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Research Article

Contamination Levels and Pathogen Distribution in Avian Habitats of Sindh and Azad Jammu & Kashmir

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ABSTRACT

The transmission of infectious pathogens among migratory and caged birds poses significant risks to avian and public health, emphasizing the need for effective disease monitoring and control. This study investigates microbial contamination in 200 samples from various caged and free-living avian habitats across Sindh Province and Azad Jammu & Kashmir (AJK), Pakistan, including feed, water, feces, litter/bedding, cages, and predatory nets. The samples were analyzed using culture, staining, biochemical profiling for isolation and identification of bacterial, fungal, and viral pathogens. The overall contamination rate was 43%, with bird feces exhibiting the highest contamination compared to samples from other sources. Litter/bedding contamination was more prevalent in Sindh compared to AJK, with significant bacterial, fungal, and viral pathogens identified, including fecal pathogens such as *E. coli* (8.14%), *Salmonella* spp. (6.98% & 5.81%), and *Chlamydia psittaci* (5.81%). Fungal contamination was more dominant in feed and litter/bedding, with *Aspergillus* fumigates identified (5.81%) in feed and (4.65%) in litter/bedding, respectively. *Candida albicans* was more prevalent in feces (4.65%), while *Cryptococcus neoformans* was detected in both feces and litter/bedding ranging (4.65 to 3.49%). Both, Newcastle disease virus and infectious bronchitis virus were more prevalent in litter/bedding and feces, occurring at (5.81%) and (3.49%), respectively. Statistical analysis revealed non- significant differences ($p > 0.05$) in contamination levels across most sample types, suggesting similar contamination trends across both regions, with some minor variations in litter/bedding samples. This study revealed that feces were a significantly higher source of bacterial pathogens compared to other sample types, while litter/bedding exhibited significant viral and fungal contamination, particularly for viruses. Fungal contamination was also notable in feed. This study underscores the drastic need for effective monitoring and control of the pathogens in caged and free-living birds to reduce risks to avian species.

Keywords: Birds, Contamination, Environment, Living Condition, Pathogens, Routes.

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INTRODUCTION

Avian species have recognized their role in ecosystems, as well as their interactions and movements. However, these bird species may reservoirs and are involved in spread pathogens to birds, animals and humans' in various regions around the globe (Smith et al., 2019; Johnson & Miller, 2020; Ahmed et al., 2021). It has been reported that more than 10, 000 avian species are recognized around the world (Benskin et al., 2009). Pakistan is well known for its diverse natural fauna, approximately more than 650 avian species distributed in the various regions (Chagas et al., 2017).

The infected cage birds are likely a source and transmission of the pathogens, thus posing risk of diseases in humans (Thakur et al., 2017; Garcia et al., 2018).

Migratory birds have been recognized to act as vectors in transmission of the pathogen in their host species (Lee et al., 2019). Infectious agents can transmit via migratory birds across the continents and adopt diverse climatic conditions (Krauss et al., 2007; Olsen et al., 2006). In addition, wild birds are identified as a spread of the pathogens in humans, animals and domesticated birds (Mihaela and Marina, 2014). Sometimes, certain bird species act as spread of pathogens without obvious clinical symptoms and signs and spread infections across the flyways (Reed et al., 2003). For example, migratory waterfowls were identified spread of highly pathogenic avian influenza viral strains including H5N8, H5N1 in different continents (Munster et al., 2007; Chen et al., 2006).

Illegal traffic and trade of wildlife and cage birds' trade were recognized as a source of transmission of infections across the regions (Karesh et al., 2005; Chomel et al., 2007). These birds may involve transmission of infections via human-mediated movement, and bypassing bio-security controls and natural barriers (Hernandez-Divers et al., 2005; Friend et al., 2012). This study is designed to evaluate sources and transmission of various pathogens including viruses, bacterial and fungal species, in birds living in caged and free-living environment.

MATERIALS AND METHODS

Study area and sample collection

A total of 200 samples were collected, including feed, water, feces, litter/dropping, predatory nets, and cages samples. Random sampling was performed for presence and transmission of pathogens including bacteria, fungi and viruses via various sources vice versa free-living migratory and caged birds in our previous study (Mohi Ud Din et al., 2024). Faecal samples from litter and bedding were aseptically obtained from the using swabs and sterile tubes. In addition, the samples were obtained from nets used for caching free living bird. The samples, comprising of sterile cotton wool swabs soaked in phosphate-buffered saline (PBS) were utilized to maintain pH levels. Sampling was conducted across various regions to cover a wide geographical distribution. These regions included Hyderabad (n=25), Thatta (n=25), Badin (n=25), Dadu (n=25), and Karachi (n=25) in Sindh Province, and Mirpur (n=25), Bhimber (n=25), and Kotli (n=25) in Azad Jammu and Kashmir (Table 1). After being collected, the samples were kept in cold chain containers and transported to the Department of Veterinary Microbiology at Sindh Agriculture University, Tandojam and Veterinary Research Institute Peshawar, Khyber Pakhtunkhwa, for further analysis.

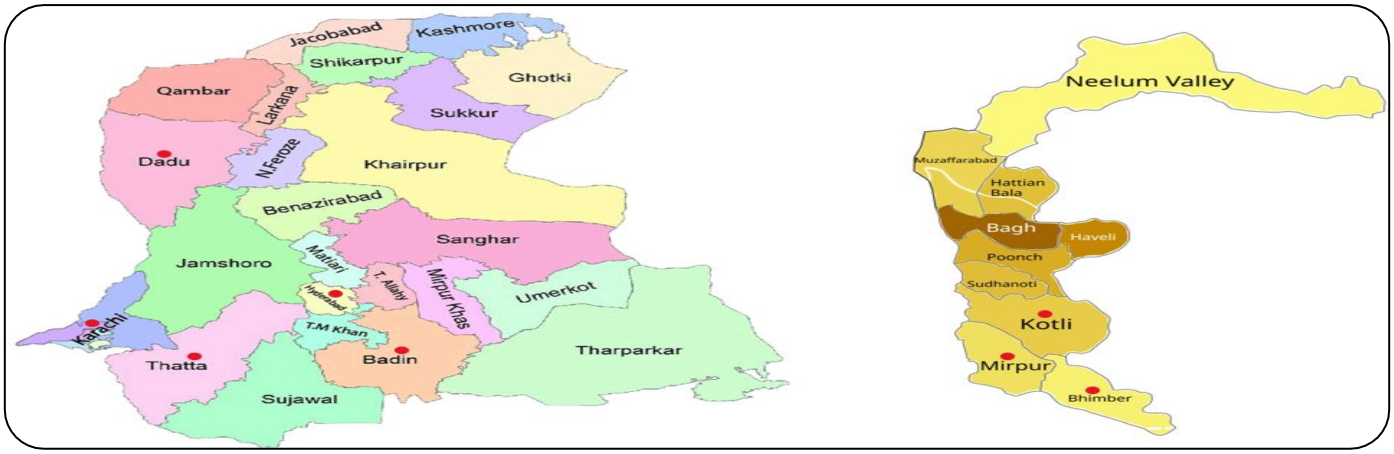

Figure 1. Map of study areas in Sindh province and Azad Jammu and Kashmir.

Media Preparation / Culture and Sub-culture for Bacterial and Fungal species

For the detection and isolation of bacterial species, the feed, water, feces, nets, cages, and litter/bedding samples were aseptically cultured onto various microbiological laboratory media; Nutrient agar (NA), Blood agar (BA), MacConkey (MAC) Agar, and Eosin Methylene Blue (EMB) Agar (Oxoid, UK).

For screening of fungal pathogens the samples were cultured on Sabouraud dextrose agar (SDA-Sigma Aldrich), Czapek dox agar (CDA-Sigma Aldrich), and Potato dextrose agar (PDA- Sigma Aldrich). Each anhydrous NA 28gm/BA 40gm/MA 50gm/EMBA 38gm, and SDA 65gm/CDA 49gm/PDA 39gm, were measured using an electronic weighing balance DA 7240 (Allied Fisher Scientific, 7240DA) for accurate and precise determination, and dissolved in 1000ml distilled water using magnetic stirrer MSH 300 (HVD, Life Science) and magnetic stirrer hot plate MSH 78-2, prepared separately (Pena et al., 2010; Forbes et al., 2017; Jorgensen et al., 2015).

The media was sterilized by autoclaving at 121°C 15 Ib/in² for 15 minutes and then after cooled at room temperature was poured into petri plates. Following the sample (s) cultivated on Petri dishes, were transferred into the incubator CTD 43 (MEDIFLOW, CTD 43) and incubated at 37°C/22°C for overnight to isolate the bacterial and fungal pathogens, respectively. Colonies were recovered from mixed cultures with the streak plate method, which included use a sterile loop to streak the sample across the agar surface, therefore segregating individual cells to form distinct colonies. Cultured microorganisms were effectively transferred from surfaces for culture by promptly inoculating agar plates with samples utilizing the swabbing method. A small, diluted sample was distributed using a sterile spreader during the spread plate process to uniformly disperse bacterial cells over the agar surface. So, the purification of culture was done by sub-culturing of the typical well-separated colony on the corresponding medium. The process was repeated for several times. These approaches enabled the successful isolation, cultivation, and examination of bacterial and fungal cultures. All infected plates were incubated aerobic and anaerobic conditions at 37°C for duration of 18 to 24 hours. Specific incubation conditions were implemented on the selective medium as per the manufacturer's guidelines.

Table 1. Sample collection from diverse sources and geographical regions.

Locations	Feed	Water	Feces	Litter/ bedding	Predatory net	Cage	Total samples
Hyderabad	8	4	5	3	2	3	25
Thatta	7	5	5	4	1	3	25
Badin	5	6	6	3	2	3	25
Dadu	6	3	7	4	2	3	25
Karachi	7	5	5	6	0	2	25
Mirpur	5	3	6	4	3	4	25
Bhimber	2	2	9	6	2	4	25
Kotli	5	4	6	5	2	3	25
Total	45	32	49	35	14	25	200

Microscopic Examination

Morphological characteristics including size, shape, color, margin, consistency, and hemolytic activity on blood agar were evaluated for colonies on different media. Further, the purity of the culture/growth was observed by examining the stained smear. Smear was made from each type of colonial growth, and identification of both bacterial and fungal pathogens were performed based on the morphological characteristics, isolated colonies underwent Gram’s staining and lacto phenol cotton blue (LPCB) staining methods. After staining and drying, slides were examined to assess the bacterial structure, arrangement and color, with gram-positive bacteria appearing purple and gram-negative bacteria red. The fungal structures were appeared blue, with a good contrast against the dark background. Giemsa's stain revealed *Chlamydia psittaci* as light blue or purple inclusions within host cells. These distinct inclusion bodies, visible under high magnification, confirmed the presence of the infection.

Culture and sub-culture of samples for viral species

In current study, Embryonated chicken’s egg technique was used to recover viruses, such as Newcastle Disease Virus (NDV), and Infectious Bronchitis Virus (IBV). The materials included 9–11 day old embryonated eggs, sterile PBS, syringes, needles, disinfectants, an incubator, and other inoculation equipment. The feed was suspended in PBS, the water was filtered, and a 10% solution of feces or bedding was prepared. The supernatant was then collected by centrifugation for inoculation. Following candling to ensure viability, the eggs were sanitized with ethanol or iodine and infected by several techniques according to the virus: the yolk sac for slow-growing viruses, the chorioallantoic membrane (CAM) for IBV, and the allantoic cavity for NDV. The eggs were sealed and incubated at 37 °C. The inoculated eggs were observed for signs and embryonic death. The eggs were kept at 4°C in order to make embryo’s death. Egg contents were collected and kept for further studies. Further isolation and identification of the viral species were performed using methods described by (OIE, 2019; Gough et al., 1988; Thammakarn et al., 2015; Spekreijse et al., 2013).

Culture, sub-culture and identification of. *Chlamydia* species

The technique was also applied to detect *Chlamydia psittaci,* an obligate intracellular pathogen (Egg Pathogen-free, 5–7-day-old embryonated chicken eggs were used) from samples of feed, water, feces, litter, nets, and cages. After inoculating *Chlamydia psittaci* into embryonated hen's eggs, Giemsa's staining was used to detect *Chlamydia psittaci* elementary bodies in yolk sac preparations. This was the most commonly used method to confirm the presence of the bacterial species (Arnaud Van Wettere, 2024).

Biochemical profile for isolation and identification of bacterial and fungal species

In accordance with the established protocols, several biochemical experiments were performed to distinguish diverse bacterial species. The biochemical profiling included the Indole test, triple sugar iron (TSI) utilization, catalase activity, Oxidase Test, Citrate Utilization Test, Urease Test, Methyl Red and Voges-Proskauer (MR-VP) reactions were performed according to procedure outlined by (Khalil & Gabbar, 1992). The biochemical assessment was performed to determine and differentiate various fungal pathogens species in the various samples. The biochemical methods including cycloheximide resistance, fatty acid esterase activity, casein hydrolysis, and cellulose hydrolase activity were performed for identification of fungal pathogens. Control strains acted as references for each biochemical test, and all experiments were performed in triplicate to ensure precision (Mohi Ud Din et al., 2024).

Statistical analysis

The statistical analyses were performed using SAS 9.4 (SAS Institute, Cary, North Carolina, USA). Collected data was compiled and analyzed using Chi-square test to know the significant differences between bacterial, fungal and viral pathogens occurrence. Chi-square analysis was performed to determine the relation between the sample categories and microbial contamination across Province Sindh and Azad Jammu and Kashmir. This test was selected to acess whether the contamination rate have any differences between across sample types and the regions and. The null hypothesis (H_0) and act as no significant difference in contamination rates, and the alternative hypothesis (H_1) poised as significant differences across the samples. The statistical analyses were performed to determine the pathogens in the samples. The relationships of the pathogens were determined using the prevalence as ratio of positive samples to total collected samples. The prevalence ratio was expressed as percentage. Confidence Intervals for each region's prevalence analyzed and calculated using proportions. The statistical differences were observed employing a Fisher's Exact test Chi-Square test with a p-value threshold of $P < 0.05$ was considered for significance.

RESULTS

Assessment of microbial contamination in avian habitat samples

Out of 200 samples associated to avian habitat comprising of six categories, including water, feed, feces, litter/Bedding, cages and predatory net exhibited 86 (43%) were positive for microbial contamination of samples obtained from regions of Sindh Province and Azad Jammu and Kashmir, Pakistan (Table 2). In the both territories, feed and water showed similar contamination rates, with AJK (n=3) positive samples and Province Sindh (n=9), resulting in a total of 12 samples positive for each category. The highest contamination level was recorded in bird feces 34 samples in the both regions. The contamination level of litter/bedding was higher in 14 samples collected from Province Sindh in comparison to AJK, 6 positive samples. Bird Cages (n=6) and predatory nets (n=2) samples exhibited relatively low contamination rate in each regions. According to Chi-Square test and their corresponding p-values indicated that feed, water, feces, litter/bedding, predatory nets and cage samples exhibited non-significant difference (p > 0.05). The findings suggested, overall, the distribution of positive samples between AJK and Sindh regions are showing similar trend across the categories, with some minor differences in litter/bedding samples.

Table 2. Distribution of positive samples in across sample categories in AJK and Sindh, Pakistan.

Sample Category	Positive samples (n)	Positive samples (n)	Total positive Samples (%)	Chi-Square Value	P-value
	Sindh Province	AJK territory			
Feed (n=12)	9	3	6%	2.34	0.671
Water (n=12)	9	3	6%	1.86	0.758
Feces (n=34)	18	16	17%	8.33	0.080
Litter/Bedding (n=20)	14	6	10%	4.53	0.335
Predatory nets (n=2)	2	0	1%	3.24	0.517
Cage (n=6)	4	2	3%	0.8	0.371
Total (n=86)	56	30	43%		

Percentage has been calculated from total number of samples (n=200)

Assessment of microbial contamination in avian habitat of different regions

The study analyzed the occurrences of different pathogens in the samples obtained from the feed, water, feces, nets, cages, and litter/bedding across different regions in Sindh and Azad Jammu & Kashmir (AJK), examining 200 samples from feed, water, cages, feces, litter/bedding and predatory nets (Table 3). In Sindh, the highest frequency of contamination was observed in Karachi with 28% in feces and 12% each in feed, water, and litter. Thatta exhibited higher contamination in litter (20%) compared to other sources, while Hyderabad having 12% in feces and 8% each in feed, water, and litter. In contrast, Dadu showed contamination mainly in feces (12%) and litter (12%). Badin had the least contamination across the Sindh region, with only 12% in water and 8% in feces. In AJK, Mirpur had the highest fecal contamination (24%), followed by 8% in litter. Bhimber exhibited 20% contamination in feces and 12% in litter, with minimal contamination in feed and water. Kotli showed a 20% contamination level in feces, and moderate contamination in all other sources. Statistical analysis revealed no significant (p>0.8888) differences among regions.

Table 3. Contamination dynamics of the pathogens across different regions of AJK and Sindh, Pakistan.

Locations		Total sample	Feed		Water		Feces		Litter/ bedding		Predatory nets		Cage		X^2 (P-value)
			Freq	%	Freq	%	Freq	%	Freq	%	Freq	%	Freq	%	
SINDH	Hyderabad	25	2	8	2	8	3	12	2	8	1	4	1	4	
	Thatta	25	3	12	1	4	3	12	5	20	0	0	0	0	
	Badin	25	0	0	3	12	2	8	1	4	0	0	1	4	
	Dadu	25	1	4	0	0	3	12	3	12	0	0	0	0	
	Karachi	25	3	12	3	12	7	28	3	12	1	4	2	8	31.22
AJK	Mirpur	25	1	4	0	0	6	24	2	8	0	0	0	0	(0.888)
	Bhimber	25	0	0	1	4	5	20	3	12	0	0	1	4	
	Kotli	25	2	8	2	8	5	20	1	4	0	0	1	4	
Total		200	12		12		34		20		2		6		

Assessment of bacterial contamination across samples

Figure 2 illustrates the presence of bacterial contaminants; including *Chlamydia psittaci*, *E. coli*, *Salmonella pullorum*, and *Salmonella enterica*, through various sample types (feed, water, feces, litter/bedding, predatory nets and cages) that were interaction and/ or contact with migratory and caged birds in Sindh and AJK. Among these, *E. coli* exhibits the highest prevalence across all sample types, with feces showing the dominant percentage (8.14%), followed by water (5.81%), predatory nets (4.65%), and feed (3.49%). *Salmonella pullorum* and *Salmonella enterica* also show notable prevalence, particularly in feces (6.98% and 5.81%, respectively). In contrast, *Chlamydia psittaci* was absent in feed, water, cages, and predatory nets but was present in feces (5.81%), and litter/bedding (3.48%). Statistical analysis revealed no significant difference (p > 0.0655) in bacterial transmission across the various sample types. Percentage has been calculated from total number of positive samples (n=86), X^2: 30.28; P-value: 0.0655.

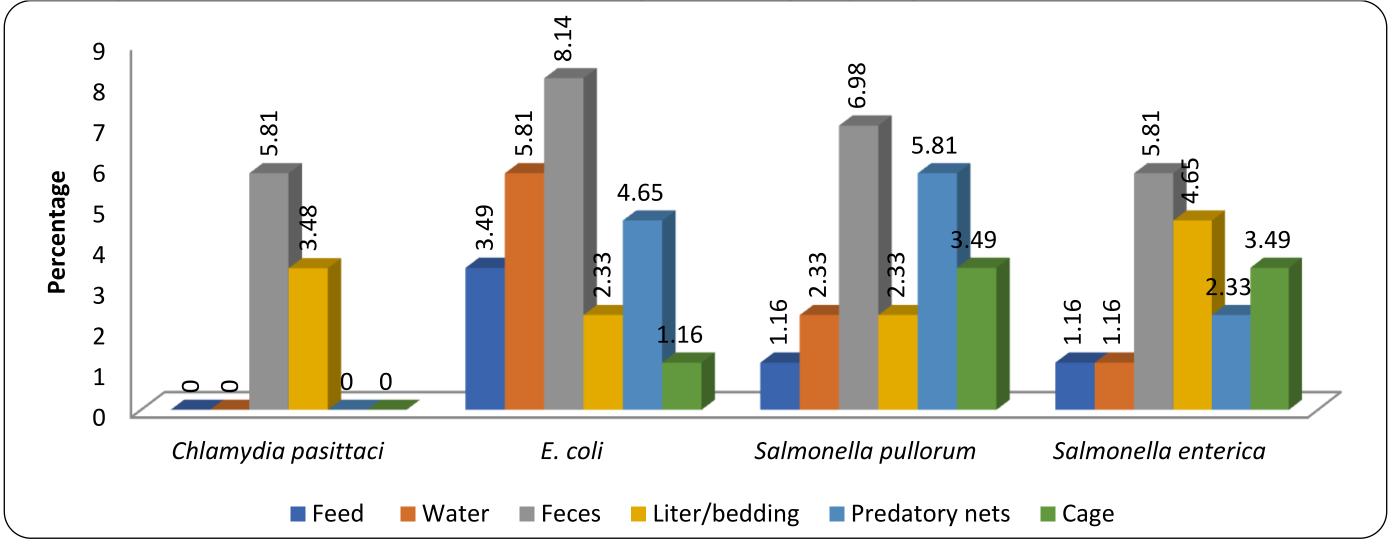

Figure 2. Bacterial contamination observed in the samples from sources interaction and/or contact to migratory/ caged birds.

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Assessment of fungal contamination across samples

Figure 3 illustrates the contamination of fungal pathogens such as; *Aspergillus fumigatus*, *Candida albicans*, and *Cryptococcus neoformans*, across various sample types (feed, water, feces, cages, litter/bedding and predatory nets) collected from different sources that were interaction and/ or contact with migratory and caged birds in Sindh and AJK. *Aspergillus fumigatus* exhibited the highest prevalence, particularly in feed (5.81%) followed by litter/bedding (4.65%) and feces (3.49%). *Candida albicans* showed highest ratio in feces (4.65%), followed by significant contamination in water (3.49%) and litter/bedding (2.33%). *Cryptococcus neoformans* were consistently present, with notable levels in feces (4.65%), litter/bedding (3.49%), and water (2.33%). Statistical analysis revealed no significant difference (p = 0.6311) in fungal transmission across these sample types.

Percentage has been calculated from total number of positive samples (n=86), X2: 12.63; P-value: 0.6311.

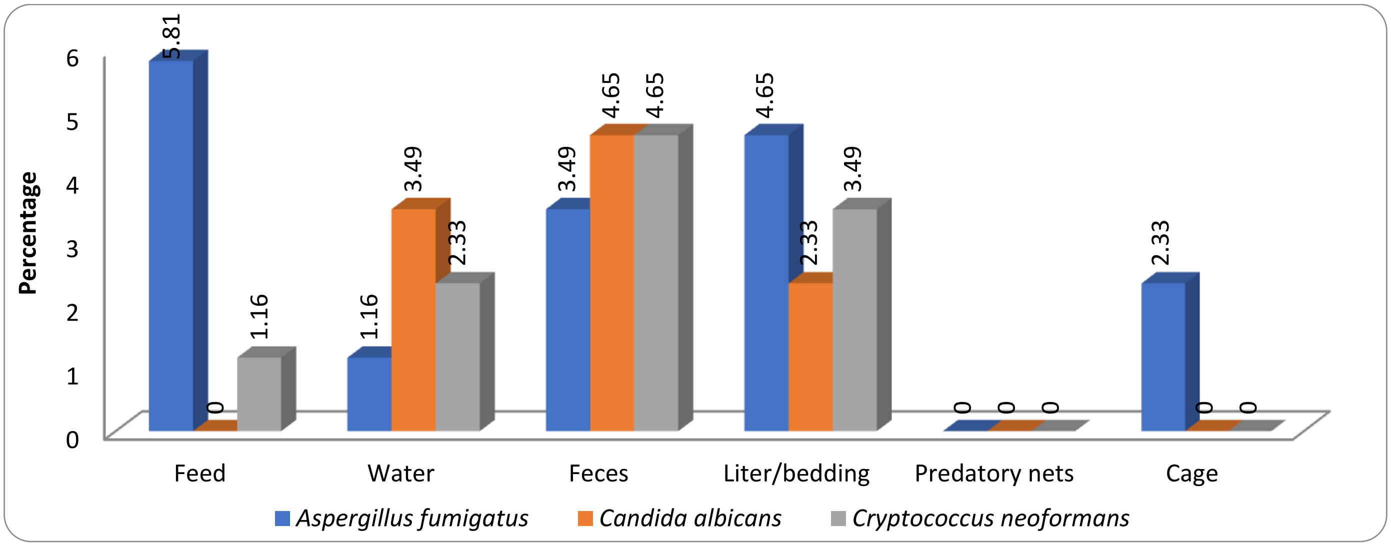

Figure 3. Contamination of fungal pathogens by migratory / caged birds from Sindh and AJK.

Assessment of viral contamination across avian habitat samples

Figure 4 illustrates the contamination of viral strains specifically the Newcastle disease virus and infectious bronchitis virus, across various sample types close to avian habitat. The results indicate that litter/bedding samples exhibited the highest prevalence of viral contamination, with NDV detected in 5.81% of samples and IBV in 2.33%. Fecal samples also showed notable contamination, with 2.33% testing positive for NDV and 3.49% for IBV. Water samples had a lower prevalence, with 1.16% testing positive for NDV and no detection of IBV. While feed, predatory nets, and cages showed no detectable viral contamination. Despite these variations, statistical analysis revealed no significant difference (p = 0.5196) in the viral presence across different sample types. Percentage has been calculated from total number of positive samples (n=86), X2: 9.13; P-value: 0.5196

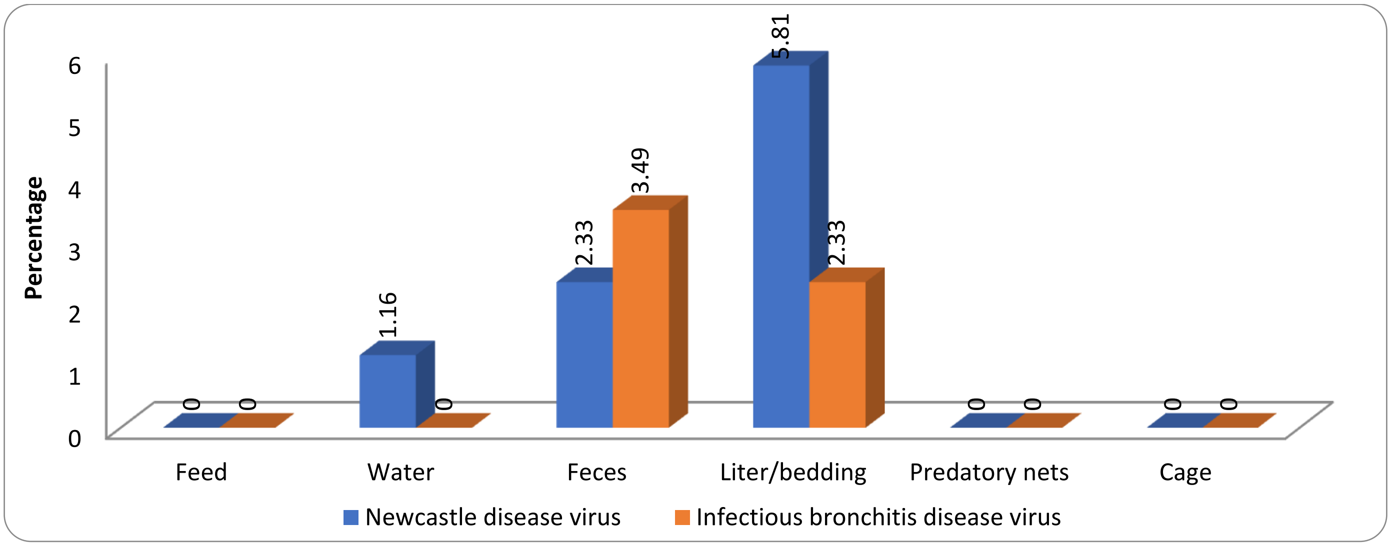

Figure 4. Contamination of viral strains from various avian habitat samples.

DISCUSSION

Pathogenic organisms pose a major risk and threat to poultry farming and associated industry. Birds typically get infected with microorganisms that come from various sources including feces, litter/bedding, feathers and housing, among others (Dumas et al., 2011). Pathogens and their toxins in the organic material may have an adverse influence on both the birds as well as workers' heath (Williams, 2009).

In this study, it was found that 43% of the samples from Sindh and AJK regions were contaminated, with feces showing the highest levels of contamination in both regions. Statistical analysis indicated consistent contamination trends across most categories, with only a slight difference observed in litter/bedding contamination between the two regions. It has been demonstrated that certain microorganisms may infect or be transferred to humans from animals vice versa, though, contact, water, food, direct or indirect environment for example *Escherichia coli* and *Staphylococcus aureus* spread and may cause zoonotic infections. In addition to the housing environment, pathogenic organisms can also be transmitted during the exhibition and marketing of pet birds (Belchior et al., 2011; Vanrompay et al., 2007). The results indicated that the highest microbial contamination detected fecal material (34 positive samples) and were the initial source of the pathogens. Contamination level in litter/bedding was found higher in Province Sindh, compared to AJK region suggesting regional differences in contamination sources. Similarly, previous research indicated the highest levels of pathogens were detected in litter during rearing birds (Witkowska et al., 2010). In fact, no statistical significant differences in pathogen prevalence across most of categories of samples obtained from bird habitats. Previously, wild avian species had been identified as vector for pathogen transmission to farm animals and contributed to contamination of pastureland, feed, water and feces (Johnston et al., 1979; Coulson et al., 1983; Fenlon et al., 1985; Graczyk et., 2008; Benskin et al., 2009). In accordance to this study, previous study reported that wild birds can act as potential fecal source of parasitic and bacterial pathogens including *Salmonella spp*, *Mycobacterium spp*, *Coxiella spp, Brucella spp*, microsporidia,and *Giardia* spp, *Cryptosporidium* spp for other animals and avian species (Ebani et al., 2021).

In this research, it was observed that *E. coli* (8.14%), was the most prevalent species across the all sample types. Several studies have shown that pathogenic *E. coli* was detected in approximately 6 to 30% in the samples obtained from different avian species (Hughes et al., 2009; Kobayashi et al., 2002; Konicek et al., 2016). Fecal samples exhibited the highest contamination levels, followed by water and predatory nets. Similarly, high rate 5.81 – 6.98% of *Salmonella enterica* and *Salmonella pullorum* were present, particularly in feces. Consistent to our findings, Batista et al., (2022) detected *E. coli* (8.9%), Salmonella spp (2.8%), and Campylobacter spp. (9.9%) in the samples obtained from free-living birds. In this study, *Chlamydia psittaci* was found in feces and litter/bedding samples. Overall, statistical analysis indicated no significant differences in contamination rate of bacterial species across the various sample types, suggesting similar risks of contamination across the sources of samples. Infected birds can shed *Chlamydia psittaci* intermittently in conjunctival secretions and droppings particularly during stress periods (Harkinezhad et al., 2009). Recently, *Chlamydia psittaci* have been detected in a pet store and breeding facility leading to mass bird exposures (Bonwitt et al., 2023).

Fungal pathogens including *Aspergillus fumigatus*, *Candida albicans* and *Cryptococcus neoformans* were detected across various sample types. The highest prevalence of *Aspergillus fumigatus* was detected in feed followed by litter/bedding, while *Candida albicans* was found in feces, water, and litter/bedding. The occurrences of *Cryptococcus neoformans* was found across almost all sample types, with the highest occurrence in feces and litter/bedding. Statistically, no significant difference was observed in fungal contamination across the sample types, suggesting that the risk of fungal contamination is relatively consistent across different habitats. It has been reported that *Aspergillus fumigatus* mostly occurs in feed and litter, whereas, humid and hot environmental conditions favor fungal growth (Khosravi et al., 2011). *Candida albicans* is an opportunistic and is capable of colonizing the gastrointestinal tract and excreted in feces. Therefore, it may contaminate water and increasing the risk of transmission. The high level presence of the pathogen in the feces corroborates earlier findings by Mahmoud et al. (2018), who demonstrated that birds shedding *Candida albicans* is a major source of environmental contamination and cross-contamination. Budgerigars and parrots droppings had been recognized as the initial and consistent source of *Cryptococcus neoformans*. Also, the pathogenic organism was detected in wooden nesting boxes (Bauwens et al. 1986).

The study revealed that Newcastle disease virus and Infectious bronchitis virus variants, were found across various sample types. The highest contamination levels of the both viral variants were present in litter/bedding and feces. Litter/bedding samples exhibited the highest prevalence of viral contamination, with NDV detected in 5.81% of samples and IBV in 2.33%. Fecal samples also showed notable contamination, with 2.33% testing positive for NDV and 3.49% for IBV. However, statistically, no significant difference (p = 0.1065) in the viral variants found across the different sample types, demonstrating tendency of risks of spread across the habitats. ND viruses are spread direct contact aerosolization of respiratory secretions with litter, water, food and feces. Birds' recovered from Newcastle disease can shed virus in the habitat. The disease is spread by direct contact with viral particles from aerosolization of respiratory secretions or feces or from food, water, or litter that is contaminated with feces. Recovered birds are believed to shed the virus indefinitely (Greenacre, 2005). IBV spread via direct contact or respiratory secretion and feces of virus particles from infected birds, premises and contaminate environment. Moreover, virus can transmit from infected birds through contaminated materials, farm visitors and utensils (Boltz et al., 2004). In this study, findings suggested that IBV and NDV viruses were shed from the infected birds through feces and litter/bedding.

CONCLUSION

In summary, microbial presence was detected in the samples from caged and free-living bird habitat in Sindh Province and Azad Jammu & Kashmir, Pakistan. Screening of the samples indicated positive for bacterial, fungal, and viral pathogens, especially in feces, litter/bedding and feed. It was revealed that feces were a significantly higher source of bacterial pathogens compared to other sample types, while litter/bedding exhibited significant viral and fungal contamination, particularly for viruses. Fungal contamination was also notable in feed.

AUTHOR CONTRIBUTIONS

All authors contributed equally to this research.

COMPETING OF INTEREST

All the authors have declared no conflict of interest. This work has not been published elsewhere, nor is it under consideration for publication. All the authors have approved its submission to this journal.

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