Elhariri et al., J Vet Sci Med Diagn 2015, 4:4
http://dx.doi.org/10.4172/2325-9590.1000168

Journal of Veterinary Science & Medical Diagnosis

Research Article

A SCITECHNOL JOURNAL

Lovebirds and Cockatiels Risk Reservoir of *Cryptococcus neoformans*, a Potential Hazard to Human Health

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Abstract

Lovebirds and cockatiels are potential carriers and/or transmitters of zoonotic diseases. Some of them could have an important impact for human health, In Egypt, the role of these birds in dispersing *C. neoformans* is not well documented, which evoked a high need to investigate the environmental ecology of this fungus in order to establish surveillance programs and applying the preventive measures for this pathogen infection. The *C. neoformans* prevalence and role of pet birds in spreading this fungal pathogen in Egypt was illustrated in this study. Two hundred Cockatiels and lovebirds excreta were collected from captive birds. The recovered isolates of *C. neoformans* species were identified by molecular identification using capsular gene specific primer CAP64. The subtyping of isolates was performed by multiplex PCR using CNa-70S/A -CNa-49S/A. Four isolates (3 and 1) from lovebirds & Cockatiels respectively were subjected to sequence analysis of Internal Transcribed Spacer (ITS) regions. Alternatively the fungal isolates were analyzed by PCR fingerprinting with uniplex PCR amplification using an oligonucleotide $(GTC)_5$. From this study it was concluded that, the excreta of these birds can play a role as a risk reservoir of *C. neoformans* in domestic and public environments and enhance their zoonotic importance to human.

Keywords

C. neoformans; Egypt; Cockatiels; Lovebirds

Introduction

The basidiomycetes yeasts of genus *Cryptococcus* include *C. neoformans/C. gattii* species complex, which is composed of two separate species *C. neoformans* and *C. deneoformans*, and five species within *C. gattii*. The most important pathogenic species *C. neoformans* and *C. gattii* show different geographical distributions. *C. neoformans* is globally distributed and has been recovered from various natural sources and with high incidence particularly in a wide variety of bird droppings, trees and soils [1].

In Africa, the isolation of 19,753 *C. neoformans* and *C. gattii* strains were reported from 25 of the 58 African countries and mainly in South Africa (79%). Environmental surveys, carried out in eight African countries (Tunisia, Egypt, Nigeria, Democratic Republic of Congo, Burundi, Zimbabwe, Botswana, and South Africa) revealed that these pathogens represented 1% of the total reported isolates in environment [2].

The most common isolate responsible for Cryptococcal infection is *C. neoformans* [3,4]. It causes mainly opportunistic infections in immunocompromised patients with underlying conditions, such as HIV, leukemia, and other cancers, or in those taking corticosteroid medications [5].

C. neoformans is primarily associated with nests and soils containing avian droppings, especially those of pigeons [1,6]. Allover, the bird guano may represent the main ecological niche for *C. neoformans* [7]. Many reports illustrated the role of the captive birds in promoting and disseminating the contamination of surrounding and public areas by Cryptococcus species [8,9]. It is suggested that the hosts acquired Cryptococcal infection via inhalation of contaminated air or excreta to the lungs with the potential to disseminate to the central nervous system and/or to other distant tissues [10].

Pet birds are bought individually or in couples, as families often do, which is a profitable business for pet shops or local breeders for their very high genetic or exotic value, these birds, commonly cockatiels and lovebirds, which are regularly sold at high prices. These birds are potential reservoir of fungal zoonotic diseases mainly Cryptococcal infection.

C. neoformans has an autochthonous environmental niche, is likely to be the source for outbreaks among various animals and humans. The worldwide increasing number of published studies for the ecology and health significance of *C. neoformans* isolates infection in human and animals. On the other hand, in Egypt there is scare information about the health significance of this fungal pathogen.

Therefore, this study was undertaken to investigate, the existence of *C. neoformans* in cockatiels and lovebirds in Egypt, depending on the *CAP64* gene molecular detection and biotyping by CNa-70S/A - CNa-49S/A. In addition, we applied the sequence analysis of the *ITS*2 region of selected isolates and fingerprinting using $(GTC)_5$ to determine the presence of *C. neoformans* from these types of bird excreta and provide insight into the environmental epidemiological life cycle of *C. neoformans* in Egypt.

Materials and Methods

Sample collection

A total 200 samples of cockatiels and lovebirds excreta was collected in clean paper envelopes from different pet bird houses and transferred directly to the laboratory. One gram from each sample was suspended in a sterilized glass bottle containing 99.0 ml of sterile physiological saline (0.85% NaCl) supplemented with chloramphenicol (10.0 mg/ml). The mixture was left at room temperature for about 10-15 min to complete dissolving of dropping, and then shaken vigorously for 4-5 min. The bottle was left for complete precipitation [11].

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Received: June 08, 2015 **Accepted:** July 23, 2015 **Published:** July 28, 2015

Isolation and phenotypic identifications

From the supernatant fluid of each prepared sample, a loopful was streaked onto plates of Sabouraud dextrose agar with chloramphenicol and incubated at 30°C for 48 hours. *C. neoformans* species were phenotypically identified by their color and microscopic morphology on Sabouraud dextrose agar (Oxoid CM41) and *Eucalyptus* leaves agar media [12]. The isolates were identified by classical mycological procedures of *C. neoformans* [6].

DNA isolation and molecular identification by capsular gene

The chromosomal DNA was isolated from *C. neoformans* isolates according to [13]. The molecular confirmatory identification of *C. neoformans* was done by using specific capsular gene primers *CAP64*. The primers for *CAP64* were designed on the basis of DNA sequences according to [14].

Molecular subtyping of *Cryptococcus neoformans* isolates

All identified isolates by *CAP64* gene were subtyped by multiplex PCR primer pairs as recommended by [15]. The two PCR primer pairs, which are specific for *C. neoformans* serotype A were used to amplify the 695-bp fragment CNa-70S (5'-ATTGCGTCCACCAAGGAGCTC-3') and CNa-70A (5'-ATTGCGTCCATGTTACG TGGC-3'). The other set of primer for serotype B is CNb-49S (5'-ATTGCGTCCAAGGTGTTGTTG-3') and CNb-49A (5' ATTGCGTCCATCCA ACCGTTATC-3'), which produce amplicon of 448-bp.

Sequencing of the ribosomal internal transcribed spacer regions

ITS1 and ITS2 regions DNA were amplified with 900nM primer ITS1 (5`-TCCGTAGGTGAACCTGCG-3`), 300nM primer ITS4 (5`-TCCTCCGCTTATTGATATGC-3`) [16]. The PCR amplification reaction was performed with a DreamTaq PCR Master Mix (2X) (Thermo, Fermentas), according to company instructions. The amplification parameters: were 95°C for 6 min, followed by 30 cycles at 95°C for 30s, 55°C for 30s, and 72°C for 30s, followed by one final extension at 72°C for 10 min. A MiniPro PCR thermal cycler (SWIFT, ESCO) was used. Negative control reactions without any template DNA were carried out simultaneously. Gel electrophoresis with 1.5% agarose gels was conducted with 1xTBE buffer (0.1 M Tris, 0.09 M boric acid, 1 mM EDTA) at 4.8 V/cm for 2 h. A 100-bp DNA ladder (JENA BioScience, Germany) was run concurrently with amplicons for sizing of the bands. Gels were stained with ethidium bromide-TBE solution for 20 min and the obtained bands were visualized using UV-trans-illuminator and photographed by a digital camera (Cleaver).

Internal Transcribed Spacer (ITS) sequence analysis

Bio edit software was used to obtain consensus sequences from aligned forward and reverse sequence reads. The obtained ITS sequences were analyzed for homology between the nucleotide sequences of the detected *C. neoformans* strains and other strains published on Gen Bank, which was done using BLAST 2.0 search programs (National Center for Biotechnology Information website. Species and genus identification were presumed for fungal isolates with scores designated in the BLAST search and The Barcode of Life Database (BOLD) [17,18].

Molecular genotyping using micro-satellite $(GTG)_5$ specific primers

PCR fingerprinting using the microsatellite $(GTG)_5$ was done by a modification of the method described [19]. PCR was performed with volumes of 50 µl containing 10 to 25ng of genomic DNA, 20 to 30ng of primer, Under the recommended buffer conditions, the PCR was performed as follows: 40 cycles of denaturation at 93°C for 20s, annealing at 50°C for 30 s, extension 72°C for 20 s and final extension 72°C for 6 min. Amplification products were analyzed by electrophoresis in 2.5 % agarose gels run in 1x TBE buffer and detected by staining with ethidium bromide under UV-trans-illuminator and photographed by a gel documentation (Cleaver microDoc). Electrophoretic bands were sized and compared with a scanner and gel image analysis software.

Results

A total of 15 (7.5%) isolates of *C. neoformans* were obtained from the 200 examined samples. Among these, 12 isolates were recovered from 143 lovebirds (8.3%) and 3 from 57 cockatiels (5.2%) (Tables 1 and 2).

As shown in Table 3, all the recovered Cryptococcus isolates were identified as *C. neoformans* strains based on all conventional and physiological characters of *C. neoformans*. Moreover, all tested isolates were positive by PCR for *CAP64* the specific capsular gene. All the 15 positive isolates were produced a 695-bp amplicon with CNa-70S/A primer pair, while no products were produced with CNa-49S/A (Figure 1).

However, the molecular subtyping of *C. neoformans* isolates was done by the sequencing of ITS region of four selected isolates representing the four major bird species in this study (Table 4). The results were compared with known sequences using the BLAST program and The Barcode of Life Database (BOLD). Three isolates of

Table 1: Collected samples isolation sources from lovebirds and cookatiel in Egypt.

Isolation source	Birds species	Bird type	Samples number	Birds origin
Australian budgerier	*Melopisittacus andulatus*	lovebird	73	Australia
English budgerier	*Melopsittacus andulatus*	lovebird	16	Australia
The Lutino Cockatiel	*Nymphicus hollandicus*	Cockatiel	18	Australia
Normal Cocktail	*Nymphicus hollandicus*	Cockatiel	23	Australia
red-faced pied lovebird	*Agapornis pullarius*	lovebird	12	Africa
Fischer's lovebird	*Agapornis fischeri*	lovebird	11	Africa
Peach-faced pied Lovebird	*Agapornis roseicollis*	lovebird	10	Africa
Indian-ring necked	*Psittacula krameri*	lovebird	9	India
Zebra Finch	*Taeniopygia guttata*	lovebird	12	Indonesia
Pied Cockatiel	*Nymphicus hollandicus*	Cockatiel	12	Australia

Table 2: Number of *C. neoformans* isolates and their recovery rates from lovebird & cockatiel excreta.

Sample type	Number	Positive *C. neoformans*	Recovery rate
lovebird	143	12	8.3 %
Cockatiel	57	3	5.2 %
Total	200	15	7.5

Table 3: Molecular typing of *Cryptococcus neoformans* isolates from lovebirds & cockatiel excreta.

Sample code	Accession No.	Isolation source	*CAP64* gene by PCR	PCR Typing		$(GTC)_5$ Profile
				CNa70S/A	CNa49S/A	
EGYCr3	KJ767784	Australian budgerier	+	+	-	II
EGY7	----------	Australian budgerier	+	+	-	II
EGY4	----------	Australian budgerier	+	+	-	I
EGY8	----------	English budgerier	+	+	-	II
EGY61	----------	red-faced pied lovebird	+	+	-	II
EGYCr2	----------	red-faced pied lovebird	+	+	-	II
EGYCr5	KJ767783	Peach-faced pied Lovebird	+	+	-	I
EGY11	----------	Peach-faced pied Lovebird	+	+	-	I
EGY2	----------	Fischer's lovebird	+	+	-	I
EGYST	KJ767782	Indian-ring necked	+	+	-	II
EGYSPA	----------	Indian-ring necked	+	+	-	II
EGY9	----------	Zebra Finch	+	+	-	II
EGYCr1	----------	Pied Cockatiel	+	+	-	II
EGY62	KJ767785	The Latino Cockatiel	+	+	-	I
EGY4	----------	Normal Cocktail	+	+	-	II

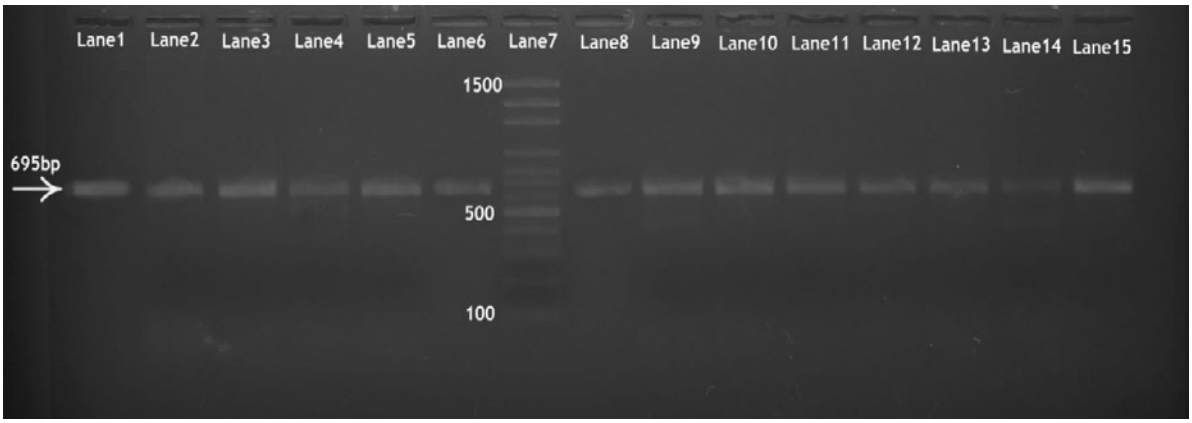

Figure 1: Agarose gel electropherosis of CNa70A/S and CNa49A/S specific PCR of all the examined *C. neoformans* isolates with production of amplicons of 695 bp for CNa70A/S, Marker 100 bp DNA ladder plus (JENA BIOSCIENCE).

Table 4: Distribution & recovery rate of *Cryptococcus neoformans* according bird's species.

Sample code	Accession No.	Isolation source	*C. neoformans* isolates number	Species		Isolation percent
				Name	Total No.	
EGYCr3	KJ767784	Australian budgerier	4	*Melopisittacus andulatus*	86	4.6 %
EGY7	----------	Australian budgerier				
EGY4	----------	Australian budgerier				
EGY8	----------	English budgerier				
EGY61	----------	red-faced pied lovebird	2	*Agapornis pullarius*	12	16.6 %
EGYCr2	----------	red-faced pied lovebird				
EGYCr5	KJ767783	Peach-faced pied Lovebird	2	*Agapornis roseicollis*	10	20 %
EGY11	----------	Peach-faced pied Lovebird				
EGY2	----------	Fischer's lovebird	1	*Agapornis fischeri*	*11*	*9 %*
EGYST	KJ767782	Indian-ring necked	2	*Psittacula krameri*	*9*	*22.2%*
EGYSPA	----------	Indian-ring necked				
EGY9	----------	Zebra Finch	1	*Taeniopygia guttata*	*12*	*8.3%*
EGYCr1	----------	Pied Cockatiel	3	*Nymphicus hollandicus*	*53*	*5.6%*
EGY62	KJ767785	The Lutino Cockatiel				
EGY4	----------	Normal Cocktail				

The sequenced *C. neoformans* strains were identified as *C. neoformans* (Serotype A) from *Melopisittacus andulatus*, *Agapornis roseicollis* and *Psittacula krameri*.

All isolates recovered from bird droppings belonged to serotype A. The comparison of ITS for the selected four strains (3 from lovebirds and 1 from cockatiel) revealed that 3 of the selected isolates

were *C. neoformans*, except one isolates from Zebra Finch, whose serotype could not be identified (EGYST- KJ767782) (Table 3).

Regarding, the prevalence of *C. neoformans* in different birds species, 10 of captive birds belonging to 8 species differed in the incidence rate of *C. neoformans* isolation according to bird species, where it was recovered at the rate of 22.2 % from *Psittacula krameri,* 20% from *Agapornis roseicollis*, 16.6% from *Agapornis pullarius,* 9% from *Agapornis fischeri* and 8.3% from *Taeniopygia guttata*. Whereas, the lower rates were recovered from *Nymphicus hollandicus* (5.6%) and *Melopisittacus andulatus* (4.6%) (Table 4).

On the other hand, the oligonucleotide primer $(GTG)_5$ was used to amplify the variable DNA fragments yielded from all the tested strains of *C. neoformans*. The fingerprint patterns by $(GTG)_5$ revealed a two different banding profile in between different strains and even this variation detected belonged to the same serotype A. $(GTG)_5$ produced amplicons with variable size ranged from 580 to 1131 bp for banding profile (I) (2). In case of banding profile (II) the PCR product varied between 587 to 1904 bp as shown in Table 5. Generally, all tested strains in the banding profile (I), were produced four bands and banding profile (II) were yielded five bands (Figure 2).

Discussion

The yeast of *C. neoformans* is a highly potential basidiomycete fungal pathogen for humans and animals health. The major route of acquisition of this fungus is via inhalation of infective basidiospores. Environment plays a major role in dispersing the infection with *C. neoformans* in human and animal surroundings. The global isolates which are responsible for Cryptococcus infection are identifies as *C. neoformans* that is commonly recovered from pigeon droppings, soil and decaying wood in hollow trees [1,3,4,20].

Table 5: The sizes of diagnostic bands for the two major banding profiles produced by $(GTG)_5$.

No. of major bands	Accession No. of four tested strains			
	KJ767782	KJ767783	KJ767784	KJ767785
1st Band	580	613	597	604
2nd Band	786	820	800	807
3rd Band*	890	933	940	920
4th Band*	1075	1131	1125	1113
5th Band			1915	1904
Banding profile	Banding profile (I)		Banding profile (II)	

Pigeons are known to be reservoirs of pathogenic yeasts, like *C. neoformans*, which is described to cause opportunistic infections in humans [21]. In Egypt, only one study focused on the role of pigeons, canaries and parrots in spreading this fungal pathogen in the environment via bird droppings and it recorded the prevalence of *C. neoformans* in bird droppings about 2.5% [22].

Role of captive and free-ranging birds as carriers and spreaders of potential pathogens for human beings is very critical, which contributes to environmental contamination and possibly to human and animal infection [23-25].

Unfortunately, in Egypt most studies on Cryptococcal infection did not correlate between environmental and clinical cases isolates. The first trial to isolate *C. neoformans* from the avian was done in Lower Egypt; *C. neoformans* was recovered by 15% of samples examined [26]. In other study, four cases of Cryptococcal meningitis in Egyptian patients were clinically diagnosed without any information about the subtype and/or variety of causative Cryptococcal agent [27].

On the other hand, with the beginning of the last century, the studies on fungal meningitis, received more attention and at 2003, a great improvement in the epidemiological data about Cryptococcus infection in Egypt, and laboratory diagnosis of Cryptococcal meningitis illustrating the role of *C. neoformans* in such infection [28-30].

In this study, 12 *C. neoformans* isolates were recovered from 143 (8.3%) Lovebirds samples and 3 isolates out of 57 (5.2%), the total of pet bird droppings yielded 15 *C. neoformans* isolates with a prevalence of 7.5% (Table 1 & 2). Also, the percentage of caged bird excreta samples which contained *C. neoformans* was comparatively lower than those detected in other study on birds with recent environmental sources associated [31,32]. Whereas our finding were in higher rate of incidence in comparison to previous work at the same area [22].

Currently, it was found that the number of *C. neoformans* positive samples were significantly recovered at higher rate from lovebirds than from cockatiel birds (Table 1). Australian or English budgerier (*Melopisittacus andulatus*) excreta had a large number of positive samples but not at significantly higher percent of recovery (4.6%) (Table 4). It is suggested that the uneven number of samples collected from lovebirds and cockatiels birds may explain the differences in number of positive samples from each order. The number of samples

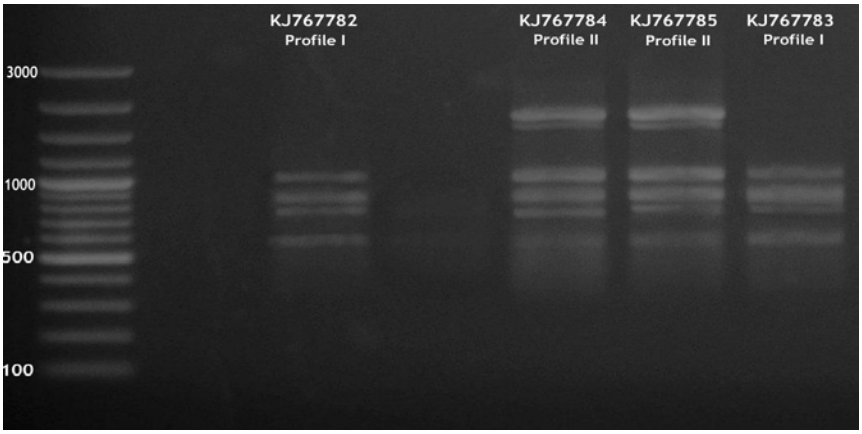

Figure 2: Micro-satellite PCR banding fingerprint using the single primer (GTG)5 KJ767782 & KJ767783 represent the banding profile (I) . KJ767784 & KJ767785 are representing the profile (II), Marker Solis BioDyne 100-3000 bp.

collected from each species was influenced by the number and types of bird's availability in pet shops.

All the recovered Cryptococcus isolates were identified as *C. neoformans* strains based on all conventional and physiological characters of *C. neoformans*. Moreover, all tested isolates were positive by PCR for *CAP64* the specific capsular gene. Molecular subtyping of *C. neoformans* isolates was done by two methods the CNa70A/S and CNa49A/S primer pair in multiplex PCR to determine the C. neoformans types (Figure 1). ITS region sequencing of selected four strains and comparing the results with known sequences using the BLAST program and the Barcode of Life Database (BOLD). ITS regions are widely used in taxonomy and molecular phylogeny because they are highly conserved region, what bring out the possibility of detecting and identifying fungi [16,33].

All examined *C. neoformans* strains were identified as *C. neoformans* serotype A according to CNa70A/S primer as shown in Table 3. These findings are similar to most of previous studies on molecular typing on this pathogen. *C. neoformans* the most dominant and prevalent worldwide [3,4,19,20] .

In Egypt, most of studies at beginning of 2003 investigated the major subtypes of *C. neoformans* either from environmental (Plant or Bird origin) or a clinical sample (Veterinary or human) is *C. neoformans* (Serotype A). [28,30,34,35]

It seems that the main reason for the high prevalence of *C. neoformans* in Egypt is attributed to its thermo-tolerance ability, the weather in Egypt is considered as temperate to hot climate, which may be reflected on isolation rate and recovery of other serovars of *C. neoformans*.

PCR fingerprinting by the microsatellite-specific sequences $(GTG)_5$ was used globally as single primer in the PCR for its discriminatory index to differentiate among unrelated isolates [19,36]. Despite the high discriminatory power, which associated with $(GTG)_5$ primer, the analysis of the PCR patterns grouped all *C. neoformans* into two major molecular types only. It is common the $(GTG)_5$ oligoprimer is characterized by production of few bands (6 to 17) and to achieve reproducible fixed results with this oligoprimer, each sample was repeated triplicate with standardised conditioning in reagents and thermal cycling conditions [19].

It appears from Table 5 that all the examined *C. neoformans* produced a conserved banding in the 3rd and 4th bands, which give a characterized profile at range 890 to 1131bp. These data confirmed that, the major *C. neoformans* genotype in Egypt form different bird species is completely related even, is related to genotype (I) or (II).

Most of captive pet birds in Egypt are imported from different regions all over the world either from Australia, Africa and Asia (Table 2). This fact reflects on the potential risk of international trade of such birds species without international regulatory measures. Boseret and coworker mentioned on this point, exotic birds like greater psittaci forms (parrots, e.g. ara or cockatoo), legally or illegally traded from for example Asia or South America, remain high in the ranking of popular pets and are also profusely represented in zoos and parks [37].

Therefore , the increasing popularity of lovebirds & cockatiels as a pet bird, and the close relationship between human beings and their pets, which may help in exposure of people to potential pathogens that may belong to the normal microbiota of these birds [38]. The greater risk of this disease was detected in children, elderly people and immuno compromised individuals after exposure to pathogenic yeasts [24].

In conclusion, this study gave a highlight and awareness about the role of pet birds as risk reservoirs for disseminating the potentially pathogenic *C. neoformans* in the environment. Moreover, the pet birds may cause a hazard to human health, particularly to immune compromised patients, children and the elderly. Therefore, further studies are urgently needed to clarify the diagnosis of such infection and its recent treatments. Current periodical examination of environmental factors related to birds as water, excreta and air must be advised to devoid the zoonosis hazard infection to human health.

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