Original Article

Bayesian evaluation of the performance of three diagnostic tests for *Chlamydophila psittaci* in humans

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Summary

Chlamydophila psittaci (*C. psittaci*) remains a significant threat to the health of farming communities in close contact with psittacine birds yet its infection burden remains poorly understood owing to the low accuracy of available diagnostic tests. This study aimed to evaluate the accuracy of chicken embryo (CEI) and mice inoculation (MI) tests and a PCR assay for the detection of *C. psittaci* in humans. Sputum specimens from 70 Egyptian individuals in contact with psittacine birds were screened for the presence of the pathogen using the three tests. A Bayesian latent class model was used to estimate the Se and Sp of the three tests. The PCR assay had a higher Se (85%; PCI 42.4% - 99.4%) than CEI (68.5%; PCI 24.6% - 95.6%) and MI (47.0%; PCI 12.3% - 85.1%) tests together with a higher Sp (98.9%; PCI 94.1% - 100%) than CEI (98.6%; PCI 93.8% - 99.9%) and MI (98.6%; PCI 93.8% - 99.9%) tests. To our knowledge, this is the first attempt at evaluating the accuracy of these tests for the detection of *C. psittaci* in humans. The PCR assay clearly outperforms the inoculation tests and hence holds better promise for routine use in surveillance programs for psittacosis.

Keywords: *Chlamydophila psittaci*, PCR, Chicken embryo inoculation test, Mice inoculation test, Bayesian analysis.

Introduction

Psittacosis is a cosmopolitan zoonosis caused by an obligate intracellular gram-negative bacterium called *Chlamydophila psittaci* (*C. psittaci*) (Andersen and Vanrompay, 2008; Ciftçi et al., 2008; Dickx and Vanrompay, 2011). The bacteria can infect humans, mammals and a wide range of bird species including psittacine birds (Lierz, 2005). Psittacine

birds can persist as asymptomatic carriers of *C. psittaci* infection (Smith et al., 2005). However, under stress conditions such as transportation, malnutrition, and overcrowding, clinical disease may manifest in the form of sinusitis, respiratory difficulty, anorexia, emaciation and diarrhoea (Harkinezhad et al., 2009; Smith et al., 2005; Tully, 2006). Asymptomatic birds may intermittently shed the agent through nasal secretions and faeces, thus posing a threat to human health (Ghorbanpoor et al., 2015).

Humans contract the infection by inhaling the organism in aerosolised, respiratory secretions or dried faeces of infected birds (Harkinezhad et al., 2009). Other potential sources of exposure include mouth-to-beak contacts, bites from infected birds and practices involving dissection of dead birds or evisceration in slaughterhouses (Dickx et al., 2010). Human infections vary from inapparent infection to severe systemic disease characterised by headaches, chills, malaise, myalgia and pulmonary involvement (Beeckman and Vanrompay, 2009; Chau et al., 2015; DE Boeck et al., 2016). Owing to the potentially serious nature of the infection, control of *C. psittaci* calls for its early, rapid and accurate detection.

Culture of the organism either in mice or the yolk sac of embryonated chicken eggs has long been held as the 'gold standard' for the definitive diagnosis of *C. psittaci* infection (Vanrompay, 2000). The test is considered to have a high specificity (Sp) such that a positive result reliably points to *C. psittaci* infection. However, culture sensitivity (Se) is low and highly variable, with false negative results prompted by either intermittent shedding or loss of viability of the organism. Furthermore, the test is often slow, with growth of the organism taking as long as two weeks (Balsamo et al., 2017).

Molecular techniques such as PCR have been upheld for their: (1) rapidity in execution, which significantly reduces the duration of therapy and thus improves prognosis and (2) high Se, owing to their ability to detect both growth-inhibited and non-viable organisms (Phalen, 2006; Vanrompay, 2000). Given these merits, these techniques hold better promise (than culture) for routine use in surveillance programmes for *C. psittaci* in humans.

The conventional technique for evaluating the performance of a diagnostic test entails the application of the index test to the test results of another reference test (Dohoo et al., 2012). A major drawback of this technique is that, with an imperfect reference test, the index test's characteristics are subject to selection and/or information bias. In the absence of a reasonable reference test or a test(s) with known Se and Sp, latent class models (LCMs) provide an invaluable option for the simultaneous estimation of Se and Sp of two or more tests without any assumption about the underlying true disease status of each subject (Hui and Walter, 1980). LCMs can be fit using either maximum likelihood or Bayesian estimation methods (Enøe et al., 2000). Generally, Bayesian methods are preferred

especially when observed data are inadequate (Branscum et al., 2005). Essentially, the models rest on three key assumptions; 1) target population should consist of two or more subpopulations with differing prevalences, 2) constancy of the Se and Sp of the index tests across the subpopulations, and 3) the tests under evaluation should be conditionally independent given the disease status (Hui and Walter, 1980).

To the best of our knowledge, there are no published studies that quantify the diagnostic performance of culture and PCR for the detection of *C. psittaci* infection in humans. Hence, the objective of this study was to estimate the Se and Sp of PCR and culture (based on growth in chicken embryos and mice inoculation) tests for *C. psittaci* in humans within a Bayesian latent class analysis framework.

Material and Methods

Study population, sample size and sample collection

The required sample size was determined as specified for single proportions:

$$n = \frac{Z_{\alpha}^{2} pq}{l^{2}}$$

Where: Z_{α} (1.96) is the value which specifies the 2-tailed confidence level (95%), p is the prevalence of psittacosis set at 4.8% (with q being$1 - p$) and l is the allowable error (5%). Given these figures, a sample size of 70 individuals was derived.

A total of 70 individuals were randomly sampled from among those in close contact with psittacine birds within markets and farms spread across the different districts in Egypt during the period October 2014 – June 2015. Prior to enrolling the study subjects, the investigators explained the purpose of the study, following which informed consent was sought. Upon consenting to participation, the participants were asked to complete a questionnaire that captured details of their demographic characteristics, contact location with psittacine birds, hand washing practices, knowledge about the disease and its transmission patterns and wearing of protective clothing. Of the 70 individuals, 60 were asymptomatic while the rest displayed flu-like symptoms. Each of the study subjects submitted sputum specimens in tubes containing 0.2 ml sucrose phosphate glutamate (SPG) buffer saline which were subsequently transferred to the laboratory (Dept. of Avian and Rabbit Medicine, Faculty of Veterinary Medicine, Zagazig University, Egypt) using portable insulated ice boxes and kept frozen at -70°C.

Diagnostic techniques

Chicken embryo inoculation (CEI)

Preparation of samples for inoculation into embryonated chicken eggs was done according to Andersen (1998). Briefly, the sputum samples were centrifuged for 15 minutes at 3,000 rpm. A clear supernatant fluid was transferred under aseptic conditions using a sterile pipette to another centrifuge tube, after which centrifugation was repeated for another 15 minutes. The

clear supernatant fluid was recollected in screw capped tubes and a stock solution of antibiotics composed of 200 µg/ml of Streptomycin (Medical Professions for Veterinary Products and Fodders Addition Company, Egypt), 500 µg/ml of Vancomycin (Mylan Company), 50 µg/ml Nystatin (GlaxoSmithKline) and 80 µg/ml Gentamycin (Memphis Schering-Plough) were added to inhibit growth of microorganisms other than *Chlamydiae* (OIE, 2017). Further, the suspension was held for an hour at 4 °C and centrifuged for 15 minutes at 3,000 rpm. The final supernatant was used for inoculation into embryonated chicken egg through the yolk sac route according to Mostafa et al. (2015).

The inoculated eggs were incubated at 37 °C in a humidified incubator. Non-inoculated control eggs were labelled and incubated beside the inoculated eggs. The eggs were candled on a daily basis and the embryos that died within 3 days post inoculation were discarded. Positivity for *C. psittaci* was based on microscopic examination of *C. psittaci* inclusion bodies in Gimenez-stained infected yolk sac membranes (Fig.1) (Gimenez, 1964).

Mice inoculation (MI)

The samples were prepared as mentioned above and subsequently inoculated intraperitoneally into 3-4 week-old albino female mice, according to Dovč et al. (2007). Briefly, each mouse was inoculated with 0.2 ml of prepared sample and observed daily for 6-7 days after inoculation. Impression smears were made from the liver, spleen, lung and heart of dead or autopsied mice 7 days post-infection and stained with Giemsa stain for identification of *C. psittaci*. Positivity for *C. psittaci* was based on detection of *C. psittaci* inclusion bodies in the impression smears (Fig. 2) (Pal, 2017).

PCR assay

Chromosomal DNA from sputum samples was extracted for detection of the ompA *C. psittaci* gene (Magnino et al., 2009) using QIA amp DNA Mini Kit (Qiagen, Germany). The extracted DNA of each sample was kept frozen at -20°C until used. *C. psittaci* strain from a chicken isolate was used as a positive control and 1 ml of sterile deionized water used as a DNA negative control. The ompA region of the extracted DNA was amplified by PCR using the primers: CPsitt-F (5′-GCTACGGGTTCCGCTCT-3′) and CPsitt-R (5′TTTGTTGATYTGAATCGAAGC-3′) (Doosti and Arshi, 2011). The samples were then placed in a thermal cycler (Mastercycler gradient, Eppendorf, Germany) with an initial denaturation step for 5 min at 95°C. An amplification step was run for 30 cycles as: denaturation in 1 min at 94°C, and alignment for 1 min at 57°C, then extension for 1 min at 72°C, with a further extension step for 7 min at 72°C. PCR products were separated through agarose gel electrophoresis and visualized using ethidium bromide staining under UV light. A sample was deemed positive by PCR when the amplification band product specific for *C. psittaci* was

detected as 1041bp (Fig. 3) (Doosti and Arshi, 2011).

Population classification

To facilitate the derivation of the Se and Sp of the three tests (CEI, MI and PCR), initially, the data were stratified by location of contact with psittacine birds giving rise to two subpopulations of individuals presumed to have different *C. psittaci* prevalences (Dohoo et al., 2012). The rationale for using the contact location as a population stratifier is that *C. psittaci* transmission is anticipated to be higher within markets, considering the likelihood of high frequency encounters with a mix of potentially infected birds, hence a probable higher prevalence of the infection in markets than farms.

Statistical analysis

A Bayesian latent class model fitted in OpenBUGS v3.2.2 (Thomas et al., 2006) was used to estimate the Se and Sp of the three tests, as well as the two subpopulation prevalences. In particular, constancy of the tests' characteristics across the two subpopulations was assumed. However, granted that the two culture tests (CEI and MI) are based on identical isolation mechanisms for the pathogen but distinct from that of the PCR i.e. are conditionally correlated, we allowed for dependence between the tests by adding two conditional covariance parameters, σ_{se} and σ_{sp}, between pairs of the Se and Sp of the tests respectively as specified by Gardner et al. (2000).

Counts (O_k) of the different test combinations e.g. +, +, + were assumed to follow a multinomial distribution of the form:

$$O_k|Se_iSp_iP_k \sim multinomial(prob_k, n_k)$$

Where Se_i and Sp_i represent the respective test characteristics for test i ($i = 1,2,3$) and P_k is the specific prevalence for the k^{th} ($k = 1,2$) subpopulation. $Prob_k$ is a vector of probabilities of observing the different combinations of test results and n_k reflects the total number of individuals tested for the k^{th} subpopulation. For instance, in the 1st subpopulation for an individual testing positive to each of the three tests, $prob_1$ is given by:

$$\begin{aligned} prob_1 = (&Pr(T_1^+T_2^+T_3^+|D^+) \\ &+ Pr(T_1^+T_2^+T_3^+|D^-)) \\ &= (Se_1Se_2 + \sigma_{se})Se_3P_1 \\ &+ \big([1 - Sp_1][1 - Sp_2] \\ &+ \sigma_{sp}\big)[1 - Sp_3][1 - P_1] \end{aligned}$$

Given two subpopulations, the available data furnished 14 degrees of freedom sufficient to estimate the 10 parameters (Se and Sp of the three tests, two prevalences and two conditional covariances) – yielding an identifiable model. Non-informative priors ($beta(1,1)$) were used to fit the Bayesian model since no prior information was available for any of the aforementioned parameters. Notably, the hypothesis: $H_0: \sigma_{se}, \sigma_{sp} = 0$, was evaluated based on a Bayesian posterior probability (POPR), analogous to the frequentist $P-$value.

The model was initialised with two Markov Chain Monte Carlo chains with

different values. Each chain comprised 20,000 samples, with the first 10,000 being discarded as the burn-in. Convergence of the chains was evaluated by visual appraisal of the time series plots of selected variables and the Gelman-Rubin diagnostic plots. The posterior distribution of the subpopulation prevalences, the Se and Sp of the three tests, as well as the conditional covariances were reported as the median and the corresponding 95% posterior credibility intervals (PCI).

Results

The cross-classified counts of the binary outcomes of the three tests are displayed in Table 1. Of note, 64.3% ($n = 45$) and 35.7% ($n = 25$) of the study participants had contact with psittacine birds in markets and farms, respectively. Only the conditional covariance for Sp (σ_{sp}) was significant ($POPR = 0.98$). Nevertheless, considering the similarity in isolation mechanisms between CEI and MI tests, both covariances were retained in the model.

Table 1. Cross-classified results for chicken embryo (CEI) and mice inoculation (MI) tests as well as PCR assay by subpopulation for *Chlamydia psittaci* detection from human sputum specimens.

	Subpopulation	
Test outcome (CEI, MI, PCR)	**1 (market)**	**2 (farm)**
+ + +	0	1
+ + -	0	0
+ - +	2	0
- + +	1	0
+ - -	0	0
- + -	0	0
- - +	0	0
- - -	42	24
Total (%)	**45 (64.3)**	**25 (35.7)**

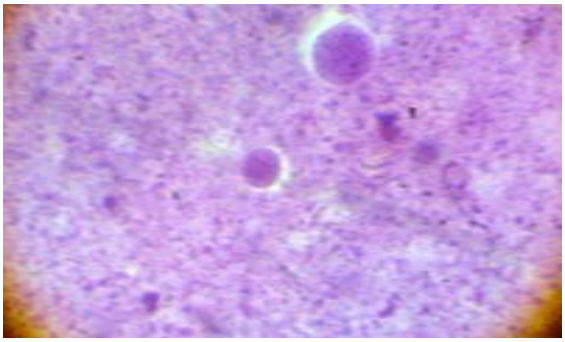

Fig. 1. *Chlamydia psittaci* inclusion bodies in the infected yolk sac membrane stained with Gimenez under an oil immersion lens (X1000).

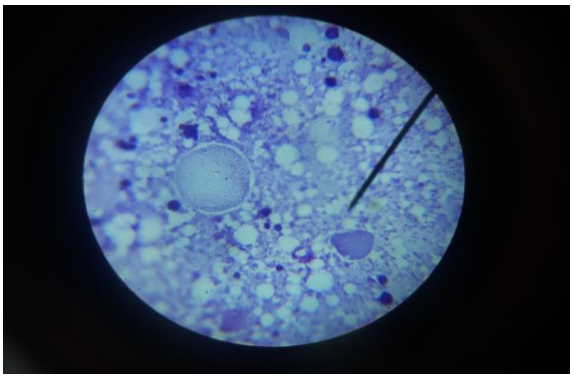

Fig. 2. *Chlamydia psittaci* inclusion bodies in the lung of mice inculcated and stained with Geimsa under an oil immersion lens.

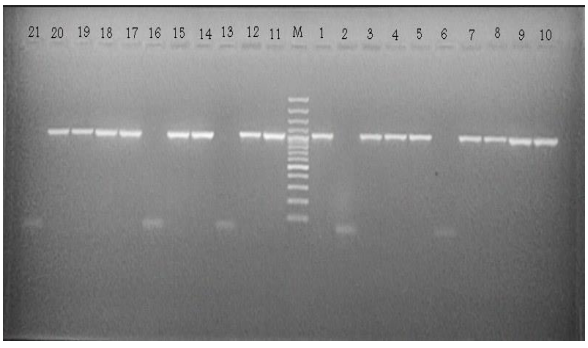

Fig. 3. PCR amplification of ompA gene from *Chlamydia psittaci* cases of the present study. Lane M: 1Kbp DNA Ladder (Fermantas); CTRL-, negative control; CTRL+, positive control (chicken *Chlamydia psittaci*) Lane 2-5: positive *C. psittaci* infected samples (ompAgene Egypt-1, ompAgene Egypt-2, ompAgene Egypt-3, ompAgene Egypt-4.Lane 1: DNA free nuclease water.

The PCR assay had a higher Se (85.0%) than either the CEI (68.5%) or the MI (47.0%) tests coupled with a higher Sp (98.9%) compared to either the CEI (98.6%) or the MI (98.6%) tests. The subpopulation-specific prevalences for *C. psittaci* were 8.1% and 6.4% for subpopulation 1 and 2, respectively (Table 2).

Table 2. The posterior median and 95% credibility intervals for the Se and Sp of Chicken embryo (CEI) and mice inoculation (MI) tests and PCR and the associated conditional covariances between the Se and Sp of CEI and MI tests.

Parameter	Median	95% PCI
Se_{CEI}	68.5	24.6 – 95.6
Sp_{CEI}	98.6	93.8 – 99.9
Se_{MI}	47.0	12.3 – 85.1
Sp_{MI}	98.6	93.8 – 99.9
Se_{PCR}	85.0	42.4 – 99.4
Sp_{PCR}	98.9	94.1 – 100.0
P_1	8.1	2.3 – 18.6
P_2	6.4	0.9 – 20.2
σ_{Se}	-0.037	-0.167 – 0.083
σ_{Sp}	0.004	0 – 0.030

Discussion

The Se and Sp of CEI, MI and PCR using latent class analysis within a Bayesian framework were estimated in the present study. According to the literature, this is the first attempt at evaluating the diagnostic performance of these three tests for the detection of *C. psittaci* in humans. The analysis demonstrates that the PCR assay clearly outperforms the culture tests in the detection of *C. psittaci* infection status in humans and thus holds better promise for routine use in surveillance programs for psittacosis. This attribute is especially desirable in the preliminary stages of a psittacosis eradication programme where a test with a high Se is indispensable to ensure that as many infected individuals as possible are promptly identified and treated. This finding is in line with previous studies (Harkinezhad et al., 2009; Verminnen et al., 2008), which showed the PCR to have higher Se and Sp compared to other traditional techniques such as culture and serology. Further, Verminnen et al. (2008) demonstrated the superiority of a nested PCR assay over micro-immunofluorescence tests, which could not detect any antibodies to *C. psittaci* in all of the infected persons involved in the study. Moreover, Opota et al. (2015) demonstrated the PCR's usefulness towards improving the routine diagnosis of psittacosis in large-scale screening programs and during outbreaks.

A positive PCR result indicates the presence of *C. psittaci* DNA without discriminating between viable and nonviable organisms (Trevejo et al., 1999). This may suggest the need to confirm the assay's positive findings by either of the culture tests (whose positivity implies the presence of an active infection) particularly when treatment is warranted. Nonetheless, the PCR assay is most preferable in cases of persistent *Chlamydia* infection where the organism may be

viable, but yet yield culture-negative results (Braukmann et al., 2012).

Our findings showed that the estimate of Se for CEI was higher (68.5%) than that of MI (47%). This disparity could be related to *C. psittaci* virulence properties, host adaptation and etiopathology. It has been shown that the invasiveness of *Chlamydia* spp. and propagation in the host are dependent on the host immune response level and the expression of bacterial factors related to virulence (Braukmann et al., 2012). Moreover, it is expected that the activity of immunity-related Guanosine Triphosphatases (IRG) proteins could be responsible for differences in host specificities of *C. psittaci* (Taylor et al., 2007; Coers et al., 2009) hence suggesting that heterogeneities between hosts' immune responses may partly explain the CEI and MI Se variability.

The notable imprecision of the tests' Se estimates as compared to their respective Sps, is not only reflective of the inadequacy of truly infected individuals necessary for Se derivation but also the absence of a true difference in the subpopulation prevalences. Toft et al. (2005) demonstrated that the precision of Se and Sp estimates increased with greater differences in the populations studied. Although the estimated prevalences were expected to differ owing to the inherent differences in *C. psittaci* transmission characteristics between farms and markets, this was not evident in the present study – a possibility of insufficient study power.

Conclusion

We The Se and Sp of the three tests: CEI, MI and PCR were simultaneously estimated without the assumption of an existing reference standard. The PCR test showed higher Se and Sp than either of the inoculation tests. Of the inoculation tests, CEI had higher Se but similar Sp to that of MI. Granted its performance, the PCR assay readily lends itself to use in routine *C. psittaci* screening programmes.

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