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Prevalence study on avian chlamydiosis, in and around Mumbai by polymerase chain reaction

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Abstract
The research work was carried out by examining swabs and/or droppings in 235 birds (belonging to 12 orders and 56 species) from the pet birds, birds were presented to Teaching Veterinary Clinical Complex, Mumbai Veterinary College, Affiliated Bai Sakarbai Dinshaw Petit Hospital for Animals, private veterinary clinics, pet shops and private aviaries in and around Mumbai. All samples were tested for *Chlamydia* spp. (Genus specific) and *Chlamydia* psittaci (Species specific) by Polymerase Chain Reaction, 7 birds (n=7/235) were tested positive for *Chlamydia* spp. but did not show positive result for *Chlamydia* psittaci. Percent prevalence of *Chlamydia* spp. was found to be 2.97% (7/235). The positive cases detected in this study were from the aviaries around Mumbai (n=7). Percent prevalence of positive cases around Mumbai is 23.33% (7/30). All the positive cases in the present study belong to Captive birds category and were adults birds. Samples tested positive for *Chlamydia* spp. where from fecal samples (n=57). 183 birds were symptomatic and 52 birds were apparently healthy from this, 2 asymptomatic birds and 5 symptomatic birds were positive for *Chlamydia* spp.

Keywords: *Chlamydia psittaci*, *Chlamydia* spp. polymerase chain reaction, birds

Introduction
Avian Chlamydiosis commonly known as Psittacosis is one of the zoonotically important disease in birds caused by a Gram-negative, obligate intracellular bacterium *Chlamydia* psittaci. The family comprises a single genus, *Chlamydia* which further comprises 11 species namely *C. abortus*, *C. avium*, *C. caviae*, *C. felis*, *C. gallinacea*, *C. muridarum*, *C. pecorum*, *C. pneumoniae*, *C. psittaci*, *C. suis*, and *C. trachomatis* (Parut *et al*. 2019) [10]. *Chlamydia* psittaci infections have been demonstrated in at least 465 different bird species, spanning 30 different bird orders (Kaleta and Taday 2003) [4] most commonly in psittacine birds.
The importance of clinical examination in birds is all about understanding the masking phenomenon where birds hide or mask signs of illness from potential predators. Symptoms of psittacosis may vary from inapparent to severe, depending on the strain, stress, age, and health status of the host. The symptoms may include rhinitis, conjunctivitis, nasal discharge, dyspnoea, diarrhea, polyuria, anorexia, lethargy, and dullness (Vanrompay *et al*. 1995) [15]. *Chlamydia* psittaci is excreted in the feces and nasal discharges of infected birds. Shedding can be exacerbated by stress factors, including reproductive activities, rearing of young, relocation, shipping, crowding, and chilling (Smith 2011) [14].
With currently available tests, pet stores can fail to identify birds infected with C. psittaci that are asymptomatic prior to sale. PCR is very sensitive and specific for detection of target DNA sequences and relies only on the presence of the organism in the specimen. A number of reports on the use of PCR techniques to detect Cp. psittaci have appeared in the literature (Hewinson *et al*., 1991, 1997; Messmer *et al*., 1997; Moroney *et al*., 1998; Olsen *et al*., 1998; Everett *et al*., 1999; McElnea and Cross, 1999) [2, 3, 7, 8, 9, 1, 6]. These tests are reported as able to detect Cp. psittaci DNA in samples of tissues, faeces and choanal and cloacal swabs and are sensitive and rapid.
Psittacosis often goes unrecognized because of the lack of distinctive symptoms and clinical suspicion. Avian Chlamydiosis is very important in avian medicine as it is a zoonotic disease and infected birds do not show any specific clinical signs. (Razmyar *et al*. 2016) [11].

Materials and Methods
Sample collection
The study was conducted in and around Mumbai. Different varieties of birds were included in the study, which may or may not have shown clinical signs and were screened for the main

etiological agent's *Chlamydia* spp (genus-specific) and *Chlamydia* psittaci (Species Specific). Anamnesis was recorded and follow-up was conducted at the places where the cases were presented. Information from owners, aviculturists, pet clinics, pet shops, and private aviaries irrespective of place, species, breed, age, sex, and health status with detail information about the birds and owners were collected.

Sample collection

A total of 235 birds included in the study were divided into 2 groups-psittaciformes (n=169) and non-psittaciformes (n=66). Further order psittaciformes was divided into 3 families-Psittaculidae (n=63), Cacatuidae (n=22) and Psittacidae (n=54). All other different orders were grouped under non-psittaciformes. Non-psittaciformes included eleven orders namely Pelecaniformes (n=6), Columbiformes (n=10), Galliformes (n=10), Strigiformes (n=1), Passeriformes (n=24), Cuculiformes (n=2), Falconiformes (n=1), Piciformes (n=1), Accipitriformes (n=9), Charadriiformes (n=1) and Anseriformes (n=1).

After restraining the bird, the sample was collected with minimum stress to the bird. Approximately 1-5gm of fresh fecal samples were aseptically collected in the small container with a sterile syringe and swabs from 3 sites (conjunctival + choanal + cloacal respectively) or only cloacal or only choanal according to bird size and health status were collected (Figure 1 & 2) and then kept in the refrigerator (4 °C) until DNA extraction.

Extraction and purification of DNA

For the extraction of DNA from faecal samples the DNA Mini kit manufactured by QIAGEN was used (Sareyyupoglu *et al*. 2007) [13]. DNA from swab was extracted using Tissue Genomic DNA Extraction Mini Kit manufactured by Favorgen (Mahmoudi *et al*. 2019) [5]. Recombinant Escherichia coli clones expressing *Chlamydia* psittaci gene culture were obtained from Hyderabad (ICAR- National Research Centre on Meat) and used as a positive control for standardization of Polymerase chain reaction.

Oligonucleotide primers

CPS and CPS_0429 primers were used in this study (Sahu *et al*., 2001) [12]. The forward and reverse primer sequences for the study is given in Table 1.

Quantification of DNA

A Nanodrop Spectrophotometer (Thermo Scientific, USA) was used to assess the purity and quantity of the extracted DNA in the Department of Animal Genetics and Breeding. 1 µl of extracted DNA was loaded into the spectrophotometer for measuring purity and concentration at 260 nm and 280nm. The quantified samples were then stored at -20 ºC until used for PCR.

Reconstitution of the primers

The lyophilized primers were reconstituted in the nuclease-free water to make a stock solution of 100 pmol/µl concentration. The reconstituted primers were stored at -20 °C. Working solution with a concentration of 10 pmol/µl was prepared using sterile nuclease-free water. Reconstituted primers (stock and working solutions) were stored at -20 °C for further use.

Table 1: Primers used for the amplification of genes coding

Organism	Primer sequence 5'-3'	Product size (bp)
*Chlamydia*ceae Family (CPS)	5' GCGTGTAAGGTTTAGATTCTTTACT 3' 5' AGCGGCCATACTACTTGCTAT 3'	167
Chlamydia psittaci (CPS_0429)	5' TGTGACATCATCAAGACTG 3' 5' GATGACAACTTCTATACCTG 3'	439

Table 2: Components of PCR reaction mixture for *Chlamydia* spp.

Sr. No	Reagent	Quantity
1	10X PCR buffer	2.5 µl
2	10mM dNTP	0.5 µl
3	25mM MgCl2	1 µl
4	CPSF	1 µl
5	CPS_0429R	1 µl
6	5U Taq Polymerase	0.2 µl
7	DNA	1 µl
8	Adjust to 25 µl using molecular grade water	

Table 3: Optimization of PCR for *Chlamydia* spp.

Cycling Condition			Cycles
Initial denaturation	95 °C	5min	1
Denaturation	95 °C	30sec	35
Annealing	55 °C	30sec	
Extension	72 °C	30sec	
Final Extension	72 °C	1min	1

Table 4: Components of PCR reaction mixture *Chlamydia* psittaci

Sr. No	Reagent	Quantity
1	10X PCR buffer	2.5 µl
2	10mM dNTP	1 µl
3	25mM MgCl2	1 µl
4	CPS_0429F	1 µl
5	CPS_0429R	1 µl
6	5U Taq Polymerase	0.2 µl
7	DNA	1 µl
8	Adjust to 25 µl using molecular grade water	

Table 5: Optimization of PCR for *Chlamydia* psittaci

Cycling Condition			Cycles
Initial denaturation	95 °C	5min	1
Denaturation	95 °C	1min	40
Annealing	50 °C	1min	
Extension	72 °C	1min	
Final Extension	72 °C	5min	1

Polymerase chain reaction

The PCR method in this study was modified from the method by Sahu *et al*. (2001) [12]. The PCR reaction mixture are shown in Table 2 and Table 4. PCR cycling conditions (Table 3 and Table 5). Reactions were performed in Thermo cycler (Biorad, USA).

Restriction endonuclease analysis

The PCR tubes were removed from the thermal cycler and stored at -5 ºC, until subjected to agarose gel electrophoresis (AGE).

After the PCR products were separated by electrophoresis, they were visualised by UV trans illumination. For this the gel slab was moved into a gel documentation system (Manufactured by Life Technologies: E-gel) for visualization and identification of the PCR products.

Results

The cases included in the study were categorised under 6 different geographical regions viz., Eastern Mumbai, Western Mumbai, Southern Mumbai, Harbour Mumbai, Navi Mumbai and around Mumbai. Out of 235 samples, 26 samples were collected from Eastern region, 87 samples from Western Region, 20 samples from Harbour region, 44 samples from Southern region, 28 samples from Navi Mumbai and 30 samples were from the regions around Mumbai. No bird was found positive for *Chlamydia* spp in Mumbai and Navi Mumbai. Whereas 7 positive cases detected in this study were from the aviaries around Mumbai. Percent prevalence of positive cases around Mumbai is 23.33% (7/30).

From the 7 positive samples for *Chlamydia* spp infection in the present study, Long Billed Corella showed higher positivity of 50% (1/2), followed by Eclectus Parrot at 33.33% (1/3) and Cockatiel 21.43% (3/14) and African Grey Parrot showed lowest positivity of 15.38% (2/13) in species-wise prevalence. While Cacatuidae had the highest family-wise prevalence (1.70%, 4/235) followed by Psittacidae with (0.8%, 2/235) and Psittaculidae with (0.43%, 1/235). Most frequent species encountered in present study was Budgerigars (n=29) followed by Cockatiels (n=14), African Grey Parrot (n=13), and Green Cheeked Conure (n=13).

Birds were categorized as Captive birds (n=178), 75.74% and Wild birds (n=57), 24.25%. Birds from pet owners, pet shops, breeders and aviaries were grouped as Captive birds. While rescued birds presented to veterinary clinics, Rescue Centres and shelters were grouped as Wild birds. All the positive cases in the present study belong to Captive birds category.

From the selected birds, adult birds were 176 (74.89%) while 59 birds (25.10%) were Juvenile, and all the positive birds for *Chlamydia* spp were adult 7/176. (3.97%). Sexing of birds was done on the basis of DNA sexing or sexual dimorphism. From the total birds, 107 birds were male, 79 birds were female and 49 birds were unidentified from which the positive birds for *Chlamydia* spp were 2 male birds and 5 female birds. The highest 98/235 (41.70%) birds were from aviaries, followed by 84/235 (35.74%) from pet owners, 33/235 (14.04%) from bird sanctuaries and 20/235 (8.51%) were from pet shops. 6 aviaries followed multiple birdcages which includes larger parrots like Macaws, Grey parrots that were reared in pair or 2-10 birds per cage while, pet owners with big parrots had individual bird cages for species like Cockatoo, Macaws, etc. Small-sized birds like passerines, budgerigars, African lovebirds were reared in colony cages in pet shops, sanctuaries, and aviaries. All the positive samples (n=7) were from the aviary.

Fecal samples were collected from 57 birds and swabs (conjunctival, cloacal and choanal) were collected from 162 birds. Whereas both fecal and swab were collected from 16 birds. 7 samples tested positive for *Chlamydia* spp from the group where only fecal samples (n=57, 12.28%) were collected while there was no positive sample detected in the group where both swab and fecal samples (n=16) were collected. None of the swabs were found positive for *Chlamydia* spp.

Fig 1: Collection of Choanal swab

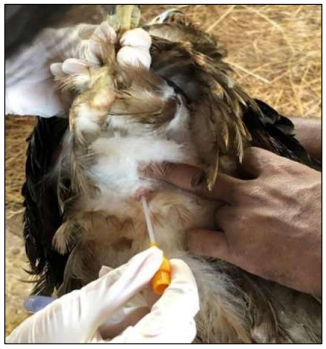

Fig 2: Collection of Cloacal swab

7 (2.97%) samples were positive for *Chlamydia* ceae family by PCR (167 bp) shown in figure 3. In the current study, polymerase chain reaction (PCR) was used for molecular detection of Avian Chlamydiosis. DNA was isolated from faeces for this study using DNA Mini kit manufactured by QIAGEN. Primer designed for *Chlamydia* ceae Family in this study were: CPSF 5’-

GCGTGTAAGGTTTAGATTCTTTACT-3′ and CPSR 5’-AGCGGCCATACTACTTGCTAT-3′ Primer used for *Chlamydia* psittaci in this study were: CPS_0429 5’-TGTGACATCATCAAGACTG-3’ and CPS_0429 5’-GATGACAACTTCTATACCTG-3’

Fig 3: *Chlamydia* spp positive band (167bp)

Out of 235 birds included in the study, 183 birds were symptomatic having clinical signs such as inappetence, diarrhea, respiratory distress, ruffled feathers, lethargy, feather loss, etc. and 52 birds were apparently healthy. Out of 183 symptomatic birds, 178 birds were negative (Figure 4) and 5 birds were positive for *Chlamydia* spp. Further from 52 non-symptomatic birds, 2 birds were positive being asymptomatic and 50 birds were negative and apparently healthy. 2 asymptomatic birds and 5 symptomatic birds were positive for *Chlamydia* spp. From 5 symptomatic birds positive for *Chlamydia* spp infection, inappetence and lethargy being the major clinical sign (21.43 percent and 21.43 percent respectively) were seen in 3 birds followed by respiratory distress (14.29 percent), diarrhoea (14.29 percent), prominent keel bone (14.29 percent) and ruffled feather (14.29 percent).

Fig 4: Conjunctivitis in Cockatiel

Out of the total samples (n=235), various clinical signs were reported in 183 birds. Pet owners of 52 symptomatic birds permitted to collect blood, consisting 47 negative and 5 positives for *Chlamydia* spp infection by PCR. Decreased Haemoglobin (Hb), Haematocrit while increased Lactate dehydrogenase (LDH) was the common finding in positive birds.

Conclusion

Prevalence of *Chlamydia* spp was found to be 2.97% (n=7/235) while no sample was found positive for *Chlamydia* psittaci. *Chlamydia* spp. can be detected with primers designed using online Integrated DNA Technology primer designing software (167 bp) received from ICAR- National Research on Meat, Hyderabad. Considering the finding of the study it may be concluded that a study with more sample size and long duration may be undertaken to estimate the prevalence of *Chlamydia* spp. in Mumbai.

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