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Application of a Nested, Multiplex PCR to Psittacosis Outbreaks

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We developed a nested, multiplex PCR for simultaneous detection of three species of chlamydiae in human and avian specimens. The PCR was designed to increase sensitivity and to circumvent inhibitors of PCR present in clinical specimens. The target sequence was the 16S rRNA gene. The first-step PCR was genus specific, and the second-step PCR was multiplexed (i.e., had multiple primer sets in the same tube) and could discriminate among *Chlamydia pneumoniae*, *Chlamydia psittaci*, and *Chlamydia trachomatis* on the basis of the molecular weight of the amplicon. The limit of detection of each of the two PCR steps was 5 inclusion-forming units. We used PCR and serologic evidence during outbreaks of psittacosis to infer that *C. psittaci* had been transmitted from birds purchased in pet stores to humans. We also used this method to test both live and dead birds from pet stores for infection with *C. psittaci*. Compared with culture, the application of PCR to avian specimens increased the rate of *C. psittaci* detection.

Among the chlamydiae, the most important human respiratory pathogens are *Chlamydia pneumoniae* and *Chlamydia psittaci*, although *Chlamydia trachomatis* can cause pneumonia in infants. *C. pneumoniae* causes approximately 10% of community-acquired pneumonias. *C. psittaci* is a common pathogen of domestic mammals and birds (12, 13). Transmission of *C. psittaci* to humans is likely underrecognized and unreported; only 38 cases were reported through national surveillance during 1994 in the United States (1).

Birds kept as pets have been an important source of human infection (6, 12). Outbreaks of human disease can occur whenever there is close and continued contact between humans and infected birds, which excrete the organism in feces and saliva (6, 8). The agent is present in tissues and secretions and is often excreted in feces by healthy birds (4, 8, 11, 14). The inhalation of infected dried bird feces is also a common method of human infection (9). Additional information on psittacosis and chlamydiosis infections can be found in a review by Vanrompay et al. (16).

Laboratory confirmation of psittacosis in humans and birds is challenging, and treatment is often empirical and predicated on the clinician's eliciting a history of bird exposure (10). Measurement of complement-fixing (CF) antibody titers and, much less commonly, recovery of *C. psittaci* from patients have been the mainstays of laboratory confirmation of psittacosis. Neither method is sensitive, and CF is very nonspecific (3, 5).

Microimmunofluorescence (MIF) is a technique that offers greater sensitivity and specificity than CF but is not as widely used (17). Both tests rely on time for antibody production to occur; thus, they are not early-detection methods. We are evaluating an ELISPOT test for earlier detection of immunoglobulin G antibody-secreting cells for chlamydiae (2).

PCR is very sensitive and specific for detection of target DNA sequences and relies only on the presence of the organism in the specimen. We are aware of only two studies that use PCR as a step toward distinguishing the above-mentioned three chlamydial species. The method of Kaltenboeck et al. (7) relies on restriction endonuclease analyses to discriminate at the species level following a second-step PCR. The method described by Tjhie et al. (15) uses hybridization probes to discriminate species following a genus PCR.

We developed a two-step PCR. The second step detects and discriminates *C. pneumoniae*, *C. psittaci*, and *C. trachomatis* either alone or in any combination simultaneously. The first-step PCR is genus specific, and the second step is multiplexed, i.e., has multiple primer sets that detect more than one target in the same tube.

With available tests, pet stores can fail to identify birds infected with *C. psittaci* that are asymptomatic prior to sale. We used PCR and serologic data during an outbreak of psittacosis to evaluate transmission of *C. psittaci* to humans from birds purchased in pet stores in Georgia. We also used this method to determine whether both live and dead birds from pet stores in West Virginia were infected with *C. psittaci*. Finally, this PCR demonstrated that a human lung sample was infected with *C. psittaci* and not with *C. pneumoniae*, which is sometimes a difficult diagnosis for clinicians to distinguish.

MATERIALS AND METHODS

Growth and purification of organisms and detection from specimens. Chlamydiae were grown as previously described (18). Briefly, stock cultures were propagated by centrifugation at 750 × *g* for 1 h at room temperature onto six-well plates of exponentially growing HEP2 cell monolayers in Eagle's minimal essential medium (MEM) supplemented with MEM nonessential amino acids, 10% fetal calf serum, and antibiotics (gentamicin and vancomycin). Then, 1 μg of cycloheximide per ml was added, and the cultures were incubated in a CO_2 incubator for 48 to 72 h. Chlamydial elementary bodies were harvested by disrupting the host monolayer with a rubber policeman in fresh Eagle's MEM with 10% fetal calf serum. The disrupted cell suspension was sonicated and partially purified by centrifugation at 500 × *g* for 10 min, followed by centrifugation on a 35% Renografin cushion (Squibb, New Brunswick, N.J.). Inclusion forming units (IFU) were measured by counting the numbers of inclusions cultured in triplicate shell vials from 10-fold serial dilutions of purified elementary bodies. IFU quantitated in this manner were used as the positive-control DNA in PCR assays.

Isolation from tissue or feces. Approximately 100 mg of minced tissue or 100 mg of feces was vortexed with 3-mm glass beads in HSC buffer (HEPES, sucrose, calcium buffer) and centrifuged at 500 × *g* for 5 min. Then, 200 μl of the supernatant was inoculated by centrifugation at 1,700 × *g* in triplicate shell vials

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of HEP2 cell monolayers. Shell vials were incubated for 72 h initially and subcultured twice at 48-h intervals if earlier passages were negative.

Serology. CF and MIF methods have been previously described (17). Initially, sera were tested by CF at state laboratories. Upon receipt at the Centers for Disease Control and Prevention (CDC), the sera were tested by MIF for all three species.

Preparation of clinical specimens. Clinical specimens included unclotted blood from EDTA tubes, throat swabs, and lung tissue from humans and feces, tissue, and cloacal swabs from birds. These specimens were prepared for PCR by using the QiaAmp Blood and QiaAmp Tissue kits (Qiagen Inc., Chatsworth, Calif.). All specimens except blood and tissue underwent a differential centrifugation of 500 × *g* for 5 min to pellet debris prior to DNA extraction according to the directions accompanying the Qiagen kits for 200-μl sample volumes. Swabs were collected into M4 chlamydia transport medium (MicroTest Inc., Snellville, Ga.) for transport to the CDC. They were vortexed vigorously prior to differential centrifugation and subsequent DNA extraction. The entire buffy coat (approximately 200 μl) was collected from 3 ml of EDTA blood and used for DNA extraction. Some PCR tubes containing specimen aliquots were randomly spiked with 5 IFU of *C. psittaci* to control for the presence of inhibitors.

PCR amplification. Samples for PCR were prepared in a class II laminar flow hood, and amplification and analysis of PCR products were each performed in separate locations. Reaction volumes of 50 μl, containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 200 μM each deoxynucleoside triphosphate, 0.01% bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.), 1.25 U of *Taq* polymerase (Boehringer Mannheim Biochemicals, Indianapolis, Ind.), 0.2 μM each outer primer, and 5 μl of sample were overlaid with 1 drop of mineral oil and placed in a Perkin-Elmer Thermalcycler (model 480; Perkin-Elmer Cetus Co., Norwalk, Conn.) for 1 cycle of 95°C for 2 min, followed by 35 cycles of 94°C for 1 min, 55°C for 30 s, and 72°C for 1 min. The nested or inner PCR mixture was similar to the first, except that it contained 1 μl of the product of the outer PCR and 0.2 μM each inner primer. The cycling conditions were identical.

Genus-specific first-step primers for 16S rRNA. Genus-specific first-step 16S rRNA primers were as follows: sense (5′→3′), ACG GAA TAA TGA CTT CGG; antisense (5′→3′), TAC CTG GTA CGC TCA ATT. The genus product was 436 bp.

Species-specific second-step 16S rRNA primers. Species-specific second-step 16S rRNA primers were as follows: *C. trachomatis* sense (5′→3′), GCA ATT GTT TCG GCA ATT G; *C. trachomatis* antisense (5′→3′), AGC GGG TAT TAA CCG CCT; *C. pneumoniae* and *C. psittaci* sense (5′→3′), ATA ATG ACT TCG GTT GTT ATT; *C. psittaci* antisense (5′→3′), TGT TTT AGA TGC CTA AAC AT; and *C. pneumoniae* antisense (5′→3′), CGT CAT CGC CTT GGT GGG CTT. Species products for *C. trachomatis*, *C. pneumoniae*, and *C. psittaci* were 412, 221, and 127 bp, respectively. Both the outer and the inner PCR mixtures were optimized with the Opti-Prime PCR Optimization kit (Stratagene, La Jolla, Calif.).

Amplification products were separated by electrophoresis through 2.5% agarose gels (1.5% Nusieve GTG agarose [FMC Bioproducts, Rockland, Maine] and 1.0% agarose [Bio-Rad, Richmond, Calif.]) in Tris-borate-EDTA and were visualized by ethidium bromide fluorescence.

RESULTS

All of the PCR primers were both sensitive and specific. Neither the outer (genus-specific) nor the inner (species-specific) primer sets cross-reacted with other respiratory pathogens. The following microorganisms were tested for cross-reactivity in the PCR assay with both the first- and second-step PCR primers: *Acinetobacter* species; *Alcaligenes faecalis*; *Bordetella pertussis*; *Corynebacterium diphtheriae*; *Corynebacterium coryneform* E6756 and E378; *Corynebacterium maruchotis* B1 G5048 and BC F124; *Corynebacterium straitium* D9110(A) and E4684; *Corynebacterium xerosis* G676 and G3375; *Ehrlichia chaffiensis*; *Flavobacterium meningosepticum*; *Haemophilus influenzae* KC 818A, KC 1050B, KC 1051C, KC 819D, KC 528E, and KC 529F; *Kingella kingae*; *Legionella pneumophila* serogroup 1; *Mycobacterium tuberculosis*; *Proteus mirabilis*; *Pseudomonas aeruginosa*; *Staphylococcus aureus*; and *Streptococcus pneumoniae*. A chlamydia-positive control of 5 IFU was included in every test to verify that the PCR was working. All of these bacteria tested negative.

We used a nested-PCR strategy to increase the sensitivity and specificity of the assay. To minimize false-positive results, we performed each step of the PCR in a separate physical location using sterile technique and a laminar flow hood. To confirm our PCR results, both positive and negative, we tested each specimen on 3 different days. We randomly spiked PCR tubes containing aliquots of clinical specimens with 5 IFU of the organism suspected of causing the outbreak to test for the presence of inhibitors. Nested-PCR assays included all five species primers so that any combination of the three chlamydial species could be identified when it was present.

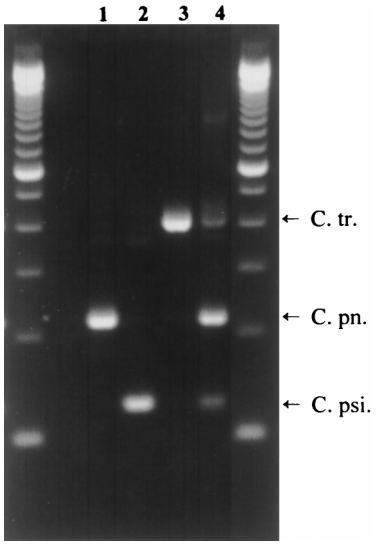

FIG. 1. Detection and discrimination of *C. pneumoniae*, *C. psittaci*, and *C. trachomatis* with multiplex PCR primers. One microliter of the genus, first-step PCR was used in the nested, multiplex PCR. A total of 5 IFU of each organism was used in the first-step PCR. The *C. pneumoniae* (C. pn.), *C. psittaci* (C. psi.), and *C. trachomatis* (C. tr.) bands are 221, 126, and 412 bp, respectively. The 100-bp ladder is from GIBCO-BRL (Gaithersburg, Md.). Lanes 1 to 3, amplification when each organism was present alone; lane 4, the three amplified bands when 5 IFU of each organism was present simultaneously.

The limit of detection of each of the primer sets, both the genus and species sets, is less than 5 IFU. An example of the sensitivity and specificity of the multiplex PCR is shown in Fig. 1. The multiplex PCR, with all five primers for detection of each *Chlamydia* organism, was run as a second-step PCR by using 1 μl of the first-step PCR product. A total of 5 IFU of each *Chlamydia* species was used in the genus, first-step PCR.

Because of reports of illness among owners of pet birds purchased from a lot confirmed to have chlamydiosis by necropsy and immunohistochemical staining, three separate groups of sick or dead birds and a few human throat swabs and blood specimens were tested for *C. psittaci*. The three groups tested were as follows. The first group included unclotted EDTA blood and/or throat specimens from some sick family members and cage droppings from their pet birds in Georgia. Only the bird specimen results are reported in Table 1, since all of the human specimens were negative for both PCR and culture. The second group consisted of 26 dead birds from a lot of birds that had confirmed chlamydiosis by immunohistochemical staining but that were unsuitable for testing by conventional methods, and the third group included 45 samples of cage droppings or cloacal swabs from birds suspected of having chlamydiosis in pet stores in West Virginia. Table 1 summarizes the PCR and culture results for the three groups of birds. All of the culture-positive specimens were also PCR positive.

Of the human serum samples from four individuals tested in the first group, one had a high MIF antibody titer specific to *C. psittaci* (1:512), which was indicative of recent exposure. None of the serum samples was tested by PCR. All of the throat swabs from these individuals were PCR negative. No human specimens from the West Virginia group were available for

TABLE 1. Comparison of multiplex PCR and culture for detection of *C. psittaci* in bird specimens[a]

Specimen group	PCR-positive result (%)	Culture-positive result (%)[b]
Group I from Georgia	50 (2 of 4)	25 (1 of 4)
Group II from Georgia	19 (5 of 26)	8 (2 of 26)
West Virginia	13 (6 of 45)	2 (1 of 45)

[a] Positive controls for PCR were 5 IFU of *C. pneumoniae* CM-1 and 5 IFU of *C. psittaci* DD34. All of the PCR-positive and culture-positive specimens were *C. psittaci* only. We detected no other chlamydial species in any of the specimens, although all of the primers for detection of all three species were included. Clinical specimens were inoculated in triplicate shell vials for culture as described in Materials and Methods. Specimens included cloacal swabs, cage droppings, and tissues from dead birds.

[b] All culture-positive specimens were also PCR positive.

testing; all culture isolates came from birds. The MIF antibody titers specific to *C. psittaci* of ill persons exposed to PCR-positive birds in the West Virginia group ranged from 1:16 to 1:512. There were no human specimens from West Virginia available for PCR testing.

Figure 2 shows the results for some of the dead birds tested from the University of Georgia. The PCR products from the genus primers on liver and lung tissues are shown in Fig. 2A. Only the lanes with the correct molecular size product (436 bp) scored as positive specimens (lanes 7 and 11). Occasionally, the product made in the first-step PCR was insufficient to be visualized on the agarose gel, because inhibitors were still present in the sample even after preparation or purification of the clinical specimen for PCR. Positive specimens could most quickly and efficiently be identified by a second-step PCR with a small fraction of the first-step PCR product.

Figure 2B shows the species products obtained when 1 μl of the genus PCR products from panel A was amplified with the nested, multiplex primers. *C. pneumoniae* was included as a positive control. Only the specimens tested in lanes 7 and 11 were positive for *C. psittaci*. No other *Chlamydia* species were detected in any of the specimens that we received from any site.

The only PCR-positive human specimen in these studies was from an outbreak of psittacosis in southeastern Australia. Four specimens of postmortem lung tissue from one patient were tested for detection and characterization of *C. psittaci*. The patient had seroconverted to *C. psittaci* by immunofluorescence and CF titers. This postmortem lung tissue was also PCR positive for *C. psittaci* in Australia when a species-specific PCR was used, but *C. pneumoniae* could not be excluded as the etiologic agent, because there was no available test. While we were unable to culture *C. psittaci* from the tissue, we did find three of the four lung specimens PCR positive only for *C. psittaci*; no *C. pneumoniae* was detected (data not shown).

DISCUSSION

The focus of these investigations was to determine if there had been transmission of *C. psittaci* from shipments of birds known to be infected to owners of these birds. Sensitive and specific tests to confirm psittacosis are lacking. We developed a nested, multiplex PCR that simultaneously distinguished and detected *C. pneumoniae*, *C. psittaci*, and *C. trachomatis*. This PCR was applied to all specimens available in these studies.

Nested PCR was used because it increases sensitivity. Clinical specimens often contain PCR inhibitors even after purification steps such as chloroform-phenol extraction. The two-step PCR usually circumvents the effects of inhibitors in clinical specimens because, while the first-step product may be too small for detection by ethidium bromide staining, enough product is synthesized for amplification and detection in the second-step or nested reaction.

The first Georgia group of specimens collected for culture and PCR consisted of unclotted blood and throat swabs from a few exposed humans. Fresh bird droppings were also collected from the corresponding households. Droppings from half of the birds tested in the first Georgia group contained *C. psittaci* as demonstrated by PCR, culture, or both. One of the ill humans exposed to a PCR-positive bird had an MIF antibody titer specific to *C. psittaci* of 1:512—a strong indication of recent exposure. In most cases, specimens were submitted from persons who had symptoms for more than 2 weeks and who had received antibiotics. This could explain why the human specimens were PCR negative.

The second Georgia group of specimens consisted of tissue from 26 dead birds from the pet stores from which the initial group of sick birds had originated. This second group was collected by the Animal Disease Eradication Veterinarian for the State of Georgia and tested at the University of Georgia

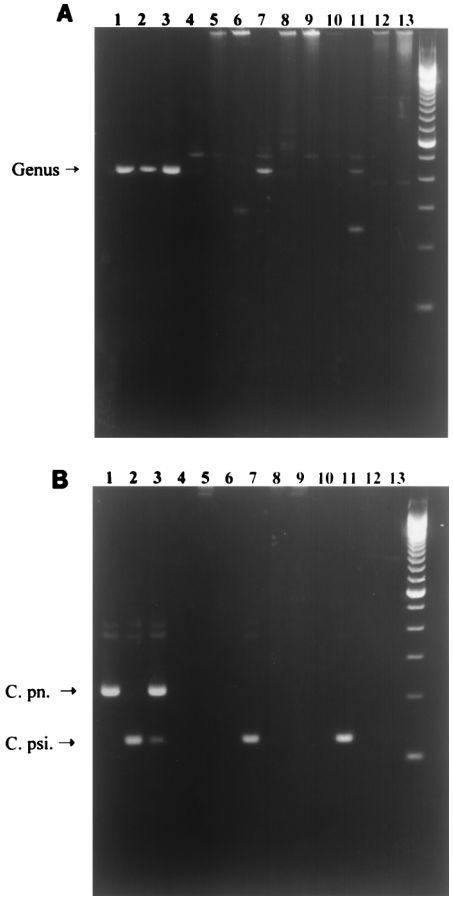

FIG. 2. (A) Genus and species amplification products of some of the bird necropsy tissues tested from Georgia pet stores. Lanes 1 to 3, the positive controls with 5 IFU of *C. pneumoniae*, 5 IFU of *C. psittaci*, and 5 IFU of each. The genus PCR product is 436 bp. The remaining lanes are liver, lung, and spleen specimens. Each lane represents tissue from a different bird. (B) Same as panel A, except that 1 μl of the genus PCR product was reamplified with the multiplex species primers. The *C. pneumoniae* (C. pn.) PCR product is 221 bp, and the *C. psittaci* (C. psi.) PCR product is 127 bp. Two tissue specimens were PCR positive for *C. psittaci.*

for the presence of *C. psittaci* by necropsy, gross inspection, traditional histochemical staining (Machiavelo and Gimenez stains), and immunohistochemical staining. Because of poor handling of carcasses prior to testing, many could not be tested properly by these techniques, and many of the birds tested negative. Tissue specimens that were unsuitable for testing by conventional methods were sent to the CDC for testing by PCR and culture techniques. In this investigation, the application of PCR to avian specimens increased the rate of detection of *C. psittaci* compared with those for culture and traditional histochemical and immunohistochemical staining.

Forty-five specimens (droppings, cloacal swabs, or tissue) from sick or dead birds from West Virginia pet stores were tested for *C. psittaci* because pet store employees and owners of sick birds were ill with a psittacosis-like illness. The results showed that persons with high MIF antibody titers specific to *C. psittaci* had been exposed to sick birds that were PCR positive for this organism.

PCR distinguished *C. psittaci* from *C. pneumoniae*, which is an important factor for clinicians making a diagnosis, since many of the tests employed in diagnosis are cross-reactive. The attending physician of the Australian patient wished to exclude a diagnosis of *C. pneumoniae* pneumonia. This was accomplished by multiplex PCR on postmortem lung tissue.

The data demonstrated that PCR detected more positive specimens than the other techniques employed in this study. This method could be useful when traditional tests fail to detect positive specimens because the specimen quality is poor, such as occurs with putrefaction. The PCR assay, unlike CF and immunohistochemical staining, is specific for chlamydiae, and it simultaneously detects and distinguishes the three species that infect humans. In the studies presented here, PCR linked transmission of *C. psittaci* from birds to humans when other tests failed or were ambiguous.

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