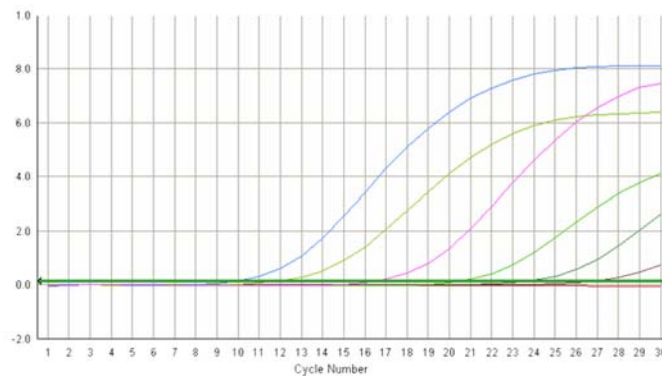


Fig. 3. The amplification curves of DNA extractions from cultures of *Chlamydia psittaci* SX5 by IQ5. The sensitivity of the newly developed FQ-PCR assays for *C. psittaci* were tested with genomic DNA extractions from cultures of *C. psittaci* SX5. Tenfold serial dilutions of DNA in the range of 1 fg to 1 μ g were prepared.

quantitative PCR assays detected 103 samples with *C. psittaci* infection and its sensitivity was 88.79%. FQ-PCR assays detected 116 samples with *C. psittaci* infection and its sensitivity was 100%. These data demonstrated the sensitivity of FQ-PCR assay was significantly higher than IHA assay and the semi-quantitative PCR assay. Besides, there were 260 negative samples. IHA assays detected 241 samples without *C. psittaci* infection among the total negative samples and its specificity was 92.69%. PCR assays detected 246 samples without *C. psittaci* infection and its specificity was 94.61%. FQ-PCR assays detected 250 samples and its specificity was 96.15%. These data also demonstrated the specificity of FQ-PCR assay was significantly higher than IHA assay and the semi-quantitative PCR assay.

DISCUSSION

The obligate intracellular bacterium *C. psittaci* is the causative agent of psittacosis, a widespread infection in psittacine birds and domestic poultry (Liao *et al.* 1997; Hsia *et al.* 2000; Circella *et al.* 2011). *C. psittaci* infections in cattle have been described worldwide and cause disease syndromes such as pneumonia, enteritis, conjunctivitis, polyarthrititis, encephalitis, abortion and subclinical infections, which lead to huge economic losses (Ronsholt 1978; Wittenbrink *et al.* 1993). So detection of *C. psittaci* in early infection is significance to prevent and control the infection of *C. psittaci*. In cattle, ELISA examinations of sera for the antibody against *C. psittaci* suggest a high level of exposure to *C. psittaci* (Souriau and Rodolakis 1986; Buendia *et al.* 2001; Anderson *et al.* 1995). The application of nested PCR to bovine clinical specimens substantiated such widespread, but mostly clinically inapparent, presumably low-level infections (Domeika *et al.* 1994; Kaltenbock *et al.* 1997). However, because of high technical demands, these PCR methods were rarely transferred from research settings to systematic epidemiological investigations and diagnostic use. FQ-PCR technique has eliminated the need of sample post-amplification handling required by the conventional PCR assay and has paved the way towards fully automated detection systems now that they usually display very high sensitivity and

broad dynamic capacity after optimization (Guo *et al.* 2009).

FQ-PCR protocols have been published for various chlamydial species, such as *Chlamydia pneumoniae* (Kuoppa *et al.* 2002; Hardick *et al.* 2004; Thurman *et al.* 2011), *Chlamydia trachomatis* (Peuchant *et al.* 2011), *Chlamydia pecorum* (DeGraves *et al.* 2003). However, there was no FQ-PCR kit to detect the *C. psittaci* infection. The major outer membrane protein (MOMP) is the main pathogenic factor during the *C. psittaci* infection (May *et al.* 1996; Zhou *et al.* 2007). In the study, we designed the specific primers and TaqMan MGB probes targeting the conserved sequence of *C. psittaci* ompA gene and established a FQ-PCR method for detecting *C. psittaci* in specimens of cattle.

The FQ-PCR assays designed in the present study proved to be specific, sensitive and efficient. The detection limit for this FQ-PCR assay was 10 fg of total DNA, with a sensitivity of 100 times higher than that of the conventional gel-based semi-quantitative PCR assay targeting the ompA gene. Besides, there was no cross-reactivity with *Chlamydia trachomatis*, *Chlamydia pneumoniae*, *Mycobacterium bovis*, *Brucella*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus*, *Streptococcus lactis*, *Klebsiella pneumoniae* and *Salmonella*, indicating that the established FQ-PCR assay had and high specificity. Moreover, examination of field samples by the IHA test kit, the semi-quantitative PCR and the established FQ-PCR revealed that the sensitivity and specificity of this FQ-PCR assay was significantly higher than IHA assay and PCR assay. These experimental results confirmed that the established FQ-PCR assay is suitable for diagnosis of *C. psittaci* infection in cattle.

In conclusion, we established a simple, quick and accurate TaqMan MGB probe-based FQ-PCR based on the sequence of *C. psittaci* ompA gene. It provides a technical support for the detection of *C. psittaci* in early infection, and it is significance to prevent and control the infection of *C. psittaci*.

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