Journal of Academia Vol. 13, Issue 2 (2025) 263 – 272

Development and Evaluation of a Novel Real-Time PCR Assay for Specific Detection of *Chlamydia psittaci* in Clinical Respiratory Samples

Nur Bainun Mohd Zin[1,2], Siti Norsuhaida Mohmd Azman[1], Noorlis Ahmad[2], Jama'ayah Mohamed Zahidi[1], Hana Farizah Zamri[1], Rohaidah Hashim[1], Mohammad Ridhuan Mohd Ali[1], Nurhamimah Zainal-Abidin[2*]

[1]*Bacteriology Unit, Infectious Disease Research Centre, Institute for Medical Research, Ministry of Health Malaysia, National Institutes of Health Complex, Bandar Setia Alam, 40170 Shah Alam, Selangor, Malaysia*
[2]*Faculty of Applied Sciences, School of Biology, Universiti Teknologi MARA (UiTM), Cawangan Negeri Sembilan, Kampus Kuala Pilah, 72000 Kuala Pilah, Negeri Sembilan, Malaysia*

[*]*Corresponding author: nurhamimah@uitm.edu.my*

Abstract

Chlamydia psittaci is an obligate intracellular Gram-negative bacterium that is zoonotic causing diseases called psittacosis in humans. Humans are usually infected through inhalation, which often leads to atypical pneumonia, with fatal outcome if left untreated. Early and accurate diagnosis is crucial for effective clinical management and containment of potential outbreaks. Nevertheless, *C. psittaci* infections are often underestimated as their clinical and laboratory presentations closely resemble those of other respiratory infections. Traditional diagnostic methods relied on serology and culture but has limitations in specificity, sensitivity and biosafety. This study aimed to develop and evaluate a rapid, specific and sensitive real-time PCR assay targeting the ompA gene for detection of *C. psittaci* in human respiratory samples. A synthetic plasmid containing an *ompA* gene fragment was used as a positive control, eliminating the need for hazardous live cultures while ensuring assay stability. Specificity testing against 28 bacterial strains revealed no cross-reactivity, and *in silico* PCR analysis against 268 bacterial genomes confirmed exclusive amplification of *C. psittaci*. Spiking experiments with human respiratory samples (n=43) demonstrated robust detection across various matrices and concentrations, with no amplification in non-spiked controls, confirming absence of false positives. The assay achieved a detection limit of 0.0002 pg (~25 DNA copies) with 97.84% amplification efficiency and an R^2 of 0.9995, indicating high precision and reproducibility. These findings establish the developed real-time PCR assay as a highly specific and sensitive diagnostic tool for *C. psittaci*, enabling rapid and accurate detection to support timely clinical management and outbreak control.

Keywords: *birds*; *diagnostic; ompA*; *psittacosis*; *zoonotic*

Article History:- Received: 22 August 2025; Revised: 13 October 2025; Accepted: 13 October 2025; Published: 31 October 2025

DOI: 10.24191/joa.v13i2.8607

Introduction

Chlamydia psittaci is an obligate intracellular Gram-negative pathogen and is the etiological agent for psittacosis. Birds serve as the primary reservoir for this bacterium, and human infection typically results in atypical pneumonia (Gu et al., 2020; Longbottom & Coulter, 2003; Ravichandran et al., 2021). Psittacosis is a zoonotic disease where the transmission to humans could be from inhalation of infectious animal particles or aerosol (Vande Weygaerde et al., 2018). Infection in humans most commonly reported among workers handling birds or poultry, highlighting occupational exposure as a significant risk factor. The clinical manifestations of psittacosis often resemble that of community acquired pneumonia, which can lead to misdiagnosis or underdiagnosis in clinical practice (Liu et al., 2023; Nieuwenhuizen et al., 2018). If the infections not promptly treated, the infection may progress rapidly, resulting in complications such as meningitis, severe pneumonia and dyspnea (Cui & Meng, 2023; Huang et al., 2023; Yao et al., 2022).

There are increasing psittacosis cases reported in China, the United States and Australia (Yao et al., 2022). Notably, in 2018, the United States experienced its largest psittacosis outbreak in three decades, which occurred in two poultry slaughterhouses (McGovern et al., 2018). In Malaysia, the study on psittacosis began in 1959 when the Institute for Medical Research (IMR) investigated a case involving a family of pigeon owners in Gerik, Perak who presented with fever, headache and cough (Tan & Babudieriz, 1977). Since then, however, there has been a lack of documented human psittacosis cases in Malaysia, suggesting underreporting or missed diagnosis (Dembek et al., 2023; Liu et al., 2023; Sheng et al., 2025).

The pathogenicity of *C. psittaci* in humans is largely determined by its ability to survive and evade host immune responses through complex host-pathogen interactions (Luu et al., 2023). This is facilitated by its biphasic developmental cycle, which alternates between the infectious, extracellular elementary bodies (EBs) and non-infectious, intracellular reticulate bodies (RBs) that are involved in replication (AbdelRahman & Belland, 2005; Bommana & Polkinghorne, 2019; Sukon et al., 2021).

Currently, serological tests such as ELISA are used for diagnosis but have low specificity due to cross-reactivity with other *Chlamydia* species especially *Chlamydia pneumonia* often resulting in false positive result (Nieuwenhuizen et al., 2018). Culture remains the standard for diagnosis but is time-consuming and required extensive safety precautions due to the pathogen's zoonotic nature (Heddema et al., 2006). Given these diagnostic limitations, the present study aimed to develop a real-time PCR assay for the rapid and specific detection of *C. psittaci* in human respiratory samples. This approach enables early diagnosis and expedite outbreak management.

Methods

Bacterial strains and clinical samples

The specificity of the developed real-time PCR was evaluated using both non-target bacterial strains and various human clinical samples. A panel of closely related and unrelated bacterial special with a total of 28 bacterial strains, provided by Bacteriology Unit, Institute for Medical Research (Table 1) were used to assess potential cross-reactivity. *In-silico* specificity of primers and probe sequences were performed in UPV/EHU website (http://insilico.ehu.es/PCR) (Table 2) (Bikandi et al., 2004; Kalendar et al., 2024). Additionally, DNA extracted from different types of human samples comprised of sputum (n=33), tracheal aspirate (n=4), bronchoalveolar lavage (n=1) and pleural fluid (n=5) (Table 3) was included to ensure that the assay does not amplify non-target sequences from human genomic DNA or the normal microbiota. In addition, these human samples were also spiked with synthetic target DNA at low concentration (0.02 pg/μl) and high concentration (200 pg/μl) to further validate assay performance in different biological matrices.

Isolation of genomic DNA

Microbial DNA from culture and clinical samples were extracted using PrimeWay Genomic DNA Extraction kit according to the manufacturer's instructions (Mohd Ali et al., 2019). For sputum samples only, an additional pre-processing step was required to reduce viscosity and improve DNA extraction efficiency. Briefly, 300 µl sputum was mixed with 300 µl of 12.5 mM 1,4-Dithiothretol (DTT) and incubated at room temperature for 1 hour (McGovern et al., 2018). DTT acts as a mucolytic agent by breaking disulfide bonds with mucin glycoproteins, therefore liquefying the samples prior to DNA extraction. Total DNA was quantified using Multiskan SkyHigh Microplate Spectrophotometer. All the DNA was stored at -20°C until further use.

Primers and probe design

The specificity primers and probe for *C. psittaci ompA* gene detection were obtained by the National Centre for Biotechnology Information (NCBI) database and checked through Basic Local Alignment Search Tool (BLAST) (https://www.ncbi.nlm.nih.gov/tools/primer-blast) (Luu et al., 2023). Primers and probe sequences were inserted in all possible combinations, potentially initiating amplification. Primers and probe pairs should only amplify the intended target, but not any unintended targets. The

oligonucleotides should have 40-60% GC content, melting point (T_m) value of 60°C-75°C, product size of 100-250 base pairs, primer length of 24-28 base pairs and self-dimer, cross-dimer, and hairpin energy value of less or equal to -4.0 kcal/mol. The forward primer sequence *ompA*-F 5'-TCAGCAGACCGGCTATTCAC-3', reverse primer sequence *ompA*-R 5'-TGCGTGTGTAGGCAAAGTCA-3', probe *ompA*-P 5'6-FAM-ACGGAATTCT/ZEN/GGTGCTGCTTAGGA-3'-IBFQ

ompA gene fragment design and cloning

C. psittaci ompA gene sequence was downloaded from the NCBI website (https://www.ncbi.nlm.nih.gov/nucleotide) (Xu et al., 2019). Adenosine (A) and thymine (T) overhangs were added to the *ompA* gene fragment for compatibility with T/A cloning vector. Chemically synthesized double-stranded DNA (gBlock) was obtained in lyophilized form from Integrated DNA Technology (UK). The gBlock gene product was resuspended in Tris-EDTA buffer according to the manufacturer's instructions to reach a final concentration of 10 ng/µl and stored at -20°C for further use. One (1) µl PCR product of *ompA* gene fragment was ligated into linearized pMiniT 2.0 vector and transformed into NEB 10-beta Competent *E. coli* (NEB#C3019) using NEB® PCR Cloning kit according to manufacturer's instructions. Successfully transformed cells were picked from LB agar plate containing 100 mg/L Ampicillin and screened by PCR using the primers listed in Table 2 to evaluate cloning of the target genes. Plasmid DNA was extracted using PrimeWay Plasmid DNA Extraction kit according to manufacturer's instructions. The plasmid DNA was stored at -20°C until further use as a positive control in the real-time PCR assay.

Real-time PCR parameters

The real-time PCR reaction was prepared containing 10 µl of 2x SensiFAST™ Probe No-ROX Mastermix, 0.3 µM primers, 0.2 µM probes, 2 µl of microbial DNA or 8 µl of human clinical samples DNA template. Ten (10) ng/µl of DNA template was used. RNA-free water was added to a final volume of 20 µl. DNA was amplified using Biorad CFX96 Touch Real-time PCR Detection System. Amplification cycling parameters included an initial denaturation at 95°C for 2 minutes, followed by 45 cycles of 95°C for 15 seconds and 60°C for 1 minute. The baseline threshold for post-amplification analysis was set at 100. Any Cq value ≤45 is considered positive. The results were analysed using CFX Maestro Software Version 2.3.

Result and Discussion

C. psittaci is classified as a Category B pathogen by the U.S. Centers for Disease Control and Prevention (CDC), therefore its handling requires biosafety level 3 (BSL-3) containment. Traditional method for isolating *C. psittaci* by cell culture as a positive control is hazardous, labor-intensive, and dependent on specific incubation conditions, making it unsuitable for routine diagnostics (Cheng et al., 2013; Okuda et al., 2011). This study employed a plasmid containing an *ompA* gene fragment as a positive control, providing a safe, stable and readily accessible alternative to live pathogen cultures (Standish et al., 2018). The synthetic plasmid developed in this study therefore ensures consistent assay performance, a key in implementing PCR as a rapid and routine diagnostic method.

The specificity of the developed real-time PCR assay was evaluated using a comprehensive panel of non-target bacterial strains and various types of human clinical samples. DNA was extracted from a total of 28 bacterial strains including both closely related and unrelated organisms, to assess potential cross-reactivity (Table 1). No amplification was detected in any non-target bacterial strain, confirming the high specificity of the assay. In addition, *in silico* PCR analysis against 268 bacterial genomes further confirmed that only *C. psittaci* sequences were amplified using the designed *ompA* primers and probe, supporting the high specificity of the assay (Table 2) (Kalendar et al., 2024).

Our findings demonstrate that the developed real-time PCR assay is highly specific for *C. psittaci*, consistent with previous reports employing species-targeted molecular assays. However, in the study by (Heddema et al., 2006), the primers and probe designed for the *ompA* gene also amplified and detected three closely related *Chlamydia* species namely *C. abortus*, *C. felis*, and *C. caviae* due to

sequence homology within the target region. This cross-reactivity required subsequent sequence analysis to distinguish the species. In contrast, the primers and probe in the present study were carefully designed and validated, both *in silico* and *in vitro*, to ensure exclusivity for *C. psittaci*, eliminating the need for additional confirmatory testing and makes the diagnosis more rapid.

To further evaluate performance in clinically relevant matrices, human samples comprised of sputum (n=33), tracheal aspirate (n=4), bronchoalveolar lavage (n=1) and pleural fluid (n=5) were spiked with a low and high concentration of synthetic target DNA in parallel without spiked control and tested under identical real-time PCR conditions (Dong et al., 2016). All reactions were performed in triplicate, with positive amplification of *ompA* gene observed only in the spiked controls (Table 3). Successful amplification of *ompA* gene in all spiked samples regardless of the concentration indicates that the assay able to detect the target sequence even in the presence of potential PCR inhibitors inherent to these sample types. The absence of amplification in non-spiked controls confirms that the assay produces no false-positive results in the absence of target DNA nor amplify background DNA or contaminants.

The sensitivity of the assay was determined using five replicates of plasmid control DNA prepared at varying concentrations (Table 4) (Angen et al., 2021). Analysis of the standard curve revealed a slope of -3.3749 which is very close to the theoretical value of -3.32, corresponding to maximum amplification efficiency. The calculated reaction efficiency was 97.84%, falling within the optimal range of 90-100%, with a correlation coefficient (R^2) of 0.9995 close to 1, reflecting high linearity and repeatability (Figure 1, Table 5). Successful amplification was observed at concentrations as low as 0.0002 pg (~25 DNA copies), with a Cq cut-off value of ≤45 (Table 4). These results indicate that the assay is capable of detecting extremely low quantities of target DNA with high precision and reproducibility. Taken together, these findings confirm that the developed real-time PCR assay is both highly specific and highly sensitive, making it a robust tool for accurate detection of *C. psittaci* in clinical samples.

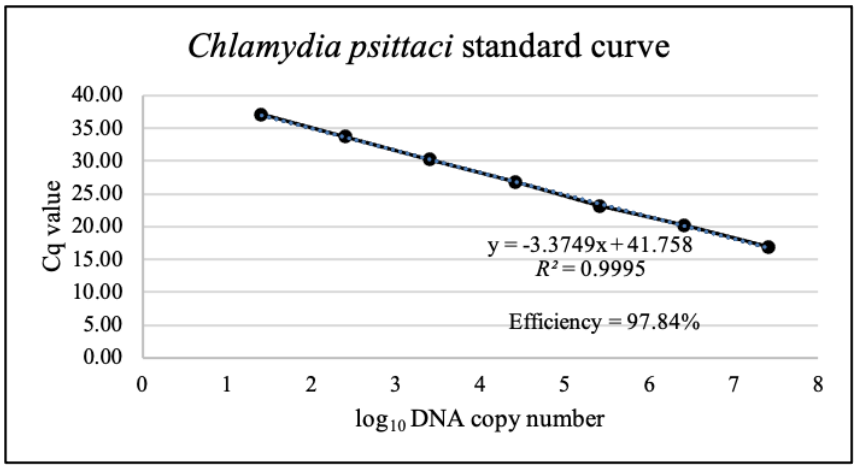

Figure 1. Standard curve of plasmid DNA in tenfold serial dilution.

Table 1. Specificity of *C. psittaci ompA* primers and probe against various bacterial strains.

Microorganism	Source	No of sample	PCR result
Acinetobacter baumannii	IMR archive	1	Negative
Bacillus subtilis	IMR archive	1	Negative
Chlamydia pneumoniae	IMR archive	1	Negative
Enterococcus faecalis	IMR archive	1	Negative
Enterococcus faecium	IMR archive	1	Negative
Escherichia coli	ATCC 25922	1	Negative
Haemophilus influenzae	IMR archive	1	Negative
Klebsiella pneumoniae	IMR archive	1	Negative
Legionella pneumophila	ATCC 33152	1	Negative
Leptospira serovar Worsolfdi	IMR archive	1	Negative
Methicillin-resistant *Staphylococcus aureus* (MRSA)	IMR archive	1	Negative
Mycobacterium tuberculosis	IMR archive	1	Negative
Mycoplasma pneumoniae	IMR archive	1	Negative
Orientia tsutsugamushi (Scrub typhus)	IMR archive	1	Negative
Proteus mirabilis	IMR archive	1	Negative
Proteus vulgaris	IMR archive	1	Negative
Pseudomonas aeruginosa	IMR archive	1	Negative
Rickettsia typhi (Endemic typhus)	IMR archive	1	Negative
Salmonella typhimurium	IMR archive	1	Negative
Shigella flexneri	IMR archive	1	Negative
Staphylococcus argenteus	IMR archive	1	Negative
Stenotrophomonas maltophilia	IMR archive	1	Negative
Streptococcus agalactiae	IMRCC S 816/95A	1	Negative
Streptococcus pneumoniae	ATCC 49619	1	Negative
Streptococcus pyogenes	IMR archive	1	Negative
Tick typhus strain TT118	IMR archive	1	Negative
Vibrio cholerae	IMR archive	1	Negative
Vibrio parahaemolyticus	IMR archive	1	Negative

Table 2. *In-silico* PCR for specificity of primers with bacterial strains.

Microorganism	No of strains	Result
Acinetobacter sp.	19	Negative
Bacillus sp.	80	Negative
Bacteroides sp.	9	Negative
Bartonella sp.	9	Negative
Bordetella sp.	10	Negative
Brucella sp.	20	Negative
Burkholderia sp.	42	Negative
Campylobacter sp.	29	Negative
Chlamydia sp.	8	Negative except *Chlamydia psittaci*
Clostridium perfringens	1	Negative
Coxiella sp.	6	Negative
Enterococcus sp.	2	Negative
Enterobacteriaceae bacterium strain	1	Negative
Escherichia coli	1	Negative
Francisella tularensis	1	Negative
Gardnerella vaginalis	1	Negative
Haemophilus sp.	3	Negative
Klebsiella pneumoniae	1	Negative
Legionella pneumophila	1	Negative
Leptospira	1	Negative
Listeria	1	Negative
Mycobacterium sp.	3	Negative
Mycoplasma pneumoniae	1	Negative
Neisseria	1	Negative
Pasteurella multocida	1	Negative
Pseudomonas	1	Negative
Rickettsia rickettsii	1	Negative
Salmonella sp.	3	Negative
Serratia marcescens	1	Negative
Shigella dysenteriae	1	Negative
Staphylococcus sp.	2	Negative
Stenotrophomonas maltophilia	1	Negative
Streptococcus sp.	4	Negative
Vibrio sp.	2	Negative

Table 3. Cq values from real-time PCR amplification of the *ompA* gene in non-spiked and spiked human respiratory samples

Sample	DNA concentration, ng/µl	Non-spiked		Spiked with 0.02 pg/µl		Spiked with 200 pg/µl	
		C. psittaci ompA, Cq	IAC, Cq	*C. psittaci ompA,* Cq	IAC, Cq	*C. psittaci ompA,* Cq	IAC, Cq
BAL_1	32.0	0.00	22.82	32.36	23.07	17.38	22.41
PF_1	23.1	0.00	35.24	31.26	30.57	17.22	41.48
PF_2	34.5	0.00	25.45	30.86	23.50	17.04	24.43
PF_3	17.6	0.00	33.56	30.61	31.66	17.01	42.12
PF_4	18.3	0.00	34.37	31.79	27.46	16.92	38.59
PF_5	24.2	0.00	24.14	30.98	24.61	17.21	24.83
SP_1	42.6	0.00	26.09	33.82	26.76	16.20	29.29
SP_2	16.4	0.00	24.13	29.97	24.89	16.31	26.01
SP_3	46.3	0.00	25.53	30.20	25.53	16.62	30.46
SP_4	31.6	0.00	24.87	30.74	24.99	16.65	27.82
SP_5	39.1	0.00	24.00	30.18	24.29	16.17	24.44
SP_6	31.7	0.00	22.57	30.29	22.53	16.60	22.40
SP_7	19.7	0.00	30.47	29.66	30.58	16.06	41.76
SP_8	19.5	0.00	27.86	30.20	28.35	16.75	39.99
SP_9	50.0	0.00	25.55	30.05	26.03	16.83	27.91
SP_10	24.4	0.00	23.81	30.14	23.51	16.83	23.73
SP_11	22.0	0.00	28.03	29.59	27.73	16.96	32.54
SP_12	46.7	0.00	21.92	30.25	22.08	16.93	21.93
SP_13	30.6	0.00	24.81	29.91	24.62	16.93	27.78
SP_14	11.2	0.00	26.85	29.91	26.56	16.89	31.20
SP_15	24.3	0.00	24.11	29.88	24.18	16.69	25.18
SP _16	46.4	0.00	22.06	30.30	24.92	16.77	22.05
SP _17	21.3	0.00	25.37	31.24	22.44	16.93	25.88
SP _18	36.7	0.00	24.36	29.83	25.22	16.73	25.61
SP _19	30.1	0.00	22.77	29.91	24.39	17.21	23.26
SP _20	33.9	0.00	22.95	30.29	24.42	17.27	23.23
SP _21	40.3	0.00	23.43	30.46	18.77	17.63	24.29
SP _22	48.7	0.00	22.08	29.78	24.10	17.70	21.85
SP _23	40.5	0.00	22.48	29.74	23.21	17.44	22.69
SP _24	32.1	0.00	22.65	30.08	22.36	17.15	22.07
SP _25	22.2	0.00	29.12	30.06	28.97	17.33	33.35
SP _26	21.9	0.00	23.50	30.22	22.30	17.36	22.24
SP _27	28.3	0.00	24.09	30.36	24.00	17.78	24.70
SP _28	36.2	0.00	23.91	30.28	23.13	17.76	23.57
SP _29	23.0	0.00	25.10	31.03	24.27	17.44	24.62
SP _30	37.7	0.00	26.14	30.27	25.57	17.76	27.90
SP _31	30.2	0.00	29.87	29.93	30.07	17.45	36.08
SP _32	20.6	0.00	30.60	30.46	30.60	18.42	36.99
SP _33	39.9	0.00	25.09	31.40	31.99	17.35	27.59
TA _1	7.5	0.00	27.59	30.31	28.18	17.46	32.75
TA _2	7.8	0.00	26.09	30.39	26.58	17.19	28.34
TA _3	11.9	0.00	28.19	30.45	28.46	17.54	34.48
TA_4	13.9	0.00	27.63	30.03	27.54	17.77	31.78

Table 4. Analytical sensitivity of real-time PCR for the detection of *C. psittaci*.

DNA copies number	Amount, pg	Mean Cq	SD, σ	CV, %
25615503.718	200	16.94	0.25	1.47
2561550.372	20	20.21	0.43	2.11
256155.037	2	23.18	0.21	0.89
25615.504	0.2	26.81	0.20	0.76
2561.550	0.02	30.20	0.19	0.62
256.155	0.002	33.71	0.39	1.14
25.616	0.0002	37.11	1.16	3.13

Table 5. Standard curve parameters for sensitivity of real-time PCR for the detection of *C. psittaci.*

Parameter	Slope	Efficiency	Linearity, R^2
Value	-3.3749	97.84%	0.9995

Conclusion

In conclusion, the real-time PCR assay developed in this study offers a specific and sensitive method for the routine detection of *C. psittaci* in human respiratory samples. By utilizing a synthetic plasmid control targeting the ompA gene of *C. psittaci*, the assay overcomes the biosafety challenges associated with handling live *C. psittaci* cultures, enabling safer and more accessible diagnostic implementation. The designed primers and probe were validated both *in silico* and *in vitro* to ensure exclusivity for *C. psittaci*, avoiding cross-reactivity with closely related and unrelated bacterial strains. The assay demonstrated reliable performance in various human clinical sample matrices as the assay capable to amplify the target region of the spiked samples even in the presence of potential PCR inhibitors, whereby no false-positive amplification in non-spiked controls. Sensitivity testing confirmed its ability to detect as few as ~25 DNA copies with excellent efficiency and reproducibility. Collectively, these features make the assay as a robust, rapid and accurate tool for the molecular detection of *C. psittaci* in clinical samples and epidemiological applications.

Acknowledgement/Funding

We would like to thank the Director General of Health Malaysia for the permission to publish this article. We would like to thank laboratory personnel at the Bacteriology Unit, Institute for Medical Research also, Universiti Teknologi Mara, Kuala Pilah, Hospital Shah Alam and Hospital Raja Perempuan Zainab II, Kota Bharu, for their direct and indirect contribution. This study was funded by the Research Grant (NMRR-21-1639-60288), awarded to one of the authors Mohammad Ridhuan Mohd Ali.

Author Contribution

Nur Bainun Mohd Zin (NBMZ), Siti Norsuhaida Mohmd Azman (SNMA), Noorlis Ahmad (NA), Jama'ayah Mohamed Zahidi (JMZ), Hana Farizah Zamri (HFZ), Rohaidah Hashim (RH), Mohammad Ridhuan Mohd Ali (MRMA), Nurhamimah Zainal-Abidin (NZA).

Conceptualization & Methodology – SNMA, JMZ, HFZ, RH, MRMA, NZA Data curation: NBMZ, MRMA, NZA; Writing – review & editing: NBMZ, SNMA, NZA, HFZ, JMZ, MRMA; Visualization: NZA; NBMZ, MRMA; Supervision: NA, MRMA, NZA; Project administration: NBMZ, MRMA, RH; Funding acquisition: MRMA

Conflict of Interest

The authors declare that there are no conflicts of interest.

Declaration on the Use of Generative AI

During the preparation of this manuscript, the authors used *ChatGPT* (OpenAI, San Francisco, CA, USA) to assist in drafting and refining certain sections of the text, including paraphrasing, and summarizing the results and discussion. The content generated by the AI was reviewed, edited, and verified for accuracy and appropriateness by authors. The authors take full responsibility for the content of the final manuscript.

References

AbdelRahman, Y. M., & Belland, R. J. (2005). The chlamydial developmental cycle. *FEMS Microbiology Reviews, 29*(5), 949–959. https://doi.org/10.1016/j.femsre.2005.03.002

Angen, Ø., Johannesen, T. B., Petersen, R. F., Uldum, S. A., & Schnee, C. (2021). Development of a species-specific real-time PCR test for *Chlamydia psittaci* and its employment in the investigation of zoonotic transmission from racing pigeons in Denmark. *Diagnostic Microbiology and Infectious Disease*, *100*(2), 115341. https://doi.org/10.1016/j.diagmicrobio.2021.115341

Bikandi, J., Millán, R. S., Rementeria, A., & Garaizar, J. (2004). *In silico* analysis of complete bacterial genomes: PCR, AFLP-PCR and endonuclease restriction. *Bioinformatics*, *20*(5), 798–799. https://doi.org/10.1093/bioinformatics/btg491

Bommana, S., & Polkinghorne, A. (2019). Mini review: Antimicrobial control of chlamydial infections in animals: Current practices and issues. *Frontiers in Microbiology, 10*:113. https://doi.org/10.3389/fmicb.2019.00113

Cheng, Y. J., Lin, K. Y., Chen, C. C., Huang, Y. L., Liu, C. E., & Li, S. Y. (2013). Zoonotic atypical pneumonia due to *Chlamydophila psittaci*: First reported psittacosis case in Taiwan. *Journal of the Formosan Medical Association*, *112*(7), 430–433. https://doi.org/10.1016/j.jfma.2012.08.017

Cui, Z., & Meng, L. (2023). Psittacosis pneumonia: Diagnosis, treatment and interhuman transmission. In *International Journal of General Medicine, 16*, 1–6. https://doi.org/10.2147/IJGM.S396074

Dembek, Z. F., Mothershead, J. L., Owens, A. N., Chekol, T., & Wu, A. (2023). Psittacosis: An underappreciated and often undiagnosed disease. *Pathogens, 12*(9), 1165. https://doi.org/10.3390/pathogens12091165

Dong, M., Fisher, C., Añez, G., Rios, M., Nakhasi, H. L., Hobson, J. P., Beanan, M., Hockman, D., Grigorenko, E., & Duncan, R. (2016). Standardized methods to generate mock (spiked) clinical specimens by spiking blood or plasma with cultured pathogens. *Journal of Applied Microbiology*, *120*(4), 1119–1129. https://doi.org/10.1111/jam.13082

Gu, L., Liu, W., Ru, M., Lin, J., Yu, G., Ye, J., Zhu, Z. A., Liu, Y., Chen, J., Lai, G., & Wen, W. (2020). The application of metagenomic next-generation sequencing in diagnosing *Chlamydia psittaci* pneumonia: A report of five cases. *BMC Pulmonary Medicine*, *20*(1), 65. https://doi.org/10.1186/s12890-020-1098-x

Heddema, E. R., Beld, M. G. H. M., de Wever, B., Langerak, A. A. J., Pannekoek, Y., & Duim, B. (2006). Development of an internally controlled real-time PCR assay for detection of *Chlamydophila psittaci* in the LightCycler 2.0 system. *Clinical Microbiology and Infection*, *12*(6), 571–575. https://doi.org/10.1111/j.1469-0691.2006.01417.x

Huang, W., Wang, F., Cai, Q., Xu, H., Hong, D., Wu, H., Zhou, L., Hu, L., & Lu, Y. (2023). Epidemiological and clinical characteristics of psittacosis among cases with complicated or atypical pulmonary infection using metagenomic next-generation sequencing: a multi-center observational study in China. *Annals of Clinical Microbiology and Antimicrobials*, *22*(1), 80. https://doi.org/10.1186/s12941-023-00631-w

Kalendar, R., Shevtsov, A., Otarbay, Z., & Ismailova, A. (2024). In silico PCR analysis: a comprehensive bioinformatics tool for enhancing nucleic acid amplification assays. *Frontiers in Bioinformatics*, *4*, 1464197. https://doi.org/10.3389/fbinf.2024.1464197

Liu, S., Cui, Z., Carr, M. J., Meng, L., Shi, W., & Zhang, Z. (2023). *Chlamydia psittaci* should be a notifiable infectious disease everywhere. *The Lancet Microbe*, *4*(2), e62–e63. https://doi.org/10.1016/S2666-5247(22)00306-8

Longbottom, D., & Coulter, L. J. (2003). Animal chlamydioses and zoonotic implications. *Journal of Comparative Pathology*, *128*(4), 217–244.

Luu, L. D. W., Kasimov, V., Phillips, S., Myers, G. S. A., & Jelocnik, M. (2023). Genome organization and genomics in Chlamydia: whole genome sequencing increases understanding of chlamydial virulence, evolution, and phylogeny. *Frontiers in Cellular and Infection Microbiology*, 13:1178736.

https://doi.org/10.3389/fcimb.2023.1178736

McGovern, O. L., Kobayashi, M., Shaw, K. A., Szablewski, C., Gabel, J., Holsinger, C., Drenzek, C., Brennan, S., Milucky, J., Farrar, J. L., Wolff, B. J., Benitez, A. J., Thurman, K. A., Diaz, M. H., Winchell, J. M., & Schrag, S. (2018). *Use of real-time PCR for Chlamydia psittaci detection in human specimens during an outbreak of psittacosis—Georgia and Virginia, 2018. Morbidity and Mortality Weekly Report (MMWR).* U.S. Centers for Disease Control and Prevention. https://www.cdc.gov/mmwr/mmwr_continuingEducation.html

Mohd Ali, M. R., Lih Huey, L., Foo, P. C., Goay, Y. X., Ismail, A. S., Mustaffa, K. M. F., Aziah, I., Kia Kien, P., Harun, A., Ismail, N., & Yean Yean, C. (2019). Duplex TaqMan hydrolysis probe-based molecular assay for simultaneous detection and differentiation of *Burkholderia pseudomallei* and *Leptospira* spp. DNA. *BioMed Research International,* 9451791. https://doi.org/10.1155/2019/9451791

Nieuwenhuizen, A. A., Dijkstra, F., Notermans, D. W., & van der Hoek, W. (2018). Laboratory methods for case finding in human psittacosis outbreaks: A systematic review. *BMC Infectious Diseases*, *18*(1), 442. https://doi.org/10.1186/s12879-018-3317-0

Okuda, H., Ohya, K., Shiota, Y., Kato, H., & Fukushi, H. (2011). Detection of *Chlamydophila psittaci* by using SYBR Green real-time PCR. *Journal of Veterinary Medical Science, 73*(2), 137–142. https://doi.org/10.1292/jvms.10-0284

Ravichandran, K., Anbazhagan, S., Karthik, K., Angappan, M., & Dhayananth, B. (2021). A comprehensive review on avian chlamydiosis: a neglected zoonotic disease. *Tropical Animal Health and Production*, *53*(4), 414. https://doi.org/10.1007/s11250-021-02859-0

Sheng, Y., Jin, L. Y., Li, N., Zhang, Y., & Shi, Y. J. (2025). Global prevalence of psittacosis in outbreaks: a systematic review and meta-analysis. *BMC Public Health*, *25*(1), 2010. https://doi.org/10.1186/s12889-025-21612-y

Standish, I., Leis, E., Schmitz, N., Credico, J., Erickson, S., Bailey, J., Kerby, J., Phillips, K., & Lewis, T. (2018). Optimizing, validating, and field testing a multiplex qPCR for the detection of amphibian pathogens. *Diseases of Aquatic Organisms*, *129*(1), 1–13. https://doi.org/10.3354/dao03230

Sukon, P., Nam, N. H., Kittipreeya, P., Sara-in, A., Wawilai, P., Inchuai, R., & Weerakhun, S. (2021). Global prevalence of chlamydial infections in birds: A systematic review and meta-analysis. *Preventive Veterinary Medicine*, *192*. https://doi.org/10.1016/j.prevetmed.2021.105370

Tan, D. S. K., & Babudieriz, B. (1977). Ornithosis in Peninsular Malaysia (in man and pigeons). *Medical Journal of Malaysia*, *31*(3).

Vande Weygaerde, Y., Versteele, C., Thijs, E., De Spiegeleer, A., Boelens, J., Vanrompay, D., Van Braeckel, E., & Vermaelen, K. (2018). An unusual presentation of a case of human psittacosis. *Respiratory Medicine Case Reports*, *23*, 138–142. https://doi.org/10.1016/j.rmcr.2018.01.010

Xu, L., Chen, H., Canales, M., & Ciric, L. (2019). Use of synthesized double-stranded gene fragments as qPCR standards for the quantification of antibiotic resistance genes. *Journal of Microbiological Methods*, *164*. https://doi.org/10.1016/j.mimet.2019.105670

Yao, W., Chen, X., Wu, Z., Wang, L., Shi, G., Yang, Z., Zhang, Y., & Wu, B. (2022). A cluster of psittacosis cases in Lishui, Zhejiang Province, China, in 2021. *Frontiers in Cellular and Infection Microbiology*, *12*. https://doi.org/10.3389/fcimb.2022.1044984