

Evaluation of the polymerase chain reaction in comparison with other diagnostic methods for the detection of *Chlamydia psittaci*

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Abstract. Various diagnostic methods exist for the detection of *Chlamydia psittaci*. In the current study, the test performance of polymerase chain reaction (PCR) was compared with other testing methods used in the diagnosis of *C. psittaci*. Tissue and fecal specimens ($n = 119$) of avian and mammalian origin were tested by PCR and one or more of the following methods: cell culture, enzyme-linked immunosorbent assay, and direct fluorescein-conjugated monoclonal antibody staining. Several gold standards, based on results of testing methods other than PCR, were used to calculate the following test performance characteristics of PCR: sensitivity and specificity, with their 95% confidence intervals; kappa statistics, a measure of intertest agreement; and lambda statistics, a chance-corrected estimate of the sensitivity and specificity. Overall, the test performance characteristics of PCR were low compared with the other testing methods. Possible reasons for the poor test performance of PCR in the current study include destruction of the organisms during storage, interference with the PCR by other reagents, or technical errors.

Chlamydia psittaci is a gram-negative, obligate intracellular organism that produces a variety of disease syndromes in birds and mammals.¹ Infection can be clinically inapparent or result in disease of the respiratory tract, conjunctiva, serous membranes, joints, central nervous system (CNS), reproductive organs, and gastrointestinal tract. Reservoirs of the organism include the respiratory, intestinal, and genital tracts of birds and mammals, with transmission occurring via inhalation or ingestion.

The mammalian and avian subgroups of *C. psittaci* differ genetically and serologically.¹⁴ Major outer membrane proteins (MOMP) determine species and type specificity of Chlamydiae.² Antigenic and genetic differences that are related to the different disease entities and host specificity occur among the subgroups.^{1,8} Host specificity is not absolute, with animal subgroups capable of causing human infection with variable clinical outcomes.

Chlamydiosis is a zoonotic disease, and human cases must be reported by state health departments to the Centers for Disease Control and Prevention.⁴ Because of both the economic and public health considerations, prompt and accurate detection of reservoirs of infection is important. Various diagnostic methods are available, such as isolation via cell or egg culture, enzyme-linked immunosorbent assay (ELISA), and direct fluorescein-conjugated monoclonal antibody staining (FA).

In this study, the test performance of polymerase chain reaction (PCR) was compared with cell culture, ELISA, and FA for the detection of *Chlamydia* spp. Cell or egg culture, traditionally considered the gold standard for diagnosis of chlamydial infections,¹² offers a sensitive means of detection, although it is time consuming and susceptible to failure when specimens are transported under poor conditions or are heavily contaminated with antibiotic-resistant bacteria or fungi.⁹ The commercially available ELISAs are rapid and sensitive but have been noted to have a high number of false positives when testing fecal specimens.⁹ The sensitivity of the ELISA may also be negatively affected by chlamydial epitopes that are inaccessible to antibodies and have a reported minimal detection level of 1,000 organisms.^{3,12} Although both the FA and ELISA have the ability to detect nonviable organisms rapidly, the test performance of commercially available products has not been widely assessed.¹⁵

Materials and methods

Clinical specimens. The California Veterinary Diagnostic Laboratory Services (CVDLS) provided 307 clinical specimens submitted from 1987 through 1992 for testing for *C. psittaci*. One or more of the following diagnostic tests were performed by CVDLS: ELISA, FA, or cell culture. Clinical specimens were frozen in storage media after testing. Clinical specimens with available ELISA, FA, or cell culture results ($n = 119$) were selected for the study and subjected to PCR.

Reference strains. The following reference strains were obtained from the American Type Culture Collection for use as positive controls for PCR: *Chlamydia trachomatis* Trachoma serotype A, strain HAR-13 (ATTC VR571B) from human conjunctiva, and *C. psittaci*, psittacosis strain 6BC (ATTC VR 125) from a parakeet.^a

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| Test A | Test B | | |
|--------|----------------|----------------|----------------|
| | + | - | |
| + | a | b | P ₁ |
| - | c | d | Q ₁ |
| | P ₂ | Q ₂ | |

$$\kappa = I_o - I_e / 1 - I_e = 2(ad - bc) / P_1Q_2 + P_2Q_1$$

Figure 1. Formula for calculating the kappa (κ) statistic.

DNA extraction. DNA was extracted from clinical specimens and reference strains by the proteinase K/boiling method.¹⁶ For this procedure, 0.75 ml of proteinase K buffer (100 mM Tris HCl, pH 8.3, 500 mM KCl, 15 mM MgCl₂, 0.5% NP40, 0.5% Tween 20, 200 μ g/ml proteinase K) was added to 0.25 ml of each specimen and incubated at 60 C for 2 hr to overnight. Tubes were then vortexed, spun in a microfuge at 13,000 \times g for 10 sec, and then boiled in a 100 C water bath for 10 min. Tubes were cooled on ice, vortexed, and spun in a microfuge at 13,000 \times g for 10 min. DNA-containing supernatant was transferred to fresh tubes.

The second step of DNA extraction utilized phenol extraction and ethanol precipitation. Phenol/chloroform/isoamyl alcohol (25:24:1) was added to an equal volume of DNA solution and vortexed for 10 sec. Tubes were spun in a microfuge at 13,000 \times g for 15 sec and the DNA-containing aqueous layer was removed. For ethanol precipitation, an equal volume of 7.5 M ammonium acetate was added to DNA and vortexed. Two volumes of ice-cold 100% ethanol was added and the mixture was vortexed and placed on ice for 5 min. The ethanol supernatant was removed after being spun in a microfuge at 13,000 \times g for 5 min. Precipitate was washed with 1 ml 70% ethanol by inverting tubes several times and spun in a microfuge at 13,000 \times g for 5 min. Supernatant was removed and DNA was dissolved in 0.5 ml 1 \times Tris EDTA (TE) buffer.

Primers. Primers 1904 (5'TAC GGG TTC CGC TCT CTC CTT ACA 3') and 1905 (5'CAA CCA CAT TCC CAT AAA GCT CCA CG 3') were synthesized on an automated synthesizer at the University of California-Davis Protein Synthesis Lab to amplify a region of the chlamydial MOMP gene, yielding an amplified fragment of 513 bp.^b Primers were then column purified with the Bio-Rad Econo-Pac 10DG column.^c

PCR. The PCR amplification mix contained 2.5 mM each of dATP, dCTP, dGTP, and dTTP,^d 15 μ l clinical specimen or reference strain, 50 picomoles (2.3 μ l) of primer 1904, 50 picomoles (1.9 μ l) of primer 1905, 5 μ l amplification buffer C from Invitrogen's PCR optimizer kit^e [pH 8.5: 2.0 mM Mg²⁺, 300 mM Tris HCl, 75 mM (NH₄)₂SO₄], and 0.5 U Taq polymerase.^d The volume of the amplification mix

| PCR | Gold Standard | | |
|-----|-----------------|-----------------|-----------------|
| | + | - | |
| + | n ₁₁ | n ₁₂ | n _{1.} |
| - | n ₂₁ | n ₂₂ | n _{2.} |
| | n _{.1} | n _{.2} | N |

$$\lambda(se) = \frac{n_{11}/n_{1.} - n_{1.}/N}{n_{2.}/N}$$

$$\lambda(sp) = \frac{n_{22}/n_{2.} - n_{2.}/N}{n_{1.}/N}$$

Figure 2. Formula for calculating the lambda (λ) statistic.

was brought up to 50 μ l with sterile distilled water, then overlaid with 50 μ l of sterile mineral oil.

The negative control consisted of an amplification mixture identical in every way except that it contained no clinical specimen. Positive controls included manufacturer's templates for 600 bp (pH 10, 2 mM Mg²⁺) and 2,000 bp (pH 8.5, 3.5 mM Mg²⁺) fragments and primers Sp6 and T7. *Chlamydia psittaci* (442.2 μ g DNA/ml) and *C. trachomatis* (349.8 μ g DNA/ml) reference strains were also used as positive controls.

The amplification mixture was subjected to 30 thermal cycles as follows: denaturation at 94 C for 1.5 min, annealing at 55 C for 2 min, and primer extension at 72 C for 3 min⁵ on a programmable thermal controller.^f

Analysis of amplified DNA. A mixture of 10 μ l of amplified product and 1 μ l of 10X loading buffer (20% Ficoll 400, 0.1 M Na₂ ethylenediaminetetraacetic acid, pH 8, 1% sodium dodecyl sulfate, and 0.25% bromphenol blue) was examined by 1.2% agarose gel electrophoresis. A molecular weight marker, lambda DNA, HindIII, was included to aid in estimation of the size of the amplified product.⁸ The DNA was visualized by ethidium bromide staining and ultraviolet illumination.

Statistical analysis. The intertest agreement PCR and the other testing methods was examined with the kappa (κ) statistic, a proportional measure of agreement that corrects for chance, where I_o denotes the observed index of agreement and I_e the value of agreement expected on the basis of chance alone (Fig. 1).⁷ When there is complete agreement, $\kappa = 100\%$. If I_o is greater than or equal to chance agreement, $\kappa \geq 0$, and if I_o is less than or equal to chance agreement, $\kappa \leq 0$. The lambda statistic, a chance-corrected method of estimating the sensitivity (λ_{SE}) and specificity (λ_{SP}), was calculated for each of the testing methods (Fig. 2).⁶

There were 108 avian specimens, 78 tissue (e.g., liver, air

Table 1. Results of cell culture, ELISA, FA, and PCR for *Chlamydia psittaci*.*

| Test | Avian | | Mammalian | Total |
|---------|--------------|---------------|--------------|----------------|
| | Feces | Tissue | Tissue | |
| PCR | 12/30 (40%) | 33/78 (42.3%) | 3/11 (27.3%) | 48/119 (40.3%) |
| ELISA | 6/19 (31.6%) | 15/35 (42.9%) | 5/6 (8.3%) | 26/60 (43.3%) |
| FA | 2/11 (18.2%) | 40/52 (76.9%) | 3/7 (42.9%) | 45/70 (64.3%) |
| Culture | 4/19 (21.1%) | 42/72 (58.3%) | 1/5 (20%) | 47/96 (49%) |

* Data are number positive/total tested (%). ELISA = enzyme-linked immunosorbent assay; FA = direct fluorescein-conjugated monoclonal antibody staining; PCR = polymerase chain reaction.

sac, and spleen) and 30 fecal, and 11 mammalian specimens, all tissue (e.g., placenta or unknown). The overall test performance characteristics were calculated with results from all specimens, as well as for the following subsets: 1) tissue specimens from birds and mammals, 2) avian tissue and fecal specimens, and 3) avian tissue specimens. The number of mammalian specimens or avian fecal specimens was insufficient to carry out separate analyses of these subsets.

The following positive gold standards were used in the analysis: 1) culture positive ($n = 47$); 2) ELISA or FA positive, or both, and culture positive ($n = 28$); and 3) culture positive or ELISA and FA positive ($n = 51$). The third set of criteria is used by CVDLS to define a true positive.

The following negative gold standards were used in the analysis: 1) negative by culture, ELISA, and FA ($n = 7$); and 2) ELISA or FA negative, or both, and culture negative ($n = 30$).

Results

Polymerase chain reaction. Forty-eight (40.3%) of 119 specimens were PCR positive when primers 1904 and 1905 were used, including 45 (41.7%) of the 108 avian specimens and 3 (27.3%) of the 11 mammalian specimens (Table 1). Among the avian specimens, 12 (40.0%) of the 30 fecal specimens and 33 (42.3%) of the 78 tissue specimens were positive. Both reference strains used as positive controls, *C. psittaci* and *C. trachomatis*, produced a fragment of the expected size with PCR.

Eighteen (38.3%) of the 47 culture-positive specimens were PCR negative, even after the reaction was repeated and aliquots of the original PCR reaction were reamplified. Seven (14.9%) culture-positive specimens were positive by PCR only after the reaction was repeated a second time, in some cases after a second phenol extraction step.

Statistical analysis. When all results from all specimens were analyzed, the intertest agreement (κ) was highest between ELISA and FA (63.1%), followed by ELISA and PCR (39.5%), then FA versus PCR (11.3%).

Sensitivity, with its corresponding 95% confidence interval, and lambda and kappa statistics were computed for PCR, FA, and ELISA with culture as the positive gold standard (Table 2). These values were

calculated with the results of all specimens combined as well as for subsets of specimens. The test performance characteristics were highest for the ELISA, followed by FA, then PCR. The test performance characteristics did not change substantially when the specimen subsets were examined, although the 95% confidence intervals for sensitivity did increase with the decreasing sample size of the subsets.

The test performance characteristics of PCR were calculated with the following positive gold standards: 1) ELISA or FA positive, or both, and culture positive; and 2) culture positive or ELISA and FA positive (Table 3). There was no appreciable difference in the PCR test performance characteristics when the second positive gold standard was used. Again, the test performance characteristics did not change substantially when the specimen subsets were examined, although the 95% confidence intervals for sensitivity did increase with the decreasing sample size of the subsets.

Test performance characteristics of PCR were also calculated with two negative gold standards: 1) culture, ELISA, and FA negative; and 2) ELISA or FA negative, or both, and culture negative (Table 4). Overall, the specificity of the PCR was lower when the first negative gold standard was used, although the number of specimens that met the definition of this gold standard was low. When the second negative gold standard was used, λ_{SP} and κ for PCR were higher when results for all specimens were analyzed compared with the following subsets: 1) avian and mammalian tissue, and 2) avian tissue only. The increase in the 95% confidence intervals with the decreasing sample size of the subsets was again noted.

Discussion

Primers to the MOMP gene, 1904 and 1905, were used for testing clinical specimens in the current study because it was felt that their use would result in a PCR reaction that was highly specific for the genus *Chlamydia* (R. B. Walsh, University of California, Davis, personal communication, 1993). However, these primers were not specific for either of the 2 reference strains, *C. psittaci* and *C. trachomatis*. Synthesizing a

Table 2. Sensitivity (SE), with corresponding 95% confidence intervals (CIs), and lambda (λ) and kappa (κ) statistics for ELISA, FA, and PCR with cell culture as the positive gold standard.*

| | ELISA versus culture | FA versus cell culture | PCR versus cell culture |
|-------------------|----------------------|------------------------|-------------------------|
| All specimens | | | |
| SE (95% CI) | 93.3% (68.1–99.8) | 86.2% (68.3–96.1) | 53.2% (38.1–67.9) |
| λ_{SE} | 88.5% | 51.7% | 18.3% |
| κ | 72.5% | 30.8% | 19.8% |
| Avian only | | | |
| SE (95% CI) | 92.9% (66.1–99.8) | 85.7% (67.3–96.0) | 54.3% (39.0–69.0) |
| λ_{SE} | 87.9% | 49.5% | 18.5% |
| κ | 72.2% | 30.2% | 23.7% |
| Tissue only | | | |
| SE (95% CI) | 92.9% (66.1–99.8) | 86.2% (68.3–96.1) | 51.2% (35.5–66.7) |
| λ_{SE} | 87.0% | 45.9% | 16.4% |
| κ | 87.9% | 29.2% | 21.5% |
| Avian tissue only | | | |
| SE (95% CI) | 92.3% (64.0–99.8) | 85.7% (67.3–96.0) | 52.4% (36.4–68.0) |
| λ_{SE} | 86.4% | 42.9% | 16.4% |
| κ | 87.8% | 28.3% | 22.0% |

* ELISA = enzyme-linked immunosorbent assay; FA = direct fluorescein-conjugated monoclonal antibody staining; PCR = polymerase chain reaction.

primer set that is specific for a portion of the MOMP gene that is unique to *C. psittaci* seems the next logical step in maximizing the specificity of the PCR. *Chlamydia psittaci*, *C. trachomatis*, and *Chlamydia pneumoniae* have been successfully differentiated with

primers to the MOMP gene and restriction endonuclease digestion.^{10,11,13}

In the current study, the possibility that the specimen type or species of origin may affect the PCR result was controlled for by examination of specimen

Table 3. Sensitivity (SE), with corresponding 95% confidence intervals (CIs), and lambda (λ) and kappa (κ) statistics for PCR compared with two positive gold standards: 1) ELISA or FA positive or both, and culture-positive; and 2) culture-positive or ELISA and FA positive.*

| | Positive gold standard 1 | Positive gold standard 2 |
|-------------------|--------------------------|--------------------------|
| All specimens | | |
| SE (95% CI) | 53.6% (33.9–72.5) | 54.9% (40.3–68.9) |
| λ_{SE} | 23.6% | 19.3% |
| κ | 21.1% | 20.0% |
| Avian only | | |
| SE (95% CI) | 55.6% (35.3–74.5) | 55.1% (40.2–69.3) |
| λ_{SE} | 24.9% | 18.8% |
| κ | 22.1% | 22.3% |
| Tissue only | | |
| SE (95% CI) | 51.9% (32.0–71.3) | 53.2% (38.1–67.9) |
| λ_{SE} | 22.2% | 17.3% |
| κ | 23.4% | 21.5% |
| Avian tissue only | | |
| SE (95% CI) | 53.9% (33.4–73.4) | 53.3% (37.9–68.3) |
| λ_{SE} | 23.1% | 16.7% |
| κ | 23.9% | 21.9% |

* PCR = polymerase chain reaction; ELISA = enzyme-linked immunosorbent assay; FA = direct fluorescein-conjugated monoclonal antibody staining.

Table 4. Specificity (SP), with corresponding 95% confidence intervals (CIs), and lambda (λ) and kappa (κ) statistics for PCR compared with two negative gold standards: 1) culture, ELISA, and FA negative; and 2) ELISA or FA negative or both, and culture negative.*

| | Negative gold standard 1 | Negative gold standard 2 |
|-------------------|--------------------------|--------------------------|
| All specimens | | |
| SP (95% CI) | 49.2% (9.9–81.6) | 66.7% (47.2–82.7) |
| λ_{SP} | 10.2% | 15.1% |
| κ | 9.5% | 8.4% |
| Avian only | | |
| SP (95% CI) | 49.2% (9.9–81.6) | 65.5% (45.7–82.1) |
| λ_{SP} | 14.3% | 15.5% |
| κ | 14.1% | 9.9% |
| Tissue only | | |
| SP (95% CI) | 49.2% (9.9–81.6) | 61.1% (35.8–82.7) |
| λ_{SP} | 9.5% | –2.1% |
| κ | 9.9% | 0.1% |
| Avian tissue only | | |
| SP (95% CI) | 49.2% (9.9–81.6) | 58.8% (32.9–81.6) |
| λ_{SP} | 14.3% | –2.9% |
| κ | 16.8% | –1.5% |

* PCR = polymerase chain reaction; ELISA = enzyme-linked immunosorbent assay; FA = direct fluorescein-conjugated monoclonal antibody staining.

subsets. False-positive results when testing fecal specimens by ELISA and FA have been reported.⁹ In addition, antigenic differences between the avian and mammalian strains of *C. psittaci* are known.¹ However, the test performance characteristics for PCR did not differ greatly when the specimens were evaluated as subsets, suggesting that the primers bind to a portion of the MOMP gene that is common to avian and mammalian strains. A previous study that performed cloning and sequencing of *C. psittaci* MOMP genes found an 80.6% similarity between guinea pig inclusion conjunctivitis strain 1 and meningopneumonitis strain Cal 10 and 68% interspecies similarity between *C. psittaci* and *C. trachomatis*.¹⁸

The estimates of the PCR test performance characteristics compared with the second negative gold standard (ELISA or FA negative, or both, and culture negative) differed for the following 2 subsets: 1) avian and mammalian tissue specimens, and 2) avian tissue. Twenty-eight (44.4%) of 63 avian and mammalian tissue specimens were PCR negative but were positive by the gold standard. Twenty-six (43.3%) of 60 avian tissue specimens were PCR negative but were positive by the gold standard. The very small or negative estimates of λ_{SP} and κ for these subsets could be because of the small sample sizes or false-negative PCR results for some culture-positive specimens. Seven (11.1%) of the 63 avian and mammalian tissue specimens and 7 (11.7%) of the 60 avian tissue specimens were PCR positive but were negative by the gold standard. This may be the result of higher sensitivity of the PCR in detecting low numbers of organisms.

The high level of chance-corrected agreement (κ) between ELISA and FA (63.1%) could be explained in part by the requirement for large numbers of organisms for detection and their susceptibility to false-positive reactions with fecal specimens.^{9,12} However, the test performance characteristics for the subset of avian and mammalian tissue were not noticeably higher. The lower levels of agreement between ELISA and PCR (39.5%) and FA and PCR (11.3%) may be the result of false-negative PCR results for some culture-positive specimens.

Seven culture-positive specimens were falsely identified as negative by PCR until the reaction was repeated a second time, in some cases after a second phenol extraction step. This may be the result of technical error or inadequate purification of the DNA specimen. An additional 18 culture-positive specimens were PCR negative after the reaction was repeated and an aliquot of the original reaction it was reamplified. Possible reasons for the false-negative PCR results include incomplete removal of the phenol used in the second step of DNA extraction from the aqueous phase of the specimen DNA or the presence of phenol ox-

idation products that damaged and introduced breaks into the nucleic acid chains.¹⁶ Destruction of the organism during storage, technical errors, or recording errors are also possible. Any of these factors could have resulted in lower estimates of the sensitivity, κ , and λ_{SE} for PCR compared with the other testing methods. Repeating the ELISA, FA, and cell culture testing of specimens at the time of PCR testing would have been helpful in determining the effect, if any, of long term frozen storage on tissue and fecal specimens. However, because of insufficient quantity of specimens and lack of available laboratory resources, this was not possible for the current study.

Ten specimens were positive by PCR but negative by both negative gold standards. Some of these specimens may have been falsely negative by cell culture for one or more of the following reasons: cell cultures were not being inoculated by centrifugation, second passages were not performed, cultures were read only up to 3 days instead of 5, or other factors may have compromised the sensitivity of cell culture¹³ (L. Woods, CVDLS, personal communication, 1993).

Several studies have examined the use of PCR for detecting *Chlamydia* spp. infections. PCR has been documented as a highly sensitive method for the detection of *C. psittaci*, able to detect fewer than 10 organisms.¹³ However, distinguishing *C. psittaci* from *C. trachomatis* has required the use of restriction endonucleases in previous reports.^{10,11,13} Ideally, primers could be synthesized that are both highly sensitive and specific for *C. psittaci*. Further PCR studies are needed to develop and evaluate primers with these qualities.

PCR has been reported as a highly sensitive diagnostic method for chlamydiosis, able to detect a single target DNA molecule in a specimen by amplifying a specific target sequence of DNA.¹⁸ However, this high degree of sensitivity can also be a disadvantage if a specimen becomes contaminated with molecules containing the specific sequence of the target molecule, resulting in a false-positive reaction.

PCR offers advantages over other testing methods in that it is fairly rapid and does not require organisms to be present in large numbers or to be viable.¹⁷ Advancing the application of PCR in the diagnosis of chlamydial infections will require that the DNA extraction procedures, primer sequences, and amplification methodology be standardized. Standardization of these procedures would go far to ensure the credibility of the PCR and establish quality control measures for this new and promising technology.

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Sources and manufacturers

- a. American Type Culture Collection, 10801 University Boulevard, Manassas, VA 20110.
- b. S94 DNA/RNA Applied Biosystems, 850 Lincoln Center Drive, Foster City, CA 94404.
- c. Bio-Rad Laboratories, 1000 Alfred Nobel Drive, Hercules, CA 94547.
- d. Pharmacia, 95 Corporate Drive, PO Box 6995, Bridgewater, NJ 08807.
- e. Invitrogen Corporation, 1600 Faraday Avenue, Carlsbad, CA 92008.
- f. PTC-100, V2.0, MJ Research, Inc., 149 Grove Street, Watertown, MA 02472.
- g. F. Hoffmann-La Roche Ltd., Diagnostics Division, CH-4070 Basel, Switzerland.

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