Genotyping of *Chlamydia psittaci* by Real-Time PCR and High-Resolution Melt Analysis


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Human infection with *Chlamydia* (Chlamydia) *psittaci* can lead to psittacosis, a disease that occasionally results in severe pneumonia and other medical complications. *C. psittaci* is currently grouped into seven avian genotypes: A through F and E/B. Serological testing, outer membrane protein A (*ompA*) gene sequencing, and restriction fragment length polymorphism analysis are currently used for distinguishing these genotypes. Although accurate, these methods are time-consuming and require multiple confirmatory tests. By targeting the *ompA* gene, a real-time PCR assay has been developed to rapidly detect and genotype *C. psittaci* by light-upon-extension chemistry and high-resolution melt analysis. Using this assay, we screened 169 animal specimens; 98 were positive for *C. psittaci* (71.4% genotype A, 3.1% genotype B, 4.1% genotype E, and 21.4% unable to be typed). This test may provide insight into the distribution of each genotype among specific hosts and provide epidemiological and epizootiological data in human and mammalian/avian cases. This diagnostic assay may also have veterinary applications during chlamydial outbreaks, particularly with respect to identifying the sources and tracking the movements of a particular genotype when multiple animal facilities are affected.

*Chlamydia* (Chlamydia) *psittaci* is an intracellular pathogen and a member of the *Chlamydiaceae* family that is most frequently associated with *Psittaciformes* but can also infect many other avian orders and species as well as a wide range of mammalian hosts (9). *C. psittaci* has the ability to remain infectious in the environment for months, presenting a variety of public health issues, including economically devastating outbreaks in poultry farms and occasionally severe pneumonia in humans (3, 4, 10, 20, 24, 25). Transmission of this atypical respiratory pathogen can occur through direct contact with infected birds, bird feces, nasal discharges, and aerosols, causing respiratory disease in both mammals and birds (14, 19). Zoonotic infections in humans usually result from close contact with infected captive birds or free-ranging birds; human-to-human transmission has also been suggested (1, 8, 17, 19, 20). Regardless of the transmission method, infection may lead to psittacosis, a primarily respiratory disease complex that may result in severe pneumonia and a wide spectrum of other medical complications (14). Individuals with occupations associated with commercial poultry as well as those with routine contact with companion or aviary birds are considered most at risk for infection. Furthermore, laboratory-acquired infections remain a concern (13).

*C. psittaci* is currently grouped into seven avian genotypes (A through F and a newly identified genotype, E/B) and two nonavian genotypes (M56 and WC) (5). Recent reclassification of *C. psittaci* has resulted in the separation of *C. abortus* and *C. caviae* into distinct species, although these species are genetically closely related (2, 22). Identification and genotyping of *C. psittaci* in avian samples and isolates are currently achieved by serological testing and molecular methods, such as outer membrane protein A (*ompA*) gene sequencing, restriction fragment length polymorphism (RFLP), and cumbersome real-time PCR assays and microarray analysis (6, 16, 18, 23). For diagnosis in humans, culture is rarely performed because the procedure is labor-intensive and requires specialized laboratory expertise and equipment. Thus, accurate diagnosis is often delayed or missed and may result in improper treatment for patients (3). Diagnosis by molecular techniques, such as real-time PCR, is not readily available in most public health laboratories, forcing them to rely upon insensitive complement fixation or microimmunofluorescence tests for detecting *C. psittaci* antibodies in suspect cases (14). Since both complement fixation and microimmunofluorescence require acute and convalescent-phase sera, they are retrospective assays considered inadequate for a timely diagnosis. A reliable and rapid assay for detecting and genotyping *C. psittaci* strains would greatly enhance the diagnostic capability for this agent and facilitate timely treatment.

This study reports the development of a real-time PCR assay followed by high-resolution melt (HRM) analysis using light-upon-extension (LUX) chemistry to specifically detect and genotype *C. psittaci*. This assay may be useful for epidemiological studies and provide valuable information for designing public health measures during outbreaks. This method may also offer greater insight into the heterogeneity of this species. To our knowledge, this is the first report of an HRM-based real-time PCR assay that simultaneously detects and genotypes this species.

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monolayers grown in 25-cm² culture flasks in Eagle’s minimal essential medium from a recommended specimen source: conjunctival, choanal, or cloacal swabs or gia, and tested positive at the time of collection (2004 to 2007) for mammals. These specimens were previously submitted to the Infectious

VR-854), CP3 (ATCC VR-574), CT1, NJ1, MN (ATCC VR-122), and VS-225 of each culture was centrifuged at 20,000

monolayers demonstrated an approximately 70% cytopathic effect, and the re-

Vero cells in triplicate. After incubation for 72 h at 37°C in a 5% CO2 atmo-

l quantities at 10-fold dilutions were used to inoculate wells of cultures of 50-

96-well flat-bottom microtiter plates containing Vero cells (21). Frozen
growth medium with 1 ml of stock

DNA from

Reference strain isolates DD-34 (ATCC VR-854), CP3 (ATCC VR-574), CT1, NJ1, MN (ATCC VR-122), and VS-225 were tested along with 169 specimens acquired from companion and avairy birds and mammals. These specimens were previously submitted to the Infectious Diseases Laboratory at the College of Veterinary Medicine, University of Georgia, and tested positive at the time of collection (2004 to 2007) for C. psittaci or other Chlamydophila species by a PCR-based assay. All specimens were obtained from a recommended specimen source: conjunctival, choanal, or cloacal swabs or whole blood.

C. psittaci culture. C. psittaci reference strains were propagated in Vero cell monolayers grown in 25-cm² culture flasks in Eagle’s minimal essential medium (MEM) supplemented with MEM nonessential amino acids, 2 µM l-glutamine, 20 µM HEPES buffer, 10% fetal calf serum, 20 µg/ml streptomycin, and 25 µg/ml vancomycin. Confluent Vero cell monolayers were inoculated by replacing the growth medium with 1 ml of stock C. psittaci culture diluted 1:10 in MEM containing 1 µg/ml of cycloheximide. The inoculated monolayers were placed at 37°C for 2 h before an additional 4 ml of MEM containing cycloheximide was added to each flask. Cultures were incubated for 7 days at 37°C or until the monolayers demonstrated an approximately 70% cytopathic effect, and the remaining Vero cells were scraped from the flask into the medium. One milliliter of each culture was centrifuged at 20,000 × g for 60 min, and the pellet was resuspended in nuclease-free water (catalog no. P1193; Promega) and used for DNA extraction. The remaining culture was dispensed into aliquots and frozen at −70°C. Titration of cultures was performed as previously described, with 96-well flat-bottom microtiter plates containing Vero cells (21). Frozen C. psittaci cultures of 50-µl quantities at 10-fold dilutions were used to inoculate wells of Vero cells in triplicate. After incubation for 72 h at 37°C in a 5% CO2 atmosphere, the medium was removed and the cells were fixed with methanol and stained with a Chlamydia genus-specific monoclonal antibody (catalog no. 30701; Bio-Rad). Inclusions (inclusion forming units/ml) were counted using an inverted fluorescence microscope.

DNA extraction. DNA from C. psittaci cultures was extracted using a QiaAmp DNA minikit (catalog no. 51304; Qiagen, Inc.) according to the manufacturer’s instructions. The DNA was eluted into 200 µl of QiaElution buffer and stored at −70°C until tested.

LUX primer design and optimization. Three primer sets targeting the variable regions of the C. psittaci ompA gene were designed. LUX chemistry (Invitrogen) utilizes a 5-carboxyfluorescein (FAM)-labeled primer and an unlabeled primer. All primer sets were designed using the C. psittaci 6BC ompA gene (GenBank accession no. X56980), the 90/105 ompA gene (GenBank accession no. AY762608), and the 7778B15 ompA gene (GenBank accession no. AY762612). All C. psittaci primer sets and expected amplicon sizes are listed in Table 1. Ppac was designed to amplify all C. psittaci genotypes, while GTpc specifically amplifies C. psittaci genotypes. The Ppac assay demonstrated a 96% efficiency, while GTpc displayed a >99% efficiency, calculated using a standardized dilution series of quantitated DNA of C. psittaci tested in triplicate over 6 logs (200 pg to 2 fg). The average for these data is reported as the square of the coefficient of regression values (efficiency); both assays had a lower limit of detection for 200 fg. A specific C. caviae marker targeting the ompA gene of C. caviae (GenBank accession no. AF269282) was designed using the above-described chemistry with unlabeled C-cav-F (5′-CCGGTGAGACAGGAAATAAC-3′) and FAM-labeled C-cav-R (5′-ccacaagCTAAGAAAGCCCGGTTTG-3′). The 5′ lowercase letters are not part of the primer itself but correspond to a self-quenching, complementary tail: “t” represents the FAM binding location]. The expected amplicon size is 78 bp.

Real-time PCR and HRM analysis. Utilization of LUX chemistry and HRM analysis has previously been described (26). The reaction mixture for all primer sets was prepared using a SuperMix-UDG platinum quantitative PCR kit (catalog no. 11730-025; Invitrogen) containing the following components per reaction mixture: 1.25 µl of 2× master mix, final concentrations of 100 nM each of the forward and reverse primers, 0.15 µl of platinum Taq polymerase (5 U/µl), 5 ng of a template, and nuclease-free water (catalog no. P1193; Promega) added to give a final volume of 25 µl. Real-time PCR was performed with a Corbett Rotor-Gene 6000 (catalog no. 6540; Corbett Life Sciences) under the following cycling conditions: 1 cycle at 95°C for 2 min, followed by 45 cycles at 95°C for 5 s and 62°C for 15 s, with data acquired at the 62°C step in the green channel. HRM was performed between 75°C and 85°C in 0.5°C increments, with fluorescence normalization regions between 75.5°C and 76°C, before separation and at 84°C to 84.5°C after the separation. All isolates were tested in triplicate. All specimens (avian and mammalian) were screened for the presence of C. psittaci, followed by genotyping, if applicable.

Sequencing. Amplification of the ompA gene from isolate strains (DD34, CP3, CT1, NJ1, VR-122, and VS-225) and specimens (no. 3, 5, 25, 30, 31, and 83) was performed using previously published primers and newly developed primers (5′-ACTATGTGGGAAGGTGCT-3′ and ompA-R (5′-TAGACTTC ATTGTGTGATCCTG-3′) (11). The PCR mixture was prepared using a SuperMix-UDG platinum quantitative PCR kit (catalog no. 11730-025; Invitrogen) containing the following components per reaction mixture: 5 µl of 10× PCR master mix-MgCl₂, 1.5 µl 50 mM MgCl₂, final concentrations of 100 nM each of the forward and reverse primers, 0.5 µl of platinum Taq polymerase (5 U/µl), 1 µl of 10 µM PCR nucleotide mixture (catalog no. C1141; Promega), 5 ng of a template, and nuclease-free water (catalog no. P1193; Promega) added to give a final volume of 50 µl. A DNA engine dyad peltier thermocycler (catalog no. PTC-0220G; Bio-Rad) was used for amplification under the following cycling conditions: 1 cycle at 95°C for 2 min, followed by 50 cycles at 95°C for 1 min, 59°C for 1 min, and 72°C for 2 min. Samples were then purified using a QIAquick gel extraction kit (catalog no. 28700; Qiagen) after separation on a 1% agarose gel. Sequencing was performed with an ABI 3130XL instrument (catalog no. 3130XL; Applied Biosystems, Inc.) under standard conditions for an 80-cm capillary. Consensus sequences were generated using DNASTar Lasergene SeqMan Pro software and aligned with published ompA gene sequences for each genotype by using ClustalW software. The GenBank accession numbers used for alignment are as follows: AY762608, AY762609, AF269261, AY762610, AY762611, AY762612, and AY762613.

RESULTS

Real-time PCR and HRM analysis. All markers (Ppac, GTpc, and GT-F) were tested against reference strains by using real-time PCR and HRM analysis. Each was screened for specificity with a variety of bacterial and viral agents at 15 ng each and displayed no cross-reactivity (Table 2). The results of the HRM analysis for Ppac yielded similar melt curves for each genotype (Fig. 1a). Ppac also amplifies C. caviae and can easily be distinguished from C. psittaci by HRM analysis by a disso-

<table>
<thead>
<tr>
<th>Marker</th>
<th>Description</th>
<th>Sequence</th>
<th>Amplicon size (bp)</th>
</tr>
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<tbody>
<tr>
<td>Ppac</td>
<td>Forward, labeled</td>
<td>5′-gaacctcATTGTTTGGCCGTACGGT-3′</td>
<td>109</td>
</tr>
<tr>
<td></td>
<td>Reverse, unlabeled</td>
<td>5′-TCTCAGACCTTCCCAAC-3′</td>
<td>274</td>
</tr>
<tr>
<td>GTpc</td>
<td>Forward, labeled</td>
<td>5′-gaactctGTGCAACCTTGAAGCTGAG-3′</td>
<td>98</td>
</tr>
<tr>
<td></td>
<td>Reverse, unlabeled</td>
<td>5′-ACTTGGACCGTATGCTAAT-3′</td>
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a The DNA sequences for each oligonucleotide set used for detection of C. psittaci are listed.

b The 5′ lowercase letters are not part of the primer itself but correspond to a self-quenching, complementary tail: “t” represents the FAM binding location.
GENOTYPING OF C. PSITTACI BY HRM

Specimen testing. One hundred sixty-nine specimens obtained from birds and companion mammals were screened along with reference strains. Of these archived nucleic acid preparations, 107 (63.3%) were positive for chlamydial DNA, 98 (91.6%) were positive for C. psittaci, and 9 (8.4%) were positive for C. caviae. Of the positive C. psittaci samples, 70 (71.4%) were genotype A, 3 (3.1%) were genotype B, 4 (4.1%) were genotype E, and 21 (21.4%) were positive for C. psittaci (positive amplification for both Ppac and GTpc markers) but could not be typed using this assay, due to inconclusive melt curve data (Table 3). The remaining specimens were negative. All C. caviae strains were obtained from guinea pig specimens, as expected.

Sequencing analysis. The ompA gene was sequenced for all available reference strains and a subset of chlamydia-positive specimens that contained nucleic acids in sufficient amounts and of sufficient quality, acquired from the Infectious Diseases Laboratory, University of Georgia, as described above. The sequences of the target regions for this assay within the ompA gene are shown in Fig. 2. The data show that this assay correctly identified DD34 and specimens 25 and 83 as genotype A, CP3 and specimens 30 and 31 as genotype B, CT1 as genotype C, NJ1 as genotype D, Vr-122 and specimens 3 and 5 as genotype E, and VS-225 as genotype F.

DISCUSSION

Newly developed molecular methods, such as DNA microarrays, a real-time PCR assay for detecting amplified product by using minor groove binding probes and competitor oligonucleotides, and multiplex variable-number tandem repeat analysis, have greatly improved upon the traditional approaches of ompA sequencing and RFLP for genotyping C. psittaci. However, a simple, rapid, and inexpensive assay is still lacking and would be ideal (6, 7, 12, 16).

The current study reports the development and implementation of a novel diagnostic assay that is capable of rapidly genotyping C. psittaci. A three-marker panel (Ppac, GTpc, and C. psittaci) with the use of standard cycling conditions followed by a HRM, is able to reliably differentiate genotypes A through F and detect the closely related DD34 and specimens 25 and 83 as genotype A, CP3 and specimens 30 and 31 as genotype B, CT1 as genotype C, NJ1 as genotype D, Vr-122 and specimens 3 and 5 as genotype E, and VS-225 as genotype F.

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commonly been identified in pigeons and doves, again consistent with our data (9). Notably, genotypes C, D, and F were not found in any of the specimens tested, supporting the claim by others that the vast majority of naturally occurring genotypes belong to the ABE cluster (16). C. caviae was detected only in guinea pig specimens (Table 3), where it is known to exist. No specimens tested positive for C. abortus, an agent typically found in mammals, although a few psittacine infections have been reported (2, 15). Neither C. caviae nor C. abortus is considered a classic respiratory pathogen and would not likely be present in respiratory samples being tested for C. psittaci in humans (2). However, if Ppac yields a positive amplification curve while GTpc is negative, C. abortus should be considered and ompA sequencing should be performed for verification. Collectively, the experimental data underscore the utility of this assay.

The reliability of HRM analysis is dependent upon both sufficient quantity and sufficient quality of the starting template. As such, specimens with amplification curves that are not sigmoidal or have threshold cycle values of 40 or above should
be interpreted with care due to the possibility of inaccurate melt curve data. If possible, these samples should be concentrated and retested for verification. When threshold cycle values of <40 are achieved, the HRM data are remarkably consistent, reproducible, and reliable, as evidenced by the virtually identical melt curves generated in each of the triplicate wells assayed on numerous occasions and verified by subsequent sequence analysis. The quality and amount of template are insufficient to allow for reliable genotype identification.

FIG. 2. Sequence data. (a) Sequence alignment of Ppac amplicons. (b) Sequence alignment of GTpc amplicons. * indicates no consensus sequence; a dash (−) indicates identical sequences. Each genotype (**) is represented by reference strains and, where applicable, specimen sequences. Genotype A includes DD34 and specimens 25 and 83, genotype B includes CP3 and specimens 30 and 31, genotype C includes CT1, genotype D includes NJ1, genotype E includes Vr122 and specimens 3 and 5, and genotype F includes VS225 only. Underlined and bold portions of the sequences are primer binding locations. Genotype E/B is excluded due to the unavailability of the strain.
inherent limitations in any real-time PCR assay but are especially critical when HRM analysis is performed. These limitations may account for the 21.4% of the specimens that were unable to be definitively genotyped since some older specimens may not have been properly stored after submission (data not shown). Unfortunately, genotype E/B has only recently been described and was unavailable for analysis with this assay (5).

Currently, there exists no rapid and simple procedure which can discriminate among the known genotypes of \textit{C. psittaci} and have the capability of identifying new strains among the extraordinary genetic heterogeneity found within this species. The current study reports a real-time PCR assay that is able to detect and identify six known \textit{C. psittaci} genotypes, along with identifying \textit{C. caviae} and \textit{C. abortus}. This assay may aid in determining the existence of variants within this species by using the exquisite discriminatory ability of LUX chemistry followed by HRM analysis. The versatility of this assay makes it useful in many applications, such as (i) improving the timely reporting of results, aiding in epidemiological investigations; (ii) possible pathogenicity and transmission studies; (iii) prospective screening of companion birds; (iv) current and retrospective analysis of specimens collected during an outbreak; and (v) a greater characterization of this infectious agent, leading to a better understanding of \textit{C. psittaci} infection within human and avian populations. This procedure can also contribute to the development of an all-inclusive molecular typing system for this agent.

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REFERENCES


