NOTE *Bacteriology*

Detection of *Chlamydophila psittaci* by Using SYBR Green Real-Time PCR

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ABSTRACT. *Chlamydophila psittaci* is the causative agent of human psittacosis and avian chlamydiosis. This zoonotic pathogen is frequently transmitted from infected birds to humans. Therefore proper and rapid detection of *C. psittaci* in birds is important to control this disease. We developed a method for detecting *C. psittaci* by using SYBR Green Real-time PCR based on targeting the cysteine-rich protein gene (*envB*) of *C. psittaci*. This one step procedure was highly sensitive and rapid for detection and quantification of *C. psittaci* from fecal samples. This assay was also able to detect other zoonotic *Chlamydophila* species such as *C. abortus* and *C. felis*. The assay is well suited for use as a routine detection method in veterinary medicine.
KEY WORDS: avian chlamydiosis, *Chlamydophila psittaci*, real-time PCR, SYBR Green dye.

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Bacteria of the family Chlamydiaceae are obligate intracellular bacterial pathogens that cause various diseases in a wide range of animals including human. Among them, *Chlamydophila psittaci*, the causative agent of psittacosis in human and avian chlamydiosis, is the most important zoonotic pathogen, which is frequently transmitted by aerosols from infected birds. *C. psittaci* has been isolated from more than 460 avian species of 30 orders [15]. Parrot, parakeet and feral pigeon are the best known as representative natural hosts. Recently, we reported that 6.0% of pet birds (11 avian orders) in Japan were positive for *C. psittaci* [3]. Wild birds, especially pigeon, can serve as a source of human psittacosis [18, 27]. Avian chlamydiosis can exist as an inapparent infection. Hence birds can be carriers to human.

The clinical signs of psittacosis in human are influenza-like symptoms. Without appropriate treatment, this infection occasionally leads to severe respiratory disease and fatal systemic disease [28]. Therefore, proper and rapid detection of *C. psittaci* in human and birds is important to control psittacosis and avian chlamydiosis. Psittacosis is a notifiable infectious disease in Australia, the U.S., a number of European countries [13], and Japan. In Japan, since 1999, all physicians have been obliged to report psittacosis cases because it is classified as category IV under the Infectious Diseases Control Law, Japan. Hence, psittacosis should be differentiated from chlamydial pneumonia caused by *C. pneumoniae* (listed under the category V notifiable infectious diseases in Japan).

C. psittaci infection can be diagnosed by isolation of the pathogen, serological detection, or DNA detection [25]. Due to the contagiousness of this pathogen, direct isolation of the pathogen or serological test by using the purified elementary body (EB) or the *C. psittaci*-infected cells as antigens are hazardous and require specialized laboratory expertise and facilities. Therefore, microbiological diagnosis of psittacosis and avian chlamydiosis can be performed only in well-equipped laboratories. Various DNA amplification methods have been developed to detect *C. psittaci* such as conventional PCR and real-time PCR [25]. The real-time PCR assay is useful as a diagnostic test for *C. psittaci*, and has simultaneously enabled the identification and/or quantification of *Chlamydia* spp. and *Chlamydophila* spp. In addition, unlike conventional PCR, this assay can detect the pathogen in just one step, making post-PCR procedures such as electrophoresis unnecessary. That means it can be diagnosed rapidly and reduces the risk of carryover contamination.

Several studies have used real-time PCR to detect *C. psittaci*. The target genes in these studies are the major outer membrane protein (MOMP) gene (*ompA*) [10, 14, 22], 23S rRNA gene [1, 5, 7] and inclusion membrane protein A gene (*incA*) [19]. However, almost of these reports aimed at developing *C. psittaci*-specific diagnostic tests.

In this study, we chose a molecular cysteine-rich protein (*envB*) of *C. psittaci* as a target gene. We designed primers that could broadly detect animal-derived *Chlamydophila* including *C. psittaci*, *C. abortus* and *C. felis*, but that did not detect other species of *Chlamydia* or *Chlamydophila*. We also evaluated the potential of our real-time PCR system to be used as a clinical diagnostic system for *C. psittaci* and other related *Chlamydophila* spp. infections.

The chlamydial species and strains used in this study are listed in Table 1. All strains were cultivated in HeLa cells or L cells in suspension form. HeLa cells were pretreated with 30 μg/m*l* DEAE-dextran in minimal essential medium α (Wako Pure Chemical Ltd., Osaka, Japan) at room temperature for 30 min before inoculation. After inoculation of each chlamydia at a multiplicity of infection of up to 10, the infected cells were incubated in the presence of 5% CO_2 at 37°C for 60 min. The inocula were exchanged into minimal essential medium α supplemented with 5% fetal bovine

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Table 1. Chlamydia species and strains used in this study

Species	Strain	Host	Reference
C. psittaci	6BC	parakeet	[11]
	Cal-10	human	[23]
	Daruma	parakeet	[8]
	GCP-1	parrot	[23]
	Mat116	psittacine	this study
	Nose	budgerigar	this study
C. abortus	B577	sheep	[23]
C. felis	Fe/C-56	feline	[21]
C. caviae	GPIC	guinea pig	[20]
C. pecorum	Maeda	cattle	[8]
C. pneumoniae	TW183	human	[12]
C. muridarum	Nigg	mouse	[8]
C. suis	S45	swine	[16]
C. trachomatis	L2/434/Bu	human	[8]
	D/UW-3/CX	human	[2]
	E/UW-5/CX	human	[4]

serum (Invitrogen, Carlsbad, CA, U.S.A.) and 1 μg/m*l* of cycloheximide in the presence of 5% CO_2 at 37°C until formation of the mature inclusion body. *C. psittaci* elemental body (EB) was purified from infected L cells in suspension form by sucrose gradient centrifugation as described previously [9, 21]. The purified EB was diluted at 2.0 mg/m*l* protein concentration in 0.01 M Tris-HCl (pH7.2) and stored at –80°C until use.

Bacteria commonly detected in fecal samples from birds were used for testing the specificity of the real-time PCR. The bacterial species were *Proteus* sp., *Pasteurella multocida*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Serratia marcescens*, *Staphylococcus aureus*, *Escherichia coli, Streptococcus agalactiae, Salmonella enterica* biovar Pullorum, and *Yersinia enterocolitica* from the culture stock of our laboratory.

Two sets of oligonucleotide primers were designed based on the *envB* gene sequence of the *C. psittaci* 6BC with accession number M61116 (National Center of Biotechnology Information: NCBI). The entire *envB* region was amplified using a pair of primers (Clone-F: 5'-GTTCATTTGCC AGCGGGAAGATAGAGG-3'; and Clone-R: 5'-AGAAC-CACGGTTGGTTACACAAATACGG-3') for making plasmid DNA to generate a standard curve. Alignment of the *envB* gene of *C. psittaci* 6BC, *C. abortus* B577 (accession#AF111200), *C. felis* Fe/C-56 (accession#AP006861), *C caviae* GPIC (accession#U41579), *C. pecorum* W73 (accession#U76761), *C. pneumoniae* TW-183 (accession#AE009440), *C. muridarum* Nigg (accession#AE002160) and *C. trachomatis* L2/434/Bu (accession#AM884176), revealed that the region encompassing nucleotide positions 997 to 2,670 of *envB* gene was the most conserved among them (Fig. 1). Accordingly, for the real-time PCR analysis, another set of primers (Env-F: 5'-AACCTCGGATAGCAAATTAATCTGG-3'; and Env-R: 5'-ATTTGGTATAAGAGCGAAGTTCTGG-3') was designed to amplify the region of the *envB* gene (152 bp), which showed high similarity among *C. psittaci* and related *Chlamydophila* such as *C. abortus* and *C. felis* but not in other *Chlamydia* and *Chlamydophila* (Fig. 1).

To generate a standard curve for the real-time PCR assay, a PCR product containing 1,358 bp covering the part of *envB* was cloned into a pGEM T easy vector (Promega Corporation, Madison, WI, U.S.A.), resulting in pEnvB. The pEnvB was purified using a commercial kit (illustra plasmidPrep Mini Spin Kit, GE Healthcare UK Ltd., Little Chalfont, Buckinghamshine, U.K.) according to the manufacturer's instructions. The concentration of pEnvB solution was calculated from the absorbance at 260 nm measured with a spectrophotometer (GeneQuant II, Pharmacia Biotech, Piscataway, NJ, U.S.A.). Serial 10-fold dilutions of pEnvB were used in the amplification reactions.

In order to determine the detection limit of the real-time PCR analysis and to verify the accuracy of DNA extraction in field samples, feces of birds containing a known titer of *C. psittaci* EB were used as templates. Emulsion birds feces [20% (w/v) in PBS] were mixed with an equivalent amount of PBS containing 10-fold serial dilutions of EB, resulting in a 10% emulsion bird feces containing known titers of EB [10 to 10^6 inclusion forming units (IFU)].

DNA was extracted from chlamydia-infected cells, bird feces containing chlamydial EB and bacterial cultures by using a DNA extraction kit (SepaGene; Sankojunyaku Co., Ltd., Tokyo, Japan) according to the manufacturer's instructions. The DNA was dissolved in 20 μl of TE buffer (10 mM Tris-HCl, pH 8.0, and 1 mM EDTA) and stored at –30°C until used.

Real-time PCR was performed with the Thermal Cycler Dice Real Time System TP800 (Takara Bio Inc., Otsu, Japan). The reaction was carried out in 12.5 μl of SYBR *Premix EX Taq* II (Perfect Real Time: Takara Bio Inc.), 10 μM of each primer, and 2 μl of DNA extract in a final volume of 25 μl. Cycling conditions were as follows: an initial cycle of heating at 95°C for 10 sec, 40 cycles of 95°C for 5 sec and 60°C for 30 sec with data acquired at the annealing and extension. After completion of the PCR reaction, a melting curve was constructed by 95°C for 15 sec, 60°C for 30 sec, then temperature was ramped up from 60 to 95°C. The identity of PCR product was confirmed by DNA sequence analysis.

Conventional nested PCR was examined using the same samples as described previously [3].

The sensitivity and the linearity of the real-time PCR assay were assessed using serially 10-fold diluted pEnvB DNAs containing the primer-spanning region of the *envB* gene as templates in triplicate. As shown in Fig. 2a, the sensitivity of detection was linear from 1×10 to 1×10^8 copies of pEnvB per reaction by real-time PCR. Negative controls did not provide any amplification. The standard curve covered a linear range of 8 orders of magnitude [regression coefficient (R^2) was 0.99], providing an accurate measurement of starting target amount (Fig. 2b). The amplification products were verified by the melting curves analysis after PCR procedures (Fig. 2c), showing that only one melting peak (at 81.75°C) was observed. These results show that a

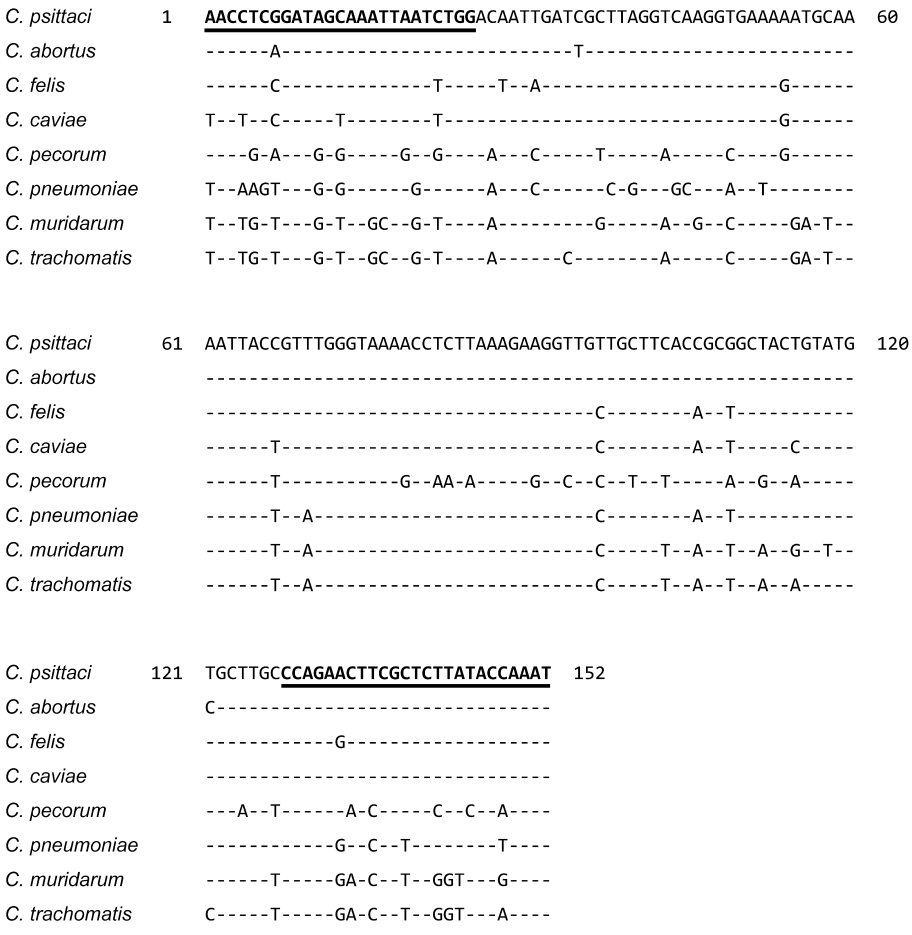

Fig. 1. Nucleotide sequence alignment of representative variant strains of *C. psittaci* 6BC, *C. abortus* B577, *C. felis* Fe/C-56, *C. caviae* GPIC, *C. pneumoniae* TW-183, *C. pecorum* W73, *C. trachomatis* L2/434/Bu, *C. muridarum* Nigg. Underscored and bold portions of the sequences are primer-binding locations.

minimum of 10 copies of the pEnvB was consistently detectable in the real-time PCR assay.

Since *C. psittaci* EB are shed in feces from infected birds, fecal samples of birds are routinely used as a source of DNA for laboratory diagnosis of *C. psittaci* infection [24]. We prepared bird feces containing known amount of EB in order to simulate clinical samples, adding with EB suspensions in PBS as a control. DNAs were extracted from the feces and PBS containing *C. psittaci* EB. The real-time PCR indicated that the cycle threshold (Ct) values of these samples were correlated with the amount of EB in both feces and PBS (data not shown).

The sensitivity of the real-time PCR was compared with that of the nested PCR routinely used in our laboratory for clinical diagnosis [3]. Template DNA samples extracted from feces and PBS containing known titers of EB as described above were analyzed by nested PCR. The detection limit of the nested PCR assay was 10^4 IFU per reaction (data not shown). In an experiment using same samples, the lower detection limit of the SYBR Green real-time PCR was only 10 IFU (data not shown).

The specificity of the real-time PCR assay was evaluated with 7 strains of *C. psittaci*, 10 strains of *Chlamydophila* and *Chlamydia* species, and bacterial culture. As a result, *C. psittaci* strains including 6BC, Borg, Cal-10, Daruma, GCP-1, Mat116, Nose, and closely related other *Chlamydophila* species such as *C. felis* and *C. abortus* were amplified by real-time PCR. All of these reactions showed a single melting peak at 81.75 ± 0.5°C (data not shown). No signal was detected from *C. pneumoniae* and *C. trachomatis* (Table 1), and also from none of bacterial culture that we examined.

In this study, we established a real-time PCR assay based on SYBR Green dye for the detection of *C. psittaci* and related *Chlamydophila* species. This assay is an effective alternative for the conventional nested PCR.

Although there are many assays for detecting *C. psittaci* [25], a simple and efficient analysis is still required by veterinarians and clinicians. Recently, real-time PCR assay has

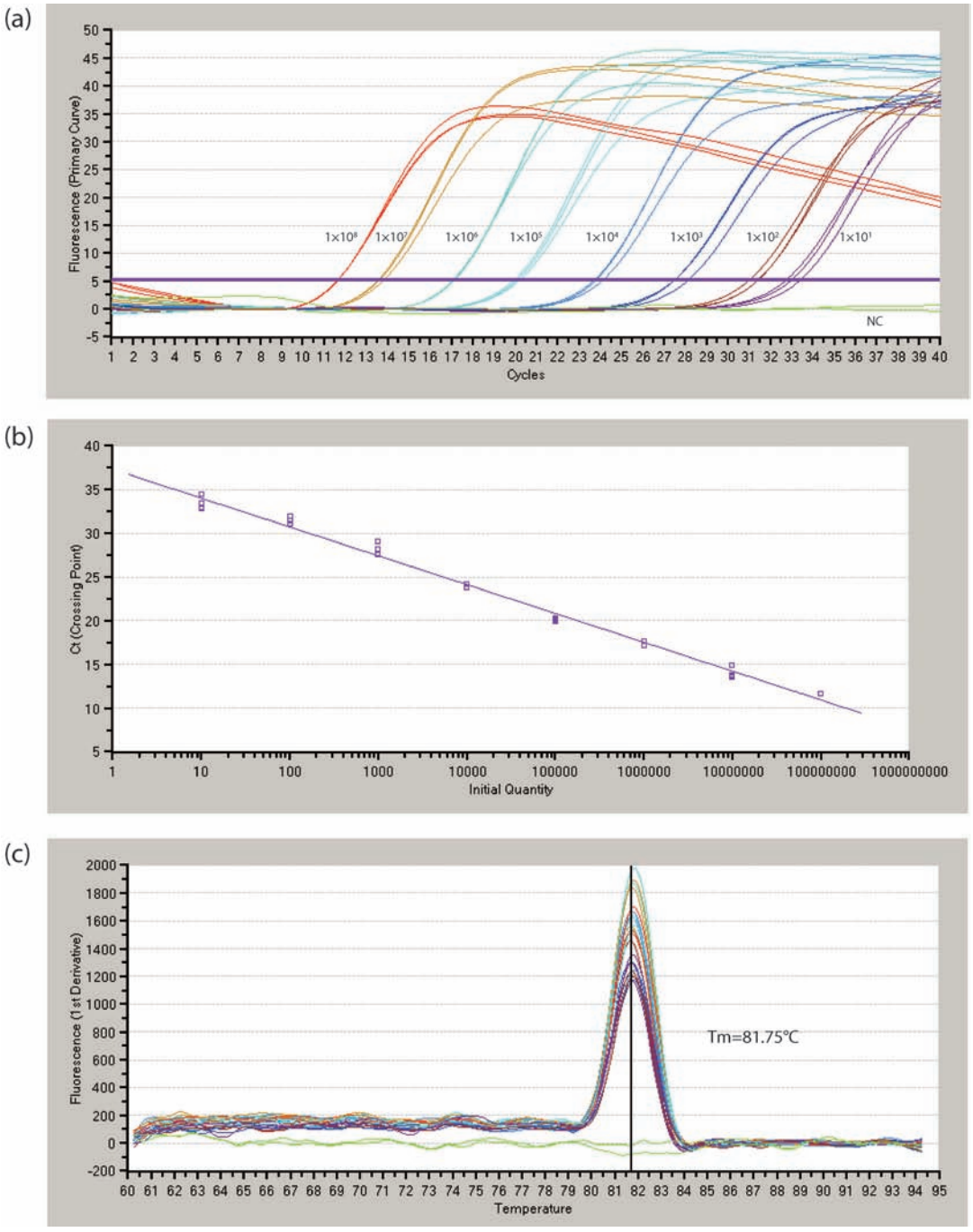

Fig. 2. Amplification curves, standard curves and melting curves of the real-time PCR assay. (a) Amplification curves were generated by fluorescence data collected at each cycle during the extension phase of the PCR. Values are triplicates of different dilutions of the pEnvB used as standard. pEnvB copy number per sample were 1×10^1, 1×10^2, 1×10^3, 1×10^4, 1×10^5, 1×10^6, 1×10^7, 1×10^8 and negative control (NC). (b) Standard curves based on serial dilutions of plasmid DNA (copy numbers are 1×10^1 to 1×10^8). The R^2 linearity value from linear regression is 0.99. (c) Melting curve analysis from the same experiment as in (a).

become more widely used as a major diagnostic test. Conventional PCR analysis including nested PCR [3] and PCR-restriction fragment length polymorphism (RFLP) [17, 26] confirm only the presence of a pathogen and take a long time for detection, because they require gel electrophoresis to confirm the presence of PCR products after PCR [25]. On

the other hand, present real-time PCR techniques can quantify and detect a pathogen more rapidly than conventional PCR. The real-time PCR method described here took only about half the time of conventional nested PCR.

Our assay uses primers that target a conserved region of the *envB* gene in *chlamydophila* spp. This *envB* region is suitable for differential diagnosis, that is, this method can distinguish from *C. psittaci* to *C. pneumoniae* or *C. trachomatis*. This primer pair amplifies closely related species such as *C. abortus* and *C. felis*. Pantchev *et al*. reported that *C. psittaci* and *C. abortus* have possibility to cause dual infections in pigs and cattle [22], and we speculate that it could also cause dual infection in avian species. Therefore, the real-time PCR established in this study which can simultaneously detect *C. psittaci* and *C. abortus*, may be a useful tool in veterinary medicine.

Fecal samples of birds are routinely used as a source of DNA for laboratory diagnosis of *C. psittaci* infection [24]. Fecal samples contain the number of different types of inhibitors of PCR [26]. These impurities might have influenced the amplification. However, it was confirmed that fecal impurities did not affect the results in this detection method. One of a general problem of PCR assay is that inhibitory substances can give a false-negative result. An internal control system may be needed to improve the accuracy of our real-time PCR [14].

Conventional nested PCR was able to detect 10^4 IFU, whereas the lower detection limit of the SYBR Green real-time PCR was only 10 IFU. Therefore, the SYBR Green PCR assay is 1,000 times more sensitive than conventional nested PCR. The short amplicons in the real-time PCR assays used in this study likely resulted in more efficient amplification and higher sensitivity. Our SYBR Green real-time PCR assay also achieved the same sensitivity as other TaqMan PCR assays compared with IFU based on Ct value [19, 22]. Ehricht *et al.* pointed out that the actual detection sensitivity depends on the integrity of the target DNA [6, 25]. The real-time PCR established in this study is applicable even to DNA samples that were extracted from avian feces.

In conclusion, the real-time PCR assay based on SYBR Green dye defined high sensitivity and rapidity and quantification for detection of *C. psittaci* from fecal samples. Early diagnostics and treatment are of importance in psittacosis. The format should emphasize use as a routine study.

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