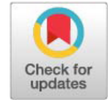

Research Article

Co-Occurrence of *Chlamydia psittaci* and *Avian influenza* H9N2 in Poultry of Pakistan

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Abstract | *Avian influenza virus* H9N2 and *Chlamydia psittaci (C. psittaci)* pose a threat to avian health by inducing respiratory distress and secondary infections, however, their co-infection remains elusive. An epidemiological study employing referral samples (n=738: 491 tissue/swab and 247 sera) was conducted to determine their co-occurrence in Pakistan. The RT-PCR results revealed average 12.21% (60/491) positive *C. psittaci*, including 47%, 33%, 20%, and 0% positivity in wild domesticated birds (WD), commercial poultry (CP), and backyard poultry (BP) and wild birds (WB), respectively. Overall sero-prevalence of *C. psittaci* was 21.05%, including 46%, 30.7%, 23% and 0% positive samples in WD, CP, BP and WB, respectively. Moreover, highest positive RT-PCR and seropositivity were found in WD of Punjab in summer. Regarding H9N2 prevalence, RT-PCR results revealed average 30.14% positivity, including 73%, 16%, 3% and 8% in CP, BP, WD and WB, respectively. The overall sero-positivity of H9N2 was 70.85%, including 36%, 32%, 20% and 12% positive detection in CP, BP, WD and WB, respectively, and with the highest sero-positivity in Sindh during winter and in KP during spring time. As for coinfection of *C. psittaci* and H9N2, average 6.51% (32/491) positivity was detected in tissues/swab and 10.53% (26/247) sero-positivity was found in serum samples. Compared to *C. psittaci* infection, higher H9N2 circulation was dominant in poultry and our study revealed that co-occurrence of H9N2 and *C. psittaci* was under estimated and demands further investigation.

Received | March 16, 2024; **Accepted** | December 10, 2024; **Published** | December 26, 2024
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Citation | Ain, N.U., N. Siddique, A.A. Malik, M.A. Abbas, S. Rafique, S. Zamir, H. Huilong, W. Yihui, Q. Hongkun and H. Cheng. 2024. Co-occurrence of *Chlamydia psittaci* and *Avian influenza* H9N2 in poultry of Pakistan. *Pakistan Journal of Agricultural Research,* 37(4): 394-402.
DOI | https://dx.doi.org/10.17582/journal.pjar/2024/37.4.394.402
Keywords | *Avian influenza* Virus, H9N2, *Chlamydia psittaci,* Coinfection, Zoonosis

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Introduction

Poultry is used as a major animal protein source worldwide, in the form of meat and eggs. The poultry sector in Pakistan is one of the most important industries as it provides more than 1.5 million employments across country. With an investment of more than Rs 750 billion, this industry is growing at an impressive rate of approximately 7.5% per annum over the last decade. Pakistan is now ranked as the 11th poultry producers of the world and has ample space for further improvement.

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Poultry sector is one of the most organized branches of the agro based sector of Pakistan. Its growth rate is 10-12% per annum (Economic Survey of Pakistan, 2021-22; Hussain *et al*., 2015). Although poultry is a vibrant and established intensive industry, however its production can be affected by various bacterial and viral infections. *Avian influenza* Virus (AIV) infection in poultry was first time reported in 1995 in Pakistan (Kausar *et al*., 2018). AIV H9N2 is considered as low pathogenic AIV (LPAIV) and has been prevailing in multiple avian species resulting in considerable economic losses in the form of respiratory infections and drop in egg production. Moreover, LPAIV targets specific hosts like humans and other mammalian species. H9N2 zoonosis has also been reported and such cases continue to raise public health concern (Beard *et al*., 1980; Kamps *et al*., 2006; Lee *et al*., 2013; Munir *et al*., 2013; Nili and Asasi, 2003; Parker *et al*., 2012; Potter, 2001). AIV outbreak took place in northern Pakistan in 1998, caused by LPAI H9N2. It resulted in egg drop, respiratory lesions and daily mortality of 2-3% (Naeem *et al*., 1999, 2003). In 2005, approximately 5-6 % mortality rate is observed in Pakistan due to AIVH9N2 (Ravichandran *et al*., 2021). AIV subtype H9N2 can be isolated from embryonated eggs of chickens (Elsayed *et al*., 2021) and serological identification is done for the detection of virus-specific antibodies using Enzyme-linked immunosorbent assays (ELISA) (Xiao *et al*., 2021). Over the last few decades, the use of molecular assays has enabled the precise detection of AIV, even at a very low pathogen count. By using Reverse Transcriptase Polymerase Chain Reaction (RT-PCR), detection and subtyping of AIV has been precisely reported (Xiao *et al*., 2021).

Avian chlamydiosis is a recurring bacterial infection in commercial and domesticated poultry. Avian Chlamydiosis outbreak caused considerable economic losses in psittacine birds and domestic poultry worldwide. The bacterium *Chlamydophila psittaci (C. psittaci)* is the etiological agent for avian chlamydiosis and human ornithosis (formerly psittacosis). *C. psittaci* infects lower respiratory tract, resulting in systemic, sometimes fatal disease in birds (Ravichandran *et al*., 2021). *C. psittaci* is a heterogeneous group with representative isolates both from mammalian and avian origin (Denamur *et al*., 1991). *C. psittaci* is a key aetiological agent in avian respiratory infections and considerable zoonotic infections (Lagae *et al*., 2014; Yang *et al*., 2007). Moreover, *C. psittaci* infects wild birds, pet birds, commercial poultry and closely contacted humans. Therefore, precautionary measures must be conducted while handling contaminated material and infected specimens (Andersen and Franson, 2017; Geigenfeind *et al*., 2010; Opota *et al*., 2015). Among caged birds, *C. psittaci* infections are most common in pigeons and doves (Smith *et al*., 2011). In order to determine the prevalence of *C. psittaci*, isolation and serological studies were conducted in routine survey. *C. psittaci* not only infects poultry and pet birds but also infect humans causing psittacosis, contributing to blindness, respiratory infections, typical pneumonia, cardiovascular diseases and death in severe situation (Chu *et al*., 2017; Longbottom and Coulter, 2003; Shewen, 1980). Until 2006, no case of infection with *C. psittaci* were reported in Punjab, Pakistan (Goldschmidt *et al*., 2006). However, previously few cases of *C. psittaci* was reported in poultry of Faisalabad, Pakistan (Siraj *et al*., 2018). The isolation and identification were carried out in specific-pathogen-free (SPF) chicken embryos and cell cultures. The serological detection of *C. psittaci* is performed with ELISA (de Freitas Raso *et al*., 2002; Lublin *et al*., 1997). Various standard and real-time PCR techniques are applied by targeting different genes for detection, identification and/or quantification of *C. psittaci* (Ehricht *et al*., 2006; Ménard *et al*., 2006). Compared with conventional PCR, real-time (RT)-PCR is more fast, sensitive and specific for diagnosis of intracellular pathogens (Opota *et al*., 2015).

In addition to *C. psittaci*, AIV H9N2 also causes respiratory distress in birds with co-infection leading to 30% mortality in chickens (Chu *et al*., 2017). Viral-bacterial co-occurrence will exacerbate disease intensity, leading to highly mortality and huge economic losses. Primarily, *C. psittaci* infection enhances the infection of AIV H9N2 by the suppression of immunity and trigger adaptive immunity in chickens (Chu *et al*., 2016, 2017; Opota *et al*., 2015; Ou *et al*., 2014). Regarding limited reports of coinfection of H9N2 and *C. psittaci* in Pakistan, we investigated referral samples (tissue/swab and sera) in order to conduct epidemiological study using RT-PCR and ELISA assay.

Materials and Methods

An epidemiological study was performed to evaluate prevalence and co-infection of AIV and *C. psittaci*

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zoonosis at National Reference Lab for Poultry Diseases, National Agricultural Research Council (NARC), Islamabad, Pakistan. For this purpose, 738 referral samples, including 491 tissue/swab and 247 serum samples were obtained from poultry of various ecological zones, including Punjab, KPK, Sindh and Islamabad Capital Territory. Shipment of samples was done in icebox to laboratory for timely diagnosis by keeping them at 0°C to -20°C until used.

AIV serological test

Serological evaluation was done using Enzyme Linked Immunosorbent Assay (ELISA)(IDEXX) and Hemagglutination Inhibition (HI) test (IDEXX) for which 247 sera were obtained from various locations. Initial screening for AIV was done using ELISA and then the ELISA positive samples were subjected to Hemagglutination Inhibition (HI) test for antibodies detection against AIV H9N2 subtype.

Serological evaluation of chlamydia

Seroprevalence of chlamydial antibodies was determined by ELISA assay (50ACP301; Biolage laboratories). The sera samples were analyzed for the detection of antibiotics against *C. psittaci* as described previously (Phalen, 2001).

Molecular detection of AIVH9N2 and C. psittaci

Presence of AIVH9N2 and *C. psittaci* was confirmed using PCR: RT-PCR and qRT-PCR (Lagae *et al.*, 2014). Processed swabs and tissues samples were subjected to RNA extraction for AIV H9N2 detection and DNA extraction for *C. psittaci* detection. Extraction of RNA was done by QIAamp Viral RNA mini kit with strict accordance to constructor's guidelines (CAT# 52906, QIAGEN, USA). DNA from processed swabs and tissue samples was extracted by FavorPrepTM Nucleic Acid Extraction Kit I based on manufacturer's instruction (CAT# FAVNK 001, FAVORGEN, Taiwan). Conventional PCR for AIV H9N2 was performed using kit, Invitrogen SuperScriptTm One step RT-PCR with Platinum Taq Cat. No 10928-042 following the manufacturer's procedure. The detection of AIV H9N2 was done by the amplification of *m* gene, *H9* and *N2* genes using the specific primers. Whereas conventional PCR for *C. psittaci* was performed by using Thermo Scientific DreamTaq Green PCR Master Mixture (2X), #K1071 adopting manufacturer's protocol (Gennari *et al.*, 2011; Lagae *et al.*, 2014). The amplified products were analyzed on agarose gel.

For both AIV H9N2 and *C. psittaci*, qRT-PCR reaction mixture was prepared using Invitrogen Super Script TM III Platinum R One Step Quantitative RT-PCR System (Cat. No. 11732-020, Invitrogen, USA). Already extracted RNA/DNA were used to carry out the procedure. Plate document was prepared on 7500 Real Time PCR equipment. PCR profile was adjusted on Real Time PCR equipment for the Matrix, H9 and *C. psittaci* genes (Ménard *et al.*, 2006) according to the specified temperature profile. The PCR was carried out in a final volume of 25µl containing 12.5µl 2X Reaction mix, 0.5µl RT/Platinum Taq mix, 5µl DNA or RNA template, 1µl of each primer, 1µl of probe, and 3.5µl nuclease free water was added up to 25µl. Different temperature profiles and different primers were used for the different genes of AIV H9N2 and *C. psittaci*.

For *m* gene of AIV sense primer 5`-AGA TGA GTC TTC TAA CCG AGG TCG-3` and anti-sense primer 5`-TGC AAA AAC ATC TTC AAG TCT CTG -3` along with the probe 5`-FAM-TCA GGC CCC CTC AAA GCC GA-TAMRA-3` were used. For detection of AIV H9N2 HA gene, sense primer 5`-ATG GGG TTT GCT GCC-3` and anti-sense primer 5`-TTA TAT ACA AAT GTT GCA YCTG -3` along with the probe 5`-FAM-TTC TGG GCC ATG TCC AAT GG-TAMRA-3` were used. Temperature profile was as follows: one cycle of cDNA synthesis at 50 °C for 20 min, one pre-denaturation cycle at 95 °C for 15 min, 40 cycles of denaturation at 95 °C for 45 sec and annealing at 54-60 °C for 30-45 sec. fluorescence pattern and CT values were the indicative of results.

C. psittaci was detected by sense primer 5`- CTG AAA CCA GTA GCT TAT AAG CGGT -3` and anti-sense primer 5`- ACC TCG CCG TTT AAC TTA ACT CC -3` along with the probe 5`-FAM-CTC ATC ATG CAA AAG GCA CGC CG-TAMRA -3` (Ehricht *et al.*, 2006). It was also detected by sense primer 5`-CTG CGC GGA TGC TAA TGG-3` and anti-sense primer 5`-CAC TAT GTG GGA AGG TGC TTCA-3` along with the probe 5`-FAM-CGC TAC TTG GTG TGAC-TAMRA -3` (Origlia et al., 2019) and sense primer 5`-CGG CGT GCC ACT TGA GA -3` and anti-sense primer 5`-GCC ATC ATG CTT GTT TCG TTT-3` along with the probe 5`-FAM-TCA TTG TCA TTA TGG TGA TTC AGG A-NFQ-MGB -3` (Ménard *et al.*, 2006). Temperature profiles were

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optimized as follows: One cycle of cDNA synthesis at 50 ˚C for 20 min, one pre-denaturation cycle at 95 ˚C for 15 min, 40 cycles of denaturation at 95 ˚C for 45 sec and annealing at 54-60 ˚C for 30-45 sec. fluorescence pattern and CT values were the indicative results.

Results and Discussion

Total 491 tissue and swab samples were subjected to RNA and DNA extraction for the molecular detection. Out of 491, 220 samples were positive for AIV, and out of these 220 positive AIV samples, only 147 samples were positive for H9N2. From total 491, 80 were positive for *Chlamydia*, whereas out of 80 positive sample, 60 were positive for psittaci specific primers (Figure 1).

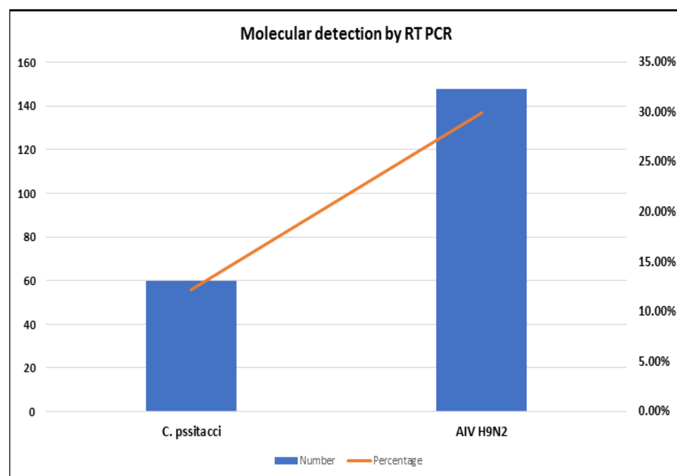

Figure 1: *Prevalence/detection of AIV (H9N2) and C. psittaci by RT-PCR. Data presented as %age.*

Prevalence of AIV H9N2 and *C. psittaci* by RT-PCR proved endemic status of H9N2 across Pakistan compared to *C. psittaci* infection (Figure 2).

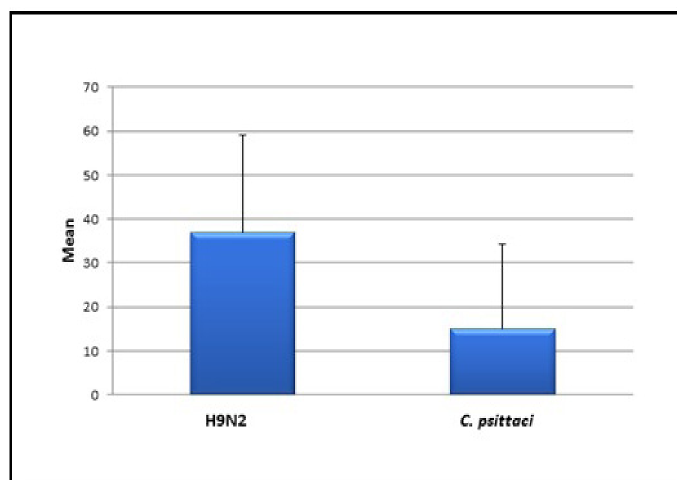

Figure 2: *Prevalence of AIV (H9N2) and C. psittaci by RT-PCR.*

A chi-square test of independence was done to determine bird type correlation between AIV (H9N2) and *C. psittaci*. The p-value was calculated at significance level of <0.05. Chi-square results showed that the relationship between the variables was statistically significant (Table 1).

Table 1: *Bird species wise variation between AIV (H9N2) and C. psittaci by RT-PCR.*

Bird species	AIV (H9N2)	Chlamydia psittaci	p value
Commercial poultry	108	20	< 0.00001
Backyard poultry	24	12	0.540809
Wild domestic	12	28	<0 .00001
Wild migratory	4	0	0.195405

Out of total 491 tissue and swab samples, 147 (29.9%) samples were found positive for AIV H9N2 and 60 (12.21%) were positive for *C. psittaci*. Among total 491 tissue and swab samples, the co-occurrence was confirmed in 32 samples (6.5%), whereas among total 207 positive samples (AIV H9N2 and *C. psittaci)*, the co-occurrence was confirmed in 32 (15.45%) samples through PCR and RT-PCR (Figure 3).

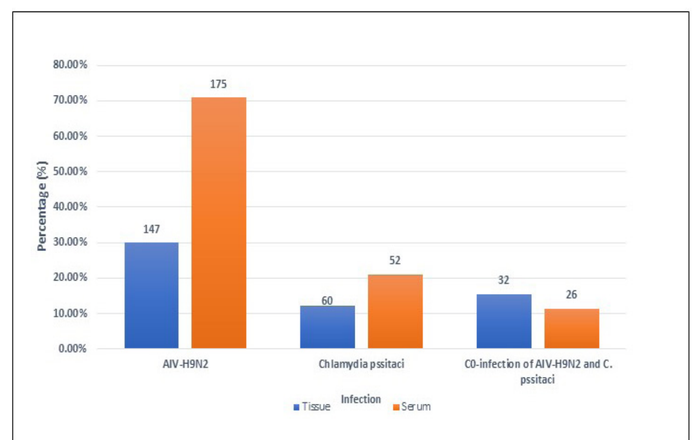

Figure 3: *Prevalence of AIV H9N2, C. psittaci and their co-infection.*

Out of 247 sera, 175 (70.85%) and 52 (21.05%) were found positive for AIV H9N2 and *C. psittaci*, respectively. Total 26 samples (10.52%) out of 247 sera were found seropositive for both AIV H9N2 and *C. psittaci*. On the other hand, among total 227 seropositive sera, 26 (11.45%) were positive for both AIV H9N2 and *C. psittaci* (Figures 3 and 4).

PCR Positive samples were further verified by qRT-PCR based on standard protocols. Samples above the threshold base line indicated positive results whereas

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samples below the threshold line were considered negative for AIV H9N2 and *C. psittaci* (Table 2).

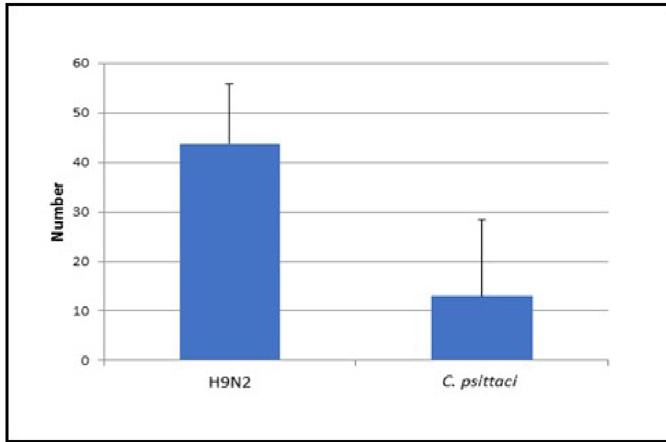

Figure 4: *Seropositive H9N2 and C. psittaci sera by ELISA (mean and standard deviation).*

Table 2: *Prevalence of AIV (H9N2) and C. psittaci by qRT-PCR.*

qRT-PCR	Sample tested (randomly selected)	Positive results	Ct-values RT-PCR
AIV H9	50	50	15.47 to 17.73
C. psittaci	60	60	10.43 to 15.63

A chi-square test of independence was used to determine seasonal correlation between AIV (H9N2) and *C. psittaci*. The p value was calculated at significance level of <0.05. Chi-square results showed that the relationship between the variables was statistically significant in 4 seasons (Table 3). Obviously, higher significance was found in summer and winter season. Approximately same ratios were found in serum samples. Out 247 samples, 175 (70.85%) sera were found positive for AIV and 52 (21.05%) sera were positive against *C. psittaci* and 26 (11.45%) samples were found to have a coinfection of AIV H9N2 and *C. psittaci*.

Table 3: *Seasonal seroprevalence between AIV (H9N2) and C. psittaci.*

Seasons	AIV (H9N2)	*C. psittaci*	p value
Spring	105	7	0.018
Summer	14	32	<0.0001
Winter	49	13	0.0001
Autumn	7	0	0

The study was conducted to evaluate the prevalence and co-infection of H9N2 and *C. psittaci* from commercial birds, backyard poultry, wild domestic and wild migratory bird species in Pakistan. From July 2018 to June 2019, total 738 (491 tissues and swab, and 247 serums) samples were collected from different ecological zones of Pakistan. H9N2 seroprevalence was 32%, 28%, 16% and 24%, respectively in KPK, Punjab, Sindh and ICT, Pakistan. Our data were different from the previous report, indicating maximum 48% in Sindh, 24% in KPK and only 2.4% in Punjab (Kausar *et al.*, 2018).

Regarding seasonal incidence, out of total 175 sera, 105 (60%), 49 (28%), 14 (8%) and 7 (4%) samples were positive during spring, winter, summer and autumn season, respectively. Slightly different results like maximum prevalence of AIV H9N2 was observed in winter approximately 75% by Kim *et al.* (2018) in his studies because during cold temperatures virus dissemination increases (Kim *et al.*, 2018). As for bird species, 63 (36%) samples were positive H9N2 from commercial poultry, 56 (32%) from the backyard poultry, wild domesticated birds contribute 35 (20%) and only 21 (12%) samples from wild migratory birds. Commercial poultry farms are handled in the intensive system with highly H9N2 infection. This was consistent with the previous report by Kim and his co-workers in commercial poultry than in backyard (Kim *et al.*, 2018). Similar findings were also reported by Ali and his colleagues in Pakistan where the prevalence of H9N2 was 6.7% and 2.7% in commercial and backyard poultry, respectively (Ali *et al.*, 2018).

As for seroprevalence of *C. psittaci*, out of 247 sera samples, 52 samples (21.05%) samples were positive. Our study was different from 77.8% positive samples performed by Yang and his colleagues (Yang *et al.*, 2007). The difference might be associated with host species, maintenance (cage or free), intensive scale, diseased birds and diagnostic assay. Moreover, seroprevalence was observed in the different provinces. From the 52 positive sera against *C. psittaci*, highest seroprevalence was observed in Punjab i.e., 36 (69.2%) samples and followed by KPK with 8 (15.3%) positive samples, whereas only 4 (7.69%) samples were found positive in Sindh and ICT. Another study during 2018 from Punjab (Faisalabad District) revealed positivity of 15.38%, 25%, 46.42%, 36.36% and 25% in chickens, ducks, pigeons, parrots and peacock, respectively (Siraj *et al.*, 2018). Based on our study, low seroprevalence was observed in other areas of Pakistan because of difference in the poultry population, temperature

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conditions and other environmental and regional factors. On other hand, maximum seroconversion was observed during summers with 32 (61.50%) positive samples, followed by 13 (25%) from winter and only 7 (13.46%) from spring whereas no seroprevalence was observed during autumn. Similarly report showed high seroprevalence in summer as compared to those samples collected in winter season (Vanrompay *et al*., 1997).

To warrant seroprevalence, out of 491 tissues and swab samples collected from different regions of Pakistan during July 2018 to June 2019, were processed for molecular detection and 147 and 60 samples were recorded positive samples by RT-PCR for H9N2, and *C. psittaci,* respectively.

RT-PCR test showed that there was 29.9% positivity for H9N2 across Pakistan. The highest positive H9N2 samples were found in commercial poultry i.e., 108 (73%) whereas backyard poultry contributed 24 (16%) to the positive samples. It was also recorded during this study that wild domestic birds contributed 12 (8%) and wild migratory birds contributed 4 (3%) to the positive H9N2 recorded samples. Similar results were also reported by Bahari where 59.3% of the RT-PCR positive samples were isolated from commercial poultry (Bahari *et al*., 2015). In contrast to this study, Muzaffar *et al.* (2018) reported prevalence of H9N2 in commercial and backyard poultry as 6.7% and 2.7%, respectively (Muzaffar *et al*., 2018). Kausar *et al* 2018 reported prevalence of 8.3% from nonvaccinated wild and domesticated birds across Pakistan (Kausar *et al*., 2018). From the 60 positive samples for *C. psittaci,* highest 28 (46.66%) samples were from wild domesticated birds followed by 20 (33.33%) from commercial poultry, 12 (20%) from backyard poultry. Similarly, tests were conducted to show high prevalence was observed in domesticated birds, including pigeons and ducks (Guo *et al*., 2016). On contrary, 5.4% positive rate was observed in commercial poultry (Lagae *et al*., 2014).

As for different regions, maximum number of H9N2 samples were positive from Sindh i.e., 56 (38%), KPK 52 (35%), Punjab 32 (22%) and from ICT 8 (5%). Contrary to our studies, the maximum i.e., 69% positive samples from Punjab, 19% from KPK and 12% from Sindh were detected (Kausar *et al*., 2018). The difference might be associated with different intensive models, bird populations and different seasons. Maximum detections of *Chlamydia psittaci* were from Punjab with 44 (73.33%) followed by KPK with 8 (13.33%) and 4 (6.6 %) prevalence was observed in Sindh and ICT. The highest positivity might be associated with large poultry population and intensive scales in Punjab.

Regarding coinfection, 32 (15.45%) samples out of 207 positive samples were positive both for AIV H9N2 and *C. psittaci* using RT-PCR method. Based on ELISA assay, 26 (11.45%) sera were found to have a coinfection of AIV H9N2 and *C. psittaci*. In a recent document, Chu and his co-workers showed combination of H9N2 with *C. psittaci* contributes to high mortality as well as severe lesions in respiratory system, leading to poultry airsacculitis and huge economic loss (Chu *et al*., 2016).

Conclusions and Recommendations

The H9N2 infection prevalence observed/recorded was 30.14% to 70.85% while *C. psittaci* infection was 12.21% and 21.05%, respectively using RT-PCR and ELISA assay in poultry from July 2018 to June 2019. More importantly, the overall coinfection rates of 15.45% and 11.45% were detected in this study from tissues and sera, respectively. Results revealed the high exposure to H9N2 and occurrence of H9N2 plus *C. psittaci* appeared as a looming threat to live birds, contributed to high risk for poultry industry and zoonotic implications. Therefore, epidemiological surveillance and vaccination campaigns are required to control the disease.

Acknowledgments

The authors gratefully acknowledge the financial and technical contribution of Ministry of Science and Technology (MoST), China and National Key Lab of Veterinary Public Health Security, College of Veterinary Medicine, China Agricultural University, Beijing, respectively. The acknowledgement is also extended to the Animal Sciences Institute, National Agricultural Research Center, Pakistan Agricultural Research Council (PARC), Islamabad.

Novelty Statement

This study provides the first evidence of Chlamydia psittaci and AIV-H9N2 co-infection in avian populations from different ecological zones of Pakistan,

revealing underestimated prevalence along with distinct seasonal and host-specific patterns. These findings highlight a critical need for targeted surveillance and control strategies for Chlamydia psittaci and AIV-H9N2 in avian populations.

Author Contributions

Naila Siddique and He Cheng: Conceptualization, writing review and editing, funding acquisition.
Noor Ul Ain, Naila Siddique, Akbar Ali Malik and Muhammad Athar Abbas: Methodology.
Noor Ul Ain, Naila Siddique, Hu Huilong and Wang Yihui: Validation.
Noor Ul Ain, Quan Hongkun, Sidra Zamir and Wang Yihui: Investigation.
Noor Ul Ain, Naila Siddique and Muhammad Athar Abbas: Data curation.
Noor Ul Ain and Akbar Ali Malik: Writing original draft preparation.
All authors have read and agreed to the published version of the manuscript.

Funding
This study was funded by the Science and Technology Partnership Program, Ministry of Science and Technology of China [grant No. KY202204001]. This study was supported in part by the Ministry of Science and Technology (MoST) [grant No. 2022YFC2304000].

Institutional review board statement
Not applicable.

Informed consent statement
Not applicable.

Data availability statement
Data contained within the article.

Conflict of interest
The authors have declared no conflict of interest.

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