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Fungal Pathogens Prevalence in Avian Species: Regional and Species-specific Variations

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ABSTRACT

Fungal pathogens are opportunistic, causing infections in caged and free-living birds with hypo-immunity. **Objectives:** To investigate the prevalence of fungal infections in avian species living in free and caged environments. **Methods:** The samples (n=600) were obtained using the simple random sampling technique (to ensure unbiased selection) from free-living and cage birds of Sindh Province and Azad Jammu and Kashmir, Pakistan. The samples were cultured on Sabouraud dextrose agar, Czapek dox agar and Potato dextrose agar and biochemical profiles. The cultures were subjected to biochemical profiles including cyclo-hexamine resistance, casein hydrolysis, fatty acid esterase activity and cellulose hydrolase activity. **Results:** Results showed that 4.16% of the samples were positive for the fungal species. Four fungal species including Aspergillus fumigatus (40.00%), Candida albicans (28.00%) Cryptococcus neoformans (24.00%) and Macrorhabdus ornithogaster (8.00%) were detected in the avian species. Significant ($p<0.05$) difference in fungal infection observed in free-living and cage birds. **Conclusions:** It was concluded that the fungal pathogens were detected in free-living and caged bird samples obtained from Sindh and Azad Jammu and Kashmir, Pakistan. The variation in infection rates among bird types and locations impacts living and environmental conditions on fungal susceptibility. It offers significant insights into fungal infections in birds and contributes to developing infection management and environmental well-being strategies.

INTRODUCTION

Fungal infections cause severe health problems to birds, whether they travel freely or caged. Fungal diseases may impact the entire population not just individual birds causing severe mortality and morbidity. To understand the epidemiology of infections in birds, it is necessary to examine the factors contributing to fungal infections in birds across different locations and occurrences. In birds, the fungal infection epidemiology is influenced by environmental factors, species susceptibility, immunological conditions and age. Illness severity and prevalence have a substantial impact on environmental conditions [1]. Fungal diseases have gained more epidemiologic attention as a consequence of the rise in fungal infections, which led to a rise in morbidity and mortality as well as significant diagnostic and treatment issues in healthcare [2]. Aspergillosis is one of the most common and harmful fungal infections in birds caused by Aspergillus species. Aspergillosis outbreaks in caged birds can be caused by inadequate ventilation, high humidity, and filthy conditions. The disease affects immunocompromised and stressed birds. In free-living birds, Aspergillus spores are commonly found in natural habitats, particularly in decaying organic matter and moist environments, which increases the risk of exposure [3]. Aspergillosis is primarily a respiratory disease, birds inhale the fungus spores and become infected, which can cause chronic or acute symptoms [4]. Candida infections are another common detrimental health issue especially,

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caused by Candida albicans in birds [5]. Free-living birds are more vulnerable to Candida infections transmitted by contaminated food or water sources; in areas where human activity has altered the natural ecosystem [6]. In caged birds, the most common transmission of this infection is usually linked to poor diet, stress and lack of husbandry practices [7]. The fungus Cryptococcus neoformans caused by Cryptococcosis is another respiratory ailment of domesticated and wild birds. This fungal infection is mostly spread by bird droppings and the transmission of illness in birds by inhaling spores of infected fouled soil [8]. Macrorhabdus ornithogaster causes macro-orhabdosis in birds which affects the gastrointestinal tract in budgerigars and finches (small passerines). Clinical symptoms of these infections include poor feather condition, diarrhea, and weight loss [9]. Risk factors like the host immune system, husbandry practices and climate have influenced fungal infection prevalence which varies by geographic location and avian species. Recently, the burden of fungal infections in domesticated and wildlife birds has increased due to bacterial resistance, emphasizing the need for preventive techniques, and improved therapeutic and diagnostic approaches in avian medicine. There is a gap in understanding the epidemiology of fungal infections in caged and free-living birds in Pakistan. Therefore, this study was conducted in two regions of Pakistan including Sindh and Azad Jammu & Kashmir to study the epidemiology of fungal infections in caged and free-living birds.

This study aims to explore the burden of fungal infections, fungal organisms and their susceptibility rate in both caged and free-living birds.

METHODS

A total of 600 (Faecal, cloacal, conjunctival, oropharyngeal/ tracheal swabs and blood) samples were obtained randomly from caged and free-living birds of regions; Hyderabad, Thatta, Badin, Dadu, and Karachi, Sindh Province, and Mirpur, Bhimber and Kotli, Azad Jammu and Kashmir. A simple random sampling method was used for data collection. This strategy provided each bird with an equal opportunity for selection (unbiased selection). The sample size was determined using the formula n=Z2P (1–P)/d2n, in which d is the margin of error (5%), P is the estimated prevalence of the target condition in the population (assumed 50% for maximum variability), and Z is the value for the desired confidence level (1.96 for 95%) [10]. Accordingly, a minimum of 384 samples were needed; however, 600 samples were gathered to ensure sufficient representation. At the time of collection, the physical condition, signs, and symptoms of the sampled birds were recorded. Fecal samples were collected aseptically from the cloacal region using cloacal swabs/sterile tubes, while for nasal, tracheal, and conjunctival samples, sterile cotton wool swabs containing phosphate buffer saline (PBS) were used to maintain the pH (Table 1).

Table 1: Sample Collection from Caged and Free-Living Avian Species

Type of Samples	Area	Caged/ Commercial Exotic Birds	Free-Living Birds	Total
Fecal, Cloacal, Conjunctival, Oropharyngeal/ Tracheal Swabs and Blood Samples	Hyderabad	50	25	75
	Thatta	50	25	75
	Badin	50	25	75
	Dadu	50	25	75
	Karachi	50	25	75
	Mirpur	50	25	75
	Bhimber	50	25	75
	Kotli	50	25	75
Total		400	200	600

Carefully, with the consent of owners, samples were taken from commercially sold healthy and sick pet avian species such as Budgies (Melopsittacus undulatus), Canaries (Serinus canaria domestica), Cockatiels (Nymphicus hollandicus), Crimson rosella (Platycercus elegans), Fisher (Agapornis fischeri), Lutino (Melopsittacus undulates), Pahari parakeet (Psittacula eupatria), Partridge (Rollulus rouloul), Pigeons (Columba livia domestica) and Quail (Synoicus ypsilophorus) for screening of fungal pathogens. During the collection of samples from pet birds, they were handled carefully to avoid causing harm or stress to the birds. The samples from free-living birds including backyard chicken (Aseel, Desi, Golden Missri and Sindhi), Bulbul (Pycnonotidae), Crow (Corvus), Dove (Columbidae), Duck (Bucephala albeola), Geese (Anser anser domesticus), Myna (Acridotheres tristi), Peacock (Pavo cristatus), Quail (Synoicus ypsilophorus) and Sparrow (Passer domesticus) were obtained using fog nets for about 09-12 hours/day in each area. The nets were inspected every hour for 06 consecutive days. The number of leaks losses and deaths in total were included. In addition, for catching free-living birds got services from experienced local bird predators. During the collection of samples from free-living birds, they were handled carefully to avoid causing harm or stress to the birds. The collected samples were brought in a cold chain container to the Department of Veterinary Microbiology, Sindh Agriculture University, Tandojam and transferred to the Veterinary Research Institute Peshawar, Khyber Pakhtunkhwa, for further processing. For surveillance of fungal infections, nasal, fecal, tracheal, and conjunctival swabs were screened by placing samples on Sabouraud dextrose agar (SDA) (Sigma-Aldrich), Czapek Dox agar (CDA) (Sigma-Aldrich) and Potato Dextrose Agar (PDA) (Sigma-Aldrich), using methods described by Pena *et al.*, [11]. Each anhydrous SDA 65gm/CDA 49gm/PDA 39gm were dissolved in 1000 ml of distilled water using magnetic

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stirrer MSH 300 (HVD, Life Science) prepared separately. The media was autoclaved at 121oC 15 lb/in^2 for 15 minutes. The media was cooled at room temperature and poured into Petri dishes. Following the sample (s) streaking/ cultured on Petri dishes and were incubated at 22oC for 24 hours. The cultural and colonial characteristics were observed for the occurrences of pathogens. Purification of culture was done by sub-culturing of typical well-separated colony on the corresponding medium. The process was repeated several times. The purity of the culture was checked by examining the stained smear. Smear was made from each type of colony, and identification of fungal species was performed based on morphological and lacto phenol cotton blue (LPCB) staining methods. The fungal growth and colony characteristics were observed and processed for further confirmation using biochemical analysis. The biochemical profiling including cycloheximide resistance, casein hydrolysis, fatty acid esterase activity and cellulose hydrolase was performed for the isolation and identification of fungal species in the free-living and caged bird samples. The PDA medium was prepared and inoculated with samples i.e. Control Plate: without cycloheximide and a test Plate: containing cycloheximide (concentration of 0.05% to 0.1%). Both plates were incubated at 25-30°C for 2-7 days. Growth was observed on the control plate but no growth on the test plate indicates cycloheximide-sensitivity. CDA media (concentration 15 mg/ml) inoculated with the samples and plates were incubated at 25–30°C for 14 days. The casein hydrolysis activity was determined by the formation of a clear zone around colonies. This proteolytic activity was an indicator (markedly clear zone) of the presence and differentiation of fungal pathogens. The agar plates were added 10 mM p-nitro phenyl acetate (p-NPA). The plates were inoculated with colonies and incubated at room temperature. The formation of a clear halo around the colony indicated high esterase activity, where the enzyme has broken down the p-NPA substrate. This activity could be noticed by the yellow-coloured p-nitro phenol product, as a halo around the fungal colony. Fungal samples were inoculated on the carboxy-methyl cellulose (CMC) agar plates. The plates were flooded with 0.1 (W/V) Congo red solutions and incubated at 30°C for 48-56 hours. Cellular activity was observed by the formation of clear zones around the fungal colonies. Statistical analysis was performed using Statistical Package for Social Science (SPSS) commercial software packages (version 17). The chi-square test was applied among the datasets to know the significant difference in prevalence (%.). p-value (probability value) is a measure which helps determine the significance of results in a hypothesis test. A value of $p<0.05$ was considered significant. A statistically significant difference in prevalence rates between caged and free-living birds was indicated by a p-value<0.05. This suggested that true changes in infection rates across different bird groups were reflected in the variance in prevalence rate, which were not the result of chance. A non-significant difference in the prevalence rates between caged and free-living birds was indicated by a p-value>0.05. This implies that regional differences in infection rates were statistically insignificant and most likely the result of chance.

RESULTS

In this study, 600 samples were examined for detection of fungal infections; out of these screened samples, 25 samples were detected positive for fungal pathogens. The rate of prevalence was calculated as 4.16% of infections in caged and free-living birds. The fungal pathogens were diagnosed positive in 17 (4.53%) and 08 (3.55%) samples in caged and free-living birds respectively (Table 2).

Table 2: Overall Prevalence of Fungal Infections in Caged and Free-Living Birds

Category	Samples Examined	Positive Samples	Frequency (%)
Caged Birds	400	17	4.53%
Free-Living Birds	200	08	3.55%
Total	600	25	4.16%

Data analyses indicated that 17 samples were recorded positive for fungal pathogens in caged birds. Among the analyzed samples obtained caged birds from Badin (n=2/50), Hyderabad (n=3/50), and Karachi (n=5/50) districts were detected positive for fungal organisms in Sindh province. The prevalence rate was calculated as 6.00%, 4.00% and 10.00% in districts Hyderabad, Badin and Karachi, respectively. The samples screened from Azad Jammu Kashmir; Bhimber (n=1) and Kotli (n=3) showed the presence of the pathogens. The prevalence rate was calculated as 2.00% and 6.00% in districts Bhimber and Kotli, respectively. A non-significant (p>0.8787) difference was observed in area-wise detection of fungal infections in caged birds (Table 3).

Table 3: Area-wise Detection of Fungal Infections in Caged Birds

Locations		Number of Samples Examined	No. of Positive Fungal Samples	Frequency (%)	X^2 (p-value)
Sindh	Hyderabad	50	3	6.00%	0.8787
	Thatta	50	2	4.00%	
	Badin	50	2	4.00%	
	Dadu	50	1	2.00%	
	Karachi	50	4	8.00%	
AJK	Mirpur	50	1	2.00%	
	Bhimber	50	2	4.00%	
	Kotli	50	2	4.00%	
Total		400	17	-	

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Data indicated that the samples (n=8) were diagnosed positive for fungal organisms in free-living birds. Among these, samples from Thatta (n=3), Badin (n=1) and Karachi (n=2) districts showed the presence of the pathogens. The samples from Bhimber (n=2) and Kotli (n=1) districts of Azad Jammu Kashmir had confirmed fungal species. The prevalence rate of the pathogens in birds was calculated as 8.00% and 4.00% in districts Bhimber and Kotli. A non-significant (p>0.5659) difference was observed in the area-wise detection of fungal infections in free-living birds (Table 4).

Table 4: Area-wise Detection of Fungal Infections in Free-living Birds

Locations		Number of Samples Examined	No. of Positive Fungal Samples	Frequency (%)	X^2 (p-value)
Sindh	Hyderabad	25	2	8.00%	0.5659
	Thatta	25	1	4.00%	
	Badin	25	0	0%	
	Dadu	25	0	0%	
	Karachi	25	2	8.00%	
AJK	Mirpur	25	0	0%	
	Bhimber	25	1	4.00%	
	Kotli	25	2	8.00%	
Total		200	8	-	

Four fungal species were identified from caged and free-living birds sampled. Aspergillus fumigatus (10), Candida albicans (07), Cryptococcus neoformans (06) and Macrorhabdus ornithogaster (02) were identified in the samples obtained from avian species. The highest prevalence rate (40.00%) was recorded for Aspergillus fumigatus followed by Candida albicans (28.00%), Cryptococcus neoformans (24.00%) and Macrorhabdus ornithogaster (8.00%). Statistically, the difference in the prevalence rate of fungal organisms detected in caged and free-living birds was significant (p<0.0017)(Table 5).

Table 5: Fungal Species Detected in the Samples of Caged and Free-living Birds

Infected Avian Species	Fungal Organisms	Pathogen Occurrence	Frequency (%)	X^2 (p-value)
Quail, Canaries, Cockatiels, Budgies, Pahari parakeets, Pigeons, Myna, Duck, Geese, Backyard chicken	Aspergillus fumigatus	10	40.00%	0.0017
Quail, Cockatiels, Lutino, Backyard chicken, Pahari parakeet	Candida albicans	07	28.00%	
Canaries, Pahari Parakeets, Pigeons, Dove	Cryptococcus neoformans	06	24.00%	
Cockatiels, Budgies	Macrorhabdus ornithogaster	02	8.00%	
Total		25	--	

Data analyses indicated that the susceptibility rate of fungal infections was higher in cockatiels and Pahari parakeets (23.52%) compared to pigeons (17.64%), canaries (11.76%), budgies (11.76%), quail (5.88%) and lutino (5.88%). Statistically, the difference in susceptibility rate of fungal infections in different caged birds in different regions of local commercial markets was non-significant (p>0.2174)(Table 6).

Table 6: Susceptibility rate of Fungal Infections in Different Caged Birds in various Regions of Local Commercial Markets

Caged Birds	No. of Positive Fungal Samples	Susceptibility Frequency (%)	X^2 (p-value)
Quail (Synoicus Ypsilophorus)	Aspergillus Fumigatus (1)	5.88%	0.2174
Canaries (Serinus Canaria Domestica)	Cryptococcus Neoformans (1) Aspergillus Fumigatus (1)	11.76%	
Cockatiels (Nymphicus Hollandicus)	Aspergillus Fumigatus (1) Candida Albicans (1) Macrorhabdus Ornithogaster (1) Candida Albicans (1)	23.52%	
Budgies (Melopsittacus Undulatus)	Aspergillus Fumigatus (1) Macrorhabdus Arnithogaster (1)	11.76%	
Pahari Parakeet (Psittacula Eupatria)	Aspergillus Fumigatus (1) Cryptococcus Neoformans (2) Candida Albicans (1)	23.52%	
Partridge *(Rollulus Rouloul)*	0	0%	
Fisher (Agapornis Fischeri)	0	0%	
Pigeons (Columba Livia Domestica)	Aspergillus Fumigatus (1) Cryptococcus Neoformans (2)	17.64%	
Lutino (Melopsittacus Undulates)	Candida Albicans (1)	5.88%	
Crimson Rosella (Platycercus Elegans)	0	0%	-
Total	17	-	-

Statistically, the difference in susceptibility rate of fungal infections in different caged birds in different regions of local commercial markets was non-significant (p>0.05). The susceptibility rate of fungal infections was higher in Backyard chicken (37.50%) compared to duck (25.00%), dove (12.50%), myna (12.50%) and geese (12.50%). Statistically, the difference in susceptibility rate of fungal infections in different free-living birds in different regions of local commercial markets was non-significant (p>0.1791) (Table 7).

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Table 7: Susceptibility rate of Fungal Infections in Free Living Birds in various Regions

Free-Living Birds	No. of Positive Fungal Samples	Susceptibility Frequency (%)	X^2 (p-value)
Quail (Synoicus Ypsilophorus)	0	0%	0.1791
Dove (Columbidae)	Cryptococcus Neoformans (1)	12.50%	
Bulbul (Pycnonotidae)	0	0%	
Crow (Corvus)	0	0%	
Sparrow (Passer Domesticus)	0	0%	
Myna (Acridotheres Tristi)	Aspergillus Fumigatus (1)	12.50%	
Peacock (Pavo Cristatus)	0	0%	
Duck (Bucephala Albeola)	Aspergillus Fumigatus (1) Candida Albicans (1)	25.00%	
Geese (Anser Anser Domesticus)	Aspergillus Fumigatus (1)	12.50%	
Backyard Chicken	Aspergillus Fumigatus (1) Candida Albicans (2)	37.50%	
Total	8	-	-

In this study, the most common risk factors associated with fungal infections in different caged and free-living birds were determined based on external environmental conditions. In the case of caged birds; housing, husbandry, humidity control, feed and water, quality and human interaction at commercial places were determined risks associated with the rate of fungal pathogens. Free-living birds may have differences in infection rate due to variations in external environmental factors including humidity and elevated temperature in Sindh Province, and heavy snowfall in Jammu and Azad Kashmir, as well as, individual conditions such as exhaustion, weakened immunity and exposure to contaminated feed residues.

DISCUSSION

The prevalence of fungal infections in the present study was estimated as 4.16% in caged and free-living avian species. Our findings are from a previous study A. fumigatus was detected in 30% of house sparrows [12] and 6-13% in captured pink-footed geese, Canada geese or herring gulls that presented as healthy carriers [13]. It has been demonstrated that the proportion of free-living birds in wild passerines was around 2.9% [14]. Discrepancies in prevalence rate are associated with study design, diagnostic methodology, sample size, environmental conditions, management approaches, and cage hygiene [15]. Several factors such as poor ventilation, high density and limited space in caged birds may pose higher infection rates. On the other hand, free-living birds may face a variety of challenges including exposure to naturally occurring fungal spores and environmental conditions that might alter infection rates [16]. In current research, four fungal species were identified from caged and free-living birds. Among these, the most prevalent was Aspergillus fumigatus followed by Candida albicans, Cryptococcus neoformans and Macrorhabdus ornithogaster. Findings are consistent with studies, which stated that Aspergillus fumigatus was a more prevalent pathogen in farmed and wild birds. Avian respiratory illnesses are mostly caused by Aspergillus fumigatus as reported by [17, 18]. According previous study demonstrated that occurrences of Candida albicans in mucosal surfaces and avian guts may increase in response to immunosuppression or stress conditions of birds [19]. Similarly, it has been observed that C. neoformans incidence was higher in several bird species [20]. The discrepancy might be explained by differences in specific bird populations studied, environmental variables, and geographical locations. Ornithogaster is a substantial pathogen, and its frequency is typically lower than that of other widely dispersed fungi such as A. fumigates [21]. In caged birds, the susceptibility to fungal infections was recorded higher in Cockatiels and Pahari parakeets than pigeons, canaries, budgies, quail and lutino. Similarly, in free-living birds, susceptibility to fungal infections was exhibited higher in Backyard chickens than ducks, doves, mynas and geese. The findings of this study are consistent with previous research that observed that pigeons are more prone to become infected due to their high density and continual interaction with secondary infections [22]. Cockatiels and Pahari parakeets are sensitive to stress and environmental conditions, which make them more prone to fungal infections [23, 24]. According to a former study, fungal infection susceptibility in many birds depends upon factors such as genetic predisposition, environment and diet [25]. Some bird species are more susceptible to infections due to factors such as close quarters, immune system differences, and environmental stress. Our results are supported by previous studies, which reported lower rates of fungal infections in canaries and other similar species [26]. Previous research studies demonstrated that quail and duck habitat conditions render them more susceptible to fungal infections [27, 28]. A prevalence of 1.7 to 3.1% was detected in grey herons, mallards and coot birds living in Guadalquivir marshes [29]. Aspergillus was detected in 9–50% and 27–31% of nocturnal heron chicks [30]. This fungal disease was recognized as the primary cause of death for 6 to 23% of common loons [31]. Aspergillosis was diagnosed in 31% of necropsied birds, mostly herring gulls [32].

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CONCLUSIONS

It was concluded that the fungal pathogens were detected in free-living and caged bird samples obtained from Sindh Province and Azad Jammu and Kashmir, Pakistan. Aspergillus fumigatus, Candida albicans, Cryptococcus neoformans and Macrorhabdus ornithogaster were detected in avian species. The variation in infection rates among bird types and locations impacts living and environmental conditions on fungal susceptibility. The research is valuable for the prevalence and distribution of fungal pathogens in avian species, emphasizing the mode of infection rates and environmental factors variation. It offers significant insights into fungal infections in birds and contributes to developing infection management and environmental well-being strategies.

Authors Contribution

Conceptualization: SHA, RA
Methodology: AMUD, MS
Formal analysis: AMUD, SHA, DHK, RA
Writing review and editing: AMUD, SHA, DHK, MS, RA

All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest

All the authors declare no conflict of interest.

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