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Detection and PCR-Based Diagnosis of *Chlamydia Psittacci* Isolated from Some Avian Species in Egypt

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Abstract:

Background: *Chlamydia psittaci* is the causative agent of human and avian psittacosis and it is an essential causative pathogen of broad spread zoonotic psittacosis**.**

Materials and methods: 233 serum samples, 330 fecal swabs and 64 tracheal swabs were obtained from ducks, ostriches, quills and pigeons of different ages and regions. *Chlamydia psittaci* was isolated using embryonated chicken eggs. Gimenez stain was used for staining the yielded yolk sac membranes. Immunoperoxidase test was used for detecting the inclusion bodies. For identification of *Chlamydia psittaci* by PCR, DNA was extracted using genomic DNA purification kit. Oligonucleotide primers were used for amplification of 16 S rRNA of *C. psittaci* by PCR and also for complete amplification of ompA of *C. psittaci*. CFT was conducted for serodiagnosis. **Results:** Positive samples showed dwarfed and congested embryos with congestion of their yolk sac vessels. Immuno-peroxidase stain positive slides showed discrete, densely labeled brown inclusion bodies was seen with in transparent back ground. The PCR results of *chlamydia psittaci* isolates showed that, in fecal samples, ostrich species had +ve % for *chlamydia psittaci* (91.7 %), pigeon (86.7 %), quail (85.7 %), duck (80 %). In the tracheal swabs, only ostrich species were positive for *chlamydia psittaci* (83.3 %). Samples of each studied species were positive for the presence of 16 S rRNA gene of *chlamydia psittaci*, and, out of 16 samples, 16 (100%) was positive for ompA. Serodiagnosis by Complement fixation test showed that, out of 233 samples, 93 samples were positive for *chlamydiae psittaci.*

Conclusion: The findings highlights the significance of *C. psittaci* diagnosis by PCR among birds to detect the possible carrier birds that can be a potential source of

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infection to other birds, or resembling a risk to individuals as being a zoonotic disease.

Keywords: *Chlamydia psittacci*, PCR, ompA, ducks, ostriches, quills and pigeons.

Introduction:

Chlamydiaceae is a family of obligate intracellular, Gram-negative, non-motile, and non-capsulated bacteria that include the well-known pathogens which mainly infect the mucosal surfaces of both humans and animals, for example, *Chlamydia trachomatis*, which causes ocular (trachoma) and genital tract infections, *C. pneumoniae* which are the causative agents of pneumonia in humans, *C. abortus* which cause significant morbidities among animals, and *C. psittacci* is the causative agent of human and avian psittacosis (Knittler & Sachse, 2015; Cheong et al., 2019). *Chlamydiaceae* species are characterized by a particular infection cycle, involving two developmental stages: the elementary bodies (EBs) that are infectious but have reduced metabolic activity, and the non-infectious reticulate bodies (RBs) that are able to divide (Gaydos & Essig, 2015).

Chlamydia psittaci is an essential causative pathogen of broad spread zoonotic psittacosis, which also recognized as ornithosis or parrot fever. This microorganism mainly infects birds, and can be transmitted to other hosts, involving humans, via a respiratory infection (Gautam & Krawiec, 2020). Serotyping of *Chlamydia psittaci* using a monoclonal antibody against major outer membrane protein (MOMP) showed a total of 6 serotypes from birds (A-F) and 2 from mammals (WC and M56), every serotype has different host specificity degrees. From psittacine birds, serotype A is mostly isolated, serotype B is mainly isolated from pigeons, C mainly from geese and ducks, while serotypes D, E, and F are mainly isolated from turkeys, pigeons and other bird species, and parrots and turkeys, respectively (Cheong et al., 2019). On the other hand, molecular genotyping for the *OmpA* gene which encodes the MOMP shows further genotypes such as E/B that is present in pigeons, together with types I that is largely genetically similar with the *C. psittaci* genotype, and J, which is largely genetically similar with the *C. abortus* (Krawiec et al., 2015).

Psittacosis is a systemic disease in psittacine birds which can be of acute, protracted, chronic, or subclinical manifestation and it represents the most important

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animal chlamydiosis of zoonotic character (Knittler, & Sachse, 2015; Borel et al., 2018).

In psittacosis, a systemic and occasionally fatal disease can occur in birds. Symptoms in birds can include rhinitis, conjunctivitis, and blepharitis with abnormal excretions. The bacteria can spread from infected birds via fecal or nasal excretions. In addition, infected birds do not always show signs of illness, meaning that humans can get infected by inhaling contaminated dust particles or the nasal discharges of infected but apparently healthy birds (De Gier et al., 2018). Symptoms in humans vary from asymptomatic to severe systemic disease. Symptoms can be influenza-like including headache, chills, fever, but severe pneumonia, endocarditis, and encephalitis could occur. Severe headache could be a distinguishing feature, with meningitis being considered in the differential diagnosis due to its severity (Chu & Durrani, 2020). People who are considered at higher risk for affecting by this disease include veterinarians, occupational exposure in the poultry industry as well as persons in pet and bird shops, (De Gier al., 2018). This study aims to spot light on the molecular characterization of *chlamydia psittaci* isolates from different avian species in Egypt and its zoonotic importance.

Materials and methods:

- **Samples:**

The present study was carried out on 233 serum samples, 330 fecal swabs and 64 tracheal swabs obtained from ducks, ostriches, quills and pigeons of different ages and regions. The study was supported by Animal Health Research institute in Egypt. The samples were collected from apparently health birds and birds suffering from diarrhea, debilitation, anorexia, respiratory manifestation and emaciation. The serum samples used for CFT were inactivated in a water bath at 56ºC for 30 minutes to remove nonspecific inhibitor. Fecal swabs were prepared according to (Edwin and Nathalie, 1979)

- **Isolation and confirmation of *Chlamydia psittaci*:**

Chlamydia psittaci was isolated using Embryonated Chicken Eggs (6-7 days fertile chicken eggs) according to Andersen and Tappe (1989). Gimenez stain was used for staining the yielded yolk sac membranes.

Immunoperoxidase test was used for confirmation of the presence of the pathogen y detecting the inclusion bodies in yolk sac impression smears according to Belloncik

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(1993) using Peroxidase-Labeled Affinity Purified Antibody to bird IgG (H+L) produced in goat obtained from KPL [Kirkegard and Perry Laboratories, Inc. Gaithersburg MD USA]. Lyophilized conjugate was stored at 2-8°C until rehydrated by adding 1 ml of reagent quality water (pyrogen free water) and stored at ¯20°C.

- **Identification of *Chlamydia* using PCR**

For PCR, harvested yolk sacs and the collected yolk sac were washed twice in physiological saline. DNA was extracted from 200 µl of this homogenate, and a genomic DNA purification kit (Gene JET Genomic DNA purification Kit (#K0722) were used for DNA extraction according to the manufacturer's protocol.

- **DNA amplification:**

Oligonucleotide primers were designed by alignment of published DNA sequence of Borel et al. (2006) that used for amplification of 16 S rRNA of *C. psittaci* by polymerase chain reaction as described in Table (1). Also Oligonucleotide primers were designed by alignment of published DNA sequence of Sachse et al. (2008) that used for complete amplification of ompA of *C. psittaci* by polymerase chain reaction using 5x FIREPol® Master Mix to amplify the ompA gene, as described in Table (1).

Table 1: Nucleotide sequences for *C. psittaci* genes-specific oligonucleotide and anticipated sizes of PCR products.

Primers	Reference	Sequence	Expected product
CTU ompA-rev	(Sachse et al., 2008)	CTU: 5'-ATGAAAAAACTC TTG AAA TCG G-3' ompA-rev:5'-TCCTTAGAATCTGAATTG AGC-3'.	1200 bp
16SIGF	(Borel et al., 2006)	59-GATGAGGCATGCAAGTCGAACG-39 59-CCAGTGTTGGCGGTCAATCTCTC-39	274 bp

- **Serodiagnosis of the collected samples by complement fixation test (CFT)**

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CFT was conducted according to **(Edwin and Nathalie, 1979)** using Amboceptor (Anti-sheep red blood cell); reference antiserum and antigen of Chlamydiae for CFT (*C. psittaci* CF test Reagent) supplied commercially from Denka Sieken Co., Ltd., Tokyo, Japan.

The serum sample was considered positive if the titer was equal or above 16 unit/ml (1:16) **(Aitken and Longbottom, 2007)**.

Results:

The results of isolated *Chlamydia psittaci* from different bird species:

As shown in Table (2), Complement fixation test showed that, out of 330 examined samples, 222 were positive for *chlamydia psittaci*, where quail was highest (60) among the avian species that had the highest +ve % (61.9%) for *chlamydia psittaci* followed by duck and pigeon (57, and 55 respectively) with +ve % of (58.7%, and 56.8% respectively), while ostrich had the lowest percent (50 representing 51.5%).

By immune-peroxidase stain, out of 330 examined samples, 203 were positive for *chlamydia psittaci*, where ostrich was the highest (59) avian species that had *chlamydia psittaci* with +ve % of (79.7%), followed by pigeon, duck and quail (54, 46, 44 respectively) with +ve % of (73%, 62.2%, and 59.5% respectively) for *chlamydia psittaci*, Table (2).

By Gimenez stain, out of 330 examined samples, 144 were positive for *chlamydia psittaci*, where ostrich was the highest avian species (43) that had the highest +ve % of *chlamydia psittaci* with +ve % of (55.1%), followed by quail, duck, and pigeon (35,35, 28 respectively) with +ve % of (48.7%, 44.9%, and 35.9% respectively) for *chlamydia psittaci* Table (2).

Table 2: Comparison of the positive results yielded by different diagnostic methods used for detection of *chlamydiae psittaci* in fecal samples in different bird samples

Test	Total no. of examined samples	Ostrich		Quail		Pigeon		Duck		Total	
		N=	%	N=	%	N=	%	N=	%	N=	%
Complement fixation test	97	50	51.5	60	61.9	55	56.8	57	58.7	222	67.3

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Immuno-peroxidase	74	59	79.7	44	59.5	54	73	46	62.2	203	61.5
Gimenez stain	78	43	55.1	38	48.7	28	35.9	35	44.9	144	43.6
Total number	330										

N= number of positive samples examined, % percentage of positive samples examined.

Incidence of *Chlamydia psittaci* recovered from fecal swab samples

Positive samples for *Chlamydia psittaci* showed dwarfed and congested embryos with congestion of their yolk sac vessels, Figure (1).

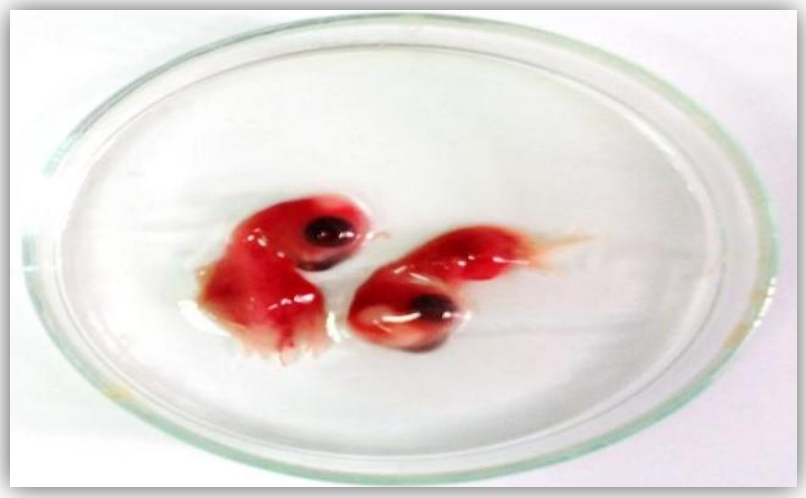

Fig.1: Growth abnormalities Dwarfism and congestion of chicken embryos inoculated with fecal swab samples

Immuno-peroxidase stain used for detection of *Chlamydia psittaci*

Immuno-peroxidase stain used for detection of *Chlamydia psittaci*, 59 (60.8%) slides in which discrete, densely labeled brown inclusion bodies was seen with in transparent back ground were positive, Figure (2).

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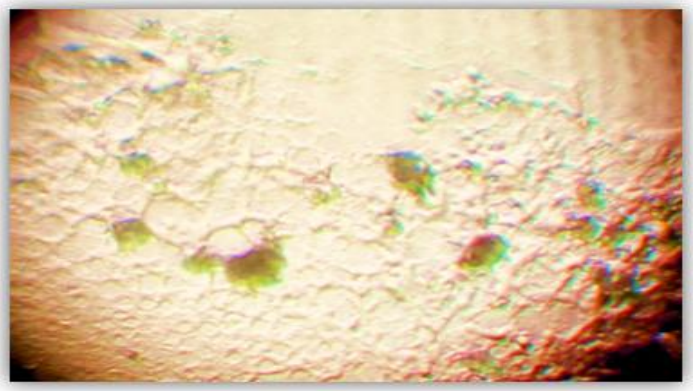

Fig.2: Impression smear of emrbryo yolk sac stained by immune-peroxidase stain showing *C. psittaci* inclusion bodies stained brown against transparent back ground.

The PCR results of *chlamydia psittaci* isolates

The PCR results of *chlamydia psittaci* isolates showed that, in fecal samples, ostrich species had the highest +ve % for *chlamydia psittaci* (91.7 %) followed by pigeon (86.7 %), quail (85.7 %), while the lowest percentage was for duck (80 %). In the tracheal swabs, only ostrich species were positive for *chlamydia psittaci* (83.3 %) while the other species did not show any +ve % for *chlamydia psittaci*, Figure (3).

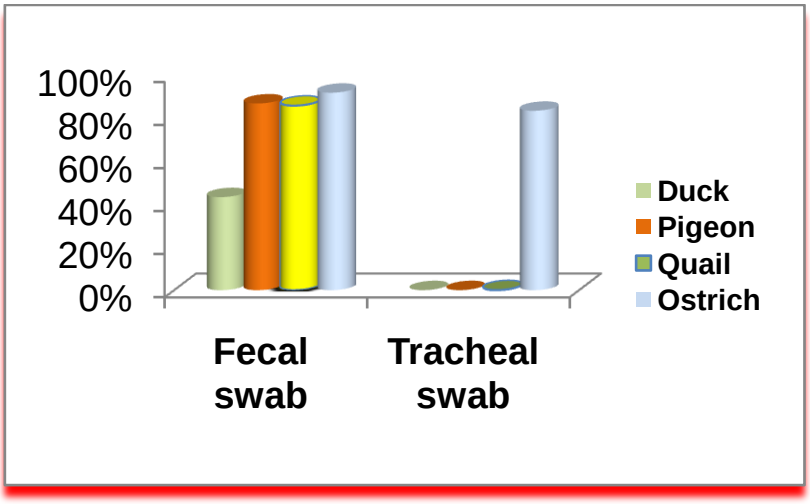

Fig.3: Comparison PCR results percentage of *chlamydia psittaci* isolates in different avian species

Results of PCR amplification ompA in yolk sac membranes of ostrich, quail, pigeon and duck

Sixteen yolk sac membrane (chlamydial isolates from fecal swabs), 4 samples of each species (ostrich, quail, pigeon and duck) from that was positive for the

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presence of 16 Sribosomal ribonucleic acid (rRNA) gene of *chlamydia psittaci*, tested for the presence of ompA of *C. psittaci*. BLASTn analysis revealed that, out of 16 samples, 16 (100%) was positive for the presence of ompA, Table (3).

Table 3: Results of PCR amplification ompA in yolk sac membranes of ostrich, quail, pigeon and duck

	Avian species							
Type of samples	Ostrich		Quail		Pigeon		Duck	
	N=	+ve%	N=	+ve%	N=	+ve%	N=	+ve%
Yolk sac membrane	4	(4) 100	4	(4) 100	4	(4) 100	4	(4) 100

N= number of examined samples.

Gel electrophoresis of ompA product

Figure (4) shows Gel electrophoresis of ompA product. Lane M; size marker with sizes (in base pairs) of weight markers indicated at the left, lanes 1, 2, 3 and 7; *chlamydia psittaci* (1200bp) isolated from ostrich, quail, pigeon and duck.

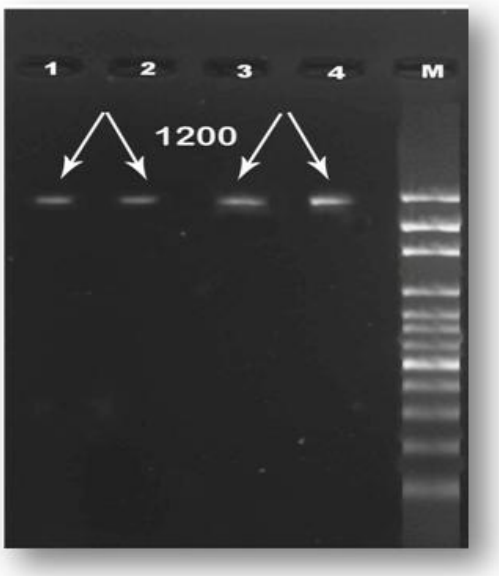

Fig. 4: Gel electrophoresis of ompA product in ostrich, quail, pigeon and duck.

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Figure (5) shows Gel electrophoresis of 16 S PCR products. Lane M; size marker with sizes (in base pairs) of weight markers indicated at the left, lanes 12, 13, 14, 15 and 16; *chlamydia psittaci* (278bp) isolated from ostrich, the most right lane is control negative.

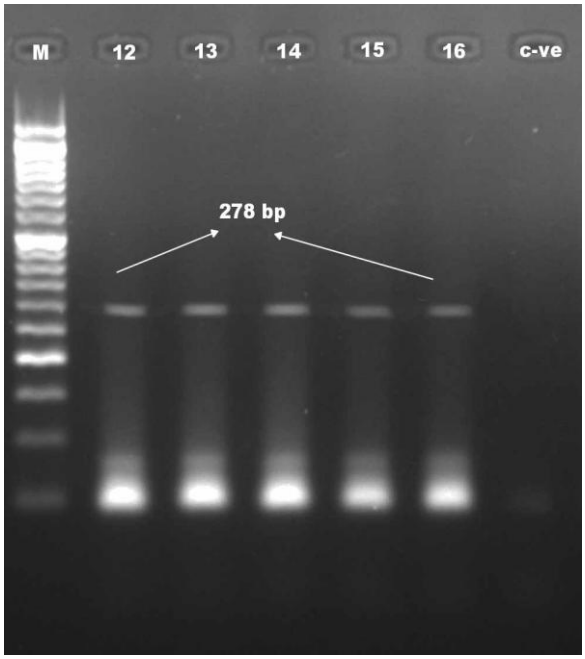

Fig. 5: Gel electrophoresis of 16 S PCR products in ostrich, the most right lane indicates control negative.

Figure (6) shows Gel electrophoresis of 16 S PCR products. Lane M; size marker with sizes (in base pairs) of weight markers indicated at the left, lanes 11, 12, 13 and 15; *chlamydia psittaci* (278bp) isolated from quail.

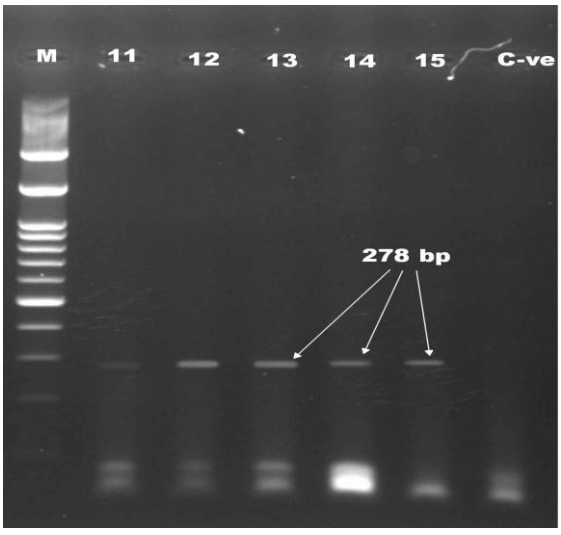

Fig.6: Gel electrophoresis of 16 S PCR productsin quail. The most right lane indicates control negative.

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Figure (7) shows Gel electrophoresis of 16 S PCR products. Lane M; size marker with sizes (in base pairs) of weight markers indicated at the left, lanes 1, 5, 6, 7, 8, 9, 10 and 11; *chlamydia psittaci* (278bp) isolated from pigeon, lanes2,3,and 4 ; negative samples.

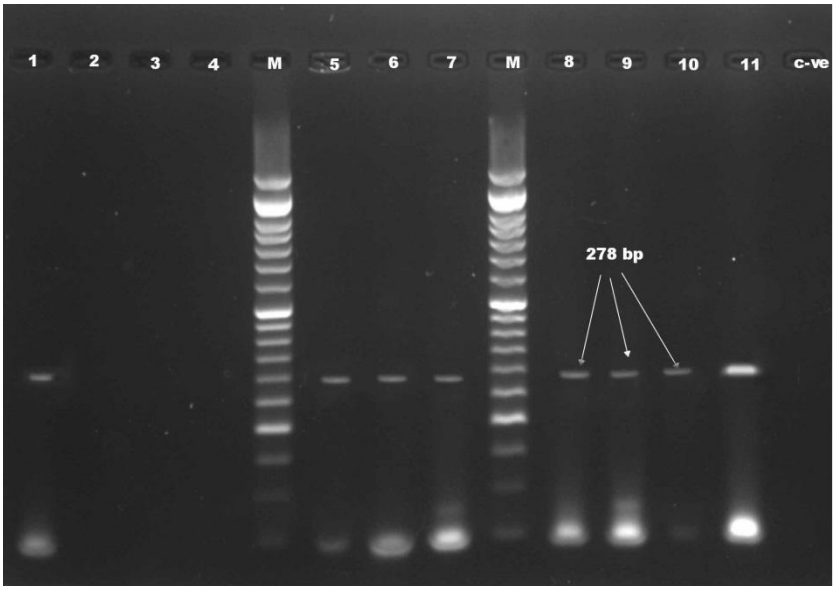

Fig. 7: Gel electrophoresis of 16 S PCR products in pigeon. The most right lane indicates control negative.

Figure (8) shows Gel electrophoresis of 16 S PCR products. Lane M; size marker with sizes (in base pairs) of weight markers indicated at the left, lanes 5, 6and 7; *chlamydia psittaci* (278bp) isolated from duck.

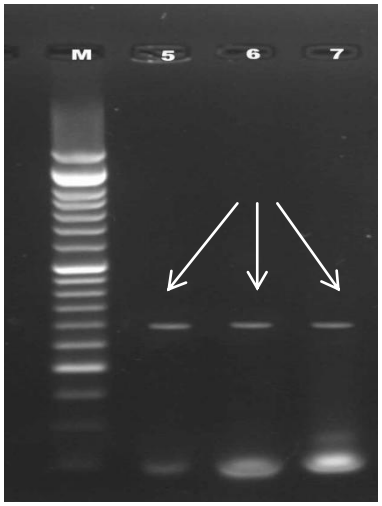

Fig. 8: Gel electrophoresis of 16 S PCR products in duck.

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Results of serodiagnosis by Complement fixation test

The results of serodiagnosis by Complement fixation test for detection of the chlamydial antibody showed that, out of 233 sample, 93 samples were positive for *chlamydiae psittaci* where duck had 35 positive sample representing the highest percentage (43.2 %) pigeon and quail representing (38.5 % and 37.8 % respectively), while no positive samples were shown by ostrich species, Table (4).

Table 4: Comparison of Complement fixation test results of chlamydial antibody detection in serum samples in different avian species.

Type of bird	Total no. of examined samples	No. of positive samples	Percentage of positive samples
Ostrich	---	---	---
Quail	74	28	37.8
Pigeon	78	30	38.5
Duck	81	35	43.2
Total	233	93	40

Discussion:

Psittacosis is still prevalent and it is considered a great cause of economic losses in the poultry industry (Sachse et al., 2015), in addition, it is also considered a high risk for zoonotic transmission to human (Szymańska-Czerwińska & Niemczuk, 2016). The present study examined four avian species, ducks, ostriches, quills and pigeons, for the presence of *chlamydia psittaci*. The present study revealed that the four examined avian species were positive for *chlamydia psittaci* in their fecal samples.

This was in line with the results of the study in Poland on some wild birds, where it was found that waterfowl seemed to possess high carriers. The water and wetland birds (particularly Anseriformes (Mallard Duck) was one of the biggest examined birds for chlamydia psittaci, in addition, Rock Pigeon were found to be positive for *chlamydia psittaci* (Krawiec, et al., 2015).

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Vorimore et al. (2015) revealed that asymptomatic *chlamydia psittaci* shedding was detected in the majority of the mule duck flocks, especially when birds were reared in open range environments.

It was stated that human cases of psittacosis related to duck breeder flocks and their offspring resulted in finding heavy shedders in the total of suspected flocks in spite of no clinical signs were shown . Due to the broad spread occurrence of *C. psittaci* on duck farms, it has become urgent to obviously detect the contamination sources so as to take proper management actions to reduce the workers' exposure to psittacosis (Vorimore et al., 2015).

AM et al. (2014) who conducted a study on the incidence of *Chlamydophila psittaci* in domestic birds in Sharkia, Egypt, revealed that *C. psittaci* was found in some domesticated animals where positive in chickens, ducks, pigeons and turkeys.

Another study in Iran on *Chlamydia psittaci* in pigeon and house sparrow and its potential infection risk for human, sowed that pigeon and sparrow were positive samples and belonged to the genotypes B and A. it was suggested that there was a high prevalence of *Chlamydia psittaci* in pigeons and sparrows in Iran causing a potential infection risk to susceptible people in public areas and parks. So, effective measures should be taken for implementing of appropriate control programmes to prevent the probable *Chlamydia psittaci* infection in human (Mahzounieh et al., 2020).

Chlamydia psittaci has been reported in over 450 bird species belonging to thirty orders as a minimum. Between the domesticated birds, *Chlamydia psittaci* is well-known to infect parrots, pigeons, chickens, turkeys, geese, ducks, game birds (such as quail), ratites (such as ostriches) (Fever, 2003).

In this study, immuno-peroxidase stain used for detection of *Chlamydia psittaci* showed brown inclusion bodies was seen with in transparent back ground. This was in agreement with Osman et al. (2011) who revealed brown inclusion bodies by impression smear of embryo yolk sac stained with immunoperoxidase stain. AM et al. (2014) also found Chlamydial inclusions confirmed in the impression smears of collected yolk sac membranes stained with Gimenez stain.

The PCR results of *chlamydia psittaci* isolates in the present study showed that, in fecal samples, ostrich species had the highest +ve % for *chlamydia psittaci* (91.7 %) followed by pigeon (86.7 %), quail (85.7 %), and duck (80 %). In the tracheal swabs, only ostrich species were positive for *chlamydia psittaci* (83.3 %)

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while the other species did not show any positivity for *chlamydia psittaci*. The study by AM et al. (2014) revealed near percentages for pigeon and duck (83.3 %).

The ompA gene was investigated as a target DNA sequence among Chlamydiacaea. In this study, 100% of the examined samples with PCR amplification ompA in ostrich, quail, pigeon and duck were positive for the presence of ompA. Gel electrophoresis of ompA product showed that *chlamydia psittaci* (1200 bp) was isolated from ostrich, quail, pigeon and duck. The findings of AM et al. revealed that the majority of the examined samples revealed the expected amplified product specific for *chlamydia psittaci* at (1041 bp). Origlia et al. (2019) sated that positive samples of *chlamydia psittaci* were genotyped by ompA gene sequences.

Conclusion

The findings highlights the significance of *C. psittaci* diagnosis by PCR among birds to detect the possible carrier birds that can be a potential source of infection to other birds, or resembling a risk to individuals who are in contact with those birds (such as workers, owners and veterinarians), and to take the important precautions to avoid the possible *chlamydia psittaci* infections as being a zoonotic disease.

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