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Emergence of *Chlamydia psittaci* in lovebirds: A new potential risk factor of Chlamydiosis.

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Abstract

Lovebirds are the most popular companion birds to human; either children or adults. To assess the potential risk of *Chlamydia psittaci* infection by such type of budgeriers in the Egyptian environment were determined by detection of the outer membrane protein A (*omp*A) gene of this pathogen in excreta and conjunctival samples of two different genera of budgeriers including ***Melopsittacus andulatus***, and ***Agapornis pullarius***. It was examined 51 fresh faecal droppings and 24 conjunctival swabs. The *omp*A gene of *C. psittaci* could be detected directly in only 27 (52.94%) of the 51 excreta while, positive samples in 13 (54.17%) of 24 of the conjunctival swabs. Negative *omp* A gene PCR samples not exclude the *C. psittaci* infection for tested samples. So, egg inoculation and yolk sac staining using Giménez stain was applied on negative samples by PCR. The results revealed that out of 24 excreta samples, chlamydial inclusions were detected in 10 samples. While from 11 swabs samples inclusions were detected in 6 samples. Totally, out of 51 excreta samples, 37 samples were positive for *C. psittaci* (72.55%) while from 24 conjunctival swabs 19 samples were positive for *C. psittaci* (79.17%).To our knowledge, this is the first study to document the potential role of lovebirds in the spread of *C. psittaci*. in Egypt. The governmental management programs and public health education should be implemented to reduce the risk of a lovebird-to-human transmission of such pathogenic agents.

Keywords: Lovebirds - *C. psittaci* – Egypt – Zoonosis – Risk - budgerier

Introduction

Chlamydia is a genus that includes important zoonotic obligate intracellular pathogens that produce acute diseases in birds and mammals, including humans. They may lead to a variety of clinical manifestations including ocular, pulmonary, genital and intestinal illness **(Rodolakis and Yousef Mohamad, 2010).**

The order Psittaciformes contains the greatest number of *Chlamydia*-positive bird species **(Kaleta and Taday, 2003).** Psittacosis is an important disease caused by *C. psittaci* **(Rohde *et al*., 2010)**, which is prevalent in poultry, pet birds and wild birds, and causes economic losses to the poultry industry and the pet trade **(Geigenfeind and Haag-Wackernagel, 2010).**

The bacterium is linked to psittacine birds such as parrots and cockatoos, the infections was called parrot-fever, but are now known as ornithosis or avian chlamydiosis **(Andersen and Vanrompay, 2000)**.

C. psittaci infections occur in at least 465 bird species, spanning 30 different bird orders. In particular, *Psittacidae* (cockatoos, parrots, parakeets and lories) and *Columbiformes* (pigeons) seem to be affected **(Beeckman and Vanrompay, 2009).**

Pet birds are known to be close friends and companions of humans, so playing an important role in human life. Unfortunately, pet birds are considered

to be a potential threat of transmitting *C. psittaci* to the owners and their family members **(Evans *et al*., 2011).** Infected birds usually remain asymptomatic and may intermittently shed the agent in nasal secretions and feces, especially when submitted to stress factors such as nutritional deficiency, prolonged transportation, overcrowding, temperature changes and/or reproduction. Infected birds can be sources of infection for other avian species and humans **(Harkinezhad *et al*., 2009)**.

Zoonotic risk of *C. psittaci* arises directly via inhalation of contaminated aerosols originating from feathers, fecal material and respiratory tract exudates. Handling the plumage, carcasses and tissues of infected birds and in rare cases, mouth-to-beak contact orbiting also carry a zoonotic risk (**Beeckman and Vanrompay, 2009).**

Recently, with the increasing popularity of lovebirds & cockatiels as a pet bird, and the close relationship between human beings and their pets, this may help in exposure of people to potential pathogens that may belong to the normal microbiota of these birds **(Brilhante *et al*., 2010).**

Globally, very limited data about the role of lovebirds in the *C. psittaci* epidemiology except few studies in China **(Zhang *et al*., 2014)** and Georgia **(Moroney *et al*., 1998)**

Chlamydia prevalence in wild birds in Egyptian environment has been reported throughout many studies (**Gamal- Eldein *et al*., 2009, El-Jakee *et al*., 2014**) but there is limited information about *Chlamydia* infection in lovebirds and it is role in Chlamydiosis spreading .In this study, the prevalence of *Chlamydia* infection in lovebirds was investigated in Giza governorate via molecular techniques.

Materials and methods

1-Samples collection:

Seventy five samples of lovebirds excreta and conjunctival swabs were collected from pet shops and love birds owners in Giza governorate and transferred directly to the laboratory Table (1).

Table 1: Number of Collected samples from different lovebirds species:

Lovebird species	Latin name	Sample Source		Samples number
		Excreta	Conjunctival swabs	
Australian budgerier	*Melopisittacus andulatus*	25	12	37
English budgerier	*Melopsittacus andulatus*	10	7	17
Red-faced pied lovebird	*Agapornis pullarius*	16	5	21
Total Number		51	24	75

2- Samples preparation.

2.1. Fecal samples preparation:

One gram from each sample was suspended in a sterilized glass bottle containing 99.0 ml of sterile physiological saline (0.85% NaCl) . 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. Fecal matter suspension was clarified by centrifugation at 3.000 rpm for 15 minutes. Supernatant were treated with antibiotics (streptomycin 2.5 mg/ml, neomycin 0.5 mg/ml and nystatin 100 units/ml) and held for 1 hour at room temperature.

2.2.Conjunctival swabs samples preparation:

Conjunctival swabs were diluted with Phosphate Buffer Saline (PBS) "pH 7.2-7.4" to 20 % and clarified by centrifugation at 3.000 rpm for 15 minutes. Supernatant were treated with antibiotics (streptomycin 2.5 mg/ml, neomycin 0.5 mg/ml and nystatin 100 units/ml), held for 1 hour at room temperature, re- centrifuged 2 times and the final supernatant was used in egg inoculation **(Edwin and Nathalie, 1979) .**

3- Direct extraction of DNA from the samples.

3.1. Extraction and purification of DNA from fecal samples.

QIAamp DNA Stool Mini Kit was used for extraction according to the instruction of the manufacturer.

3.2. Extraction and purification of DNA from swabs samples Genomic DNA was extracted from swab samples according to **McClenaghan *et al*., (1984)**

4- Direct identification of *C. psittaci* from the collected samples using PCR. (Vanrompay *et al.*, 1998).

PCR amplification of the *omp*A gene was performed using the primers **55G2-F,** 5-ATTTGGGATCGCTTCGAC-3 and **55G2-R**, 5- CCTTTATAGCCTCTTGGTTTGTG -3, and the following cycling conditions: initial denaturation at 94°C for 5 min, followed by 30 cycles of 95°C for 30 s, 50°C for 2 min and 1min of polymerization at 72°C and a final extension at 72°C for 10 min. The amplification reaction was carried out in a50-ul volume containing 12.5 ul of genomic DNA extract, 25 ul of MQ water,5 ul of Super Taq buffer(10x),1 ul of each deoxy nucleoside triphosphate (10mM) ,2.5 ul of each primer (55G2-F & 55G2-R) 20pmol/ul),and 1ml of Super Taq polymerase (15U/ml) (1/50dilutionin (Super Taqbuffer). 10 ul of the PCR mixture was subjected to electrophoresis on a 1.2%agarose gel stained with ethidium bromide and photographed under UV illumination. Amplified product of the appropriate size (1,200bp) was obtained.

5- Egg inoculation and yolk sac staining using Giménez stain.

Seven to eight days old Specific Pathogen Free (SPF) fertile chicken eggs from Koom Ousheem Al Fayyom Poultry Farm, Egypt were used for detection of chlamydiae. 200 ul of each sample was inoculated into the egg yolk sac according to **Andersen and Tappe (1989)** and the inoculated eggs were incubated at 37 °C in a humidified incubator. Non inoculated control eggs were labeled and incubated beside the inoculated eggs. The eggs were candled on a daily basis and the eggs that died within 3 days post inoculation were discarded while those died after day 3 to day 10 are opened. The yolk sac membranes were harvested and stained by Giménez stain (**Gimenez, 1964).** Embryos of specific deaths were examined for pathological changes and lesions specific for chlamydial infection.

4. Results

This study was done by the collection of excreta and conjunctival swabs of 75 love birds from two different genera. The samples were analyzed for *C. psittaci* by two parallel methods. Firstly all collected samples were subjected for the direct molecular identification targeting *Omp* A gene specific for *C. psittaci*. Then, the negative examined samples by direct PCR were further tested for presence of *C. psittaci* inclusion bodies depending on cytological examination of inoculated embryonated egg yolk and Gimenez staining.

Using PCR *omp* A was detected in 52.94% (27/51) of birds excreta in the following distribution Australian budgerier 44 % (11/25) , English budgerier 70% (7/10) and Red-faced pied lovebird 56.25% (9/16) (Table 2) with amplified product at 1,200 bp Fig (1) .

Among the conjunctival samples, 54.17% (13/24) were positive as follow 50 % (6/12) in Australian budgerier, 57.14 % (4/7) in English budgerier and 60%(3/5) in Red-faced pied lovebird (Table 2).

The negative samples for *omp* A gene (24 excreta & 11 conjunctival swabs) were further identified based on egg inoculation and Gimenez staining.

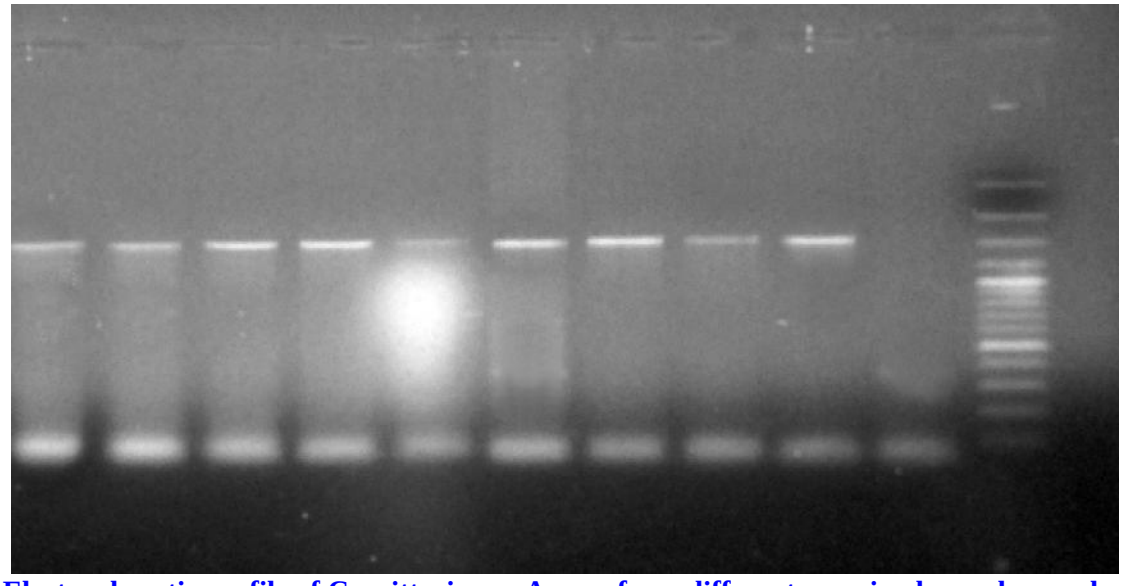


Fig. (1): Electrophoretic profile of C. psittaci omp A gene from different examined samples producing PCR product of 1200 bp, Marker GeneRuler 100 bp Plus DNA Ladder (Thermo)

Table 2: Results of direct molecular identification of collected samples by PCR targeting *Omp* A gene specific for *Chlamydia psittaci*.

Lovebird species	Excreta			Conjunctival swabs		
	Total samples number	Positive PCR	Negative PCR	Total samples number	Positive PCR	Negative PCR
Australian budgerier	25	11 44%	14	12	6 50%	6
English budgerier	10	7 7%	3	7	4 57.14	3
Red-faced pied lovebird	16	9 56.25%	7	5	3 60%	2
Total	51	27 52.94%	24	24	13 54.17%	11

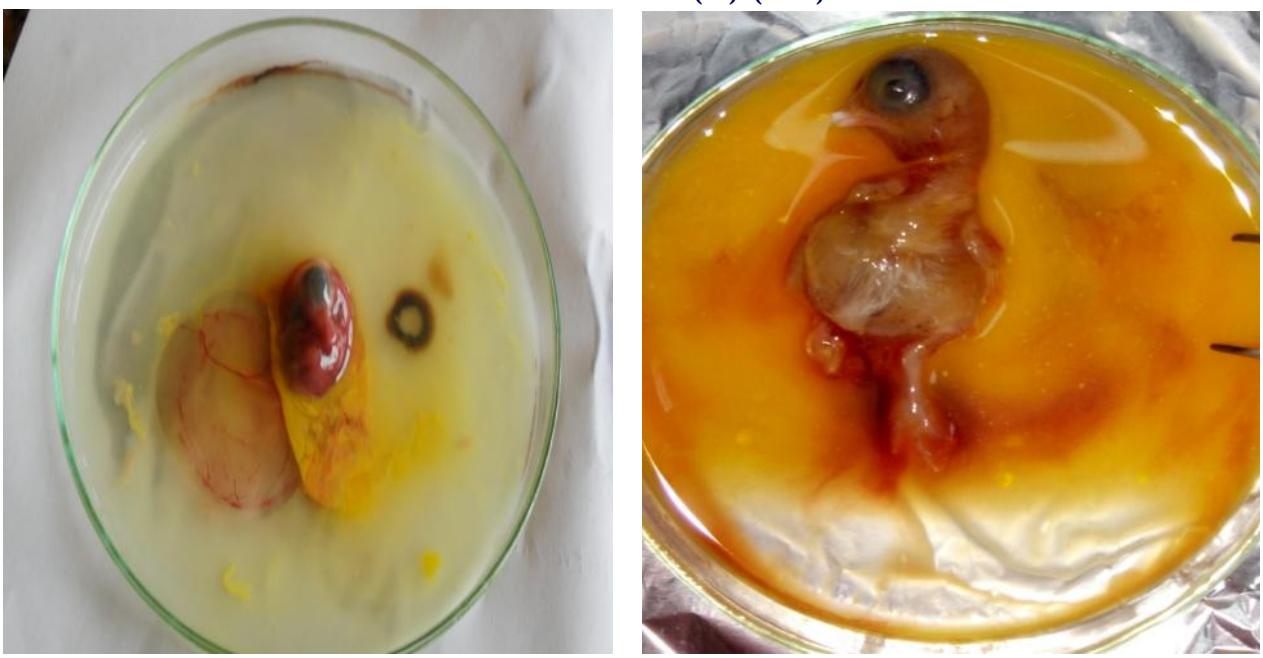
Fertile SPF eggs (7-8 days) were inoculated with prepared samples via intra yolk sac route. Positive cases were confirmed by pathological lesions encountered in the embryonic membranes in the form of congestion and severe engorgement of the blood vessels. Embryos appeared dwarfed with presence of hemorrhagic spots in the head and toes (Fig. 2). Impression smears of the collected yolk sac membranes were subjected to staining with Gimenez staining. Chlamydial inclusions appeared as small, rounded red dots against a bluish green background (Fig. 3). Out of 24 excreta samples, chlamydial inclusions were detected in 10 samples as follow 6 for Australian budgerier, 1 for English budgerier and 3 for Red-faced pied lovebird (Table 3). While from 11 swabs samples inclusions were detected in 6 samples (3 in Australian budgerier, 1 in English budgerier, 2 in Red-faced pied lovebird).Totally, out of 51 excreta samples, 37 samples were positive for *C. psittaci* (72.55%) while from 24 conjunctival swabs 19 samples were positive for *C. psittaci* (79.17%) (Table 4).

Table 3: Results of cytological examination of impression smears of the collected yolk sac membranes using Gimenez stain for PCR negative samples.

Lovebird species	Positive samples for Chlamydia inclusions	
	Excreta	Conjunctival swabs
Australian budgerier	6 / 14 42.86%	3 / 6 50%
English budgerier	1 / 3 33.33%	1 / 3 33.33%
Red-faced pied lovebird	3 / 7 42.85	2 / 2 100%
Total	10 / 24 41.67%	6 /11 54.55%

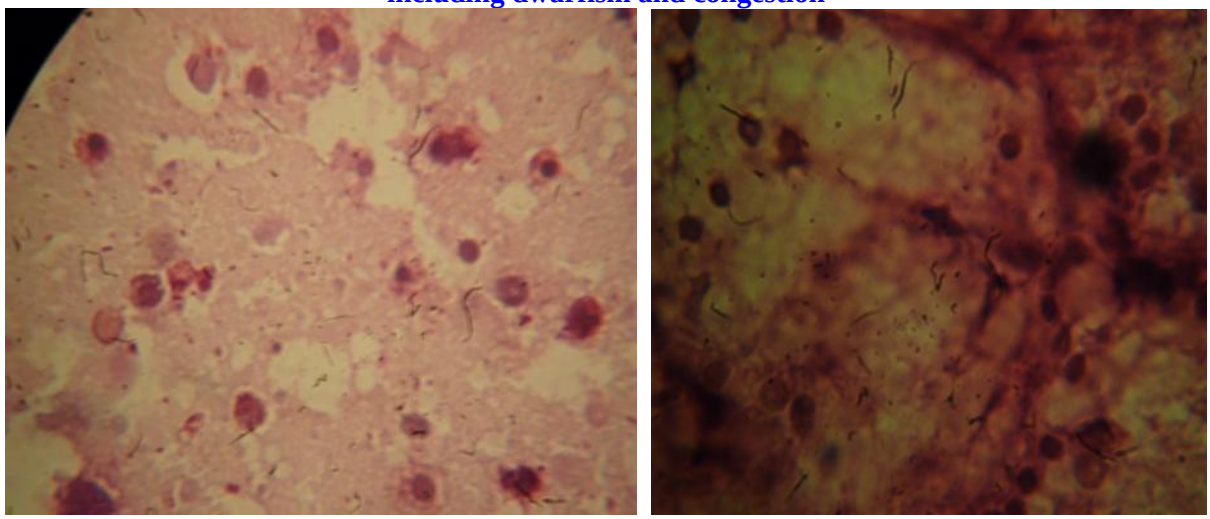
Table 4: Total result of *Chlamydia psittaci* detection from collected samples.

Lovebird species	Latin name	Positive samples		Samples number	Recovery rate (%)
		Excreta	Conjunctival swabs		
Austerilain budgerier	*Melopisittacus andulatus*	17/25	9/12	26/37	70.27
English budgerier	*Melopsittacus andulatus*	8/10	5/7	13/17	76.47
Red-faced pied lovebird	*Agapornis pullarius*	12/16	5/5	17/21	80.95
Total		37/51	19/24	56 / 75	74.67
Recovery rate (%)		72.55	79.17	74.67	



(a) **(b)**

Fig 2: (a) - (b) Chicken embryos after *C. psittaci* inoculation showing different forms of growth abnormalitis including dwarfism and congestion



(a) **(b)**

Fig 3: Chlamydial inclusions in the infected yolk sac membranes stained with Gimenez stain (a &b).

Discussion

Pet birds are potential carriers and / or transmitters of zoonotic diseases. Some of them could have an important impact on human health, like chlamydiosis, salmonellosis or even highly pathogenic avian influenza A H5N **(Boseret *et al.*, 2013).**

Unfortunately in Egypt, *C. psittaci* infections in birds were only studied in wild birds **(El-Jakee *et al.*, 2014)** or in domestic birds as turkey **(Enany *et al.*, 2009)** and chicken **(Osman *et al.*, 2007)**. No other studies investigate the prevalence of chlamydiosis in pet birds although the economic importance of them.

As pet bird or avian species could transmit *C. psittaci* infection to human via two major roles direct contact and/ or inhalation of aerosolized fecal dust, feather particles or dried respiratory tract secretions from infected birds **(Andersen and Vanrompay, 2003)**. So, this study was concerned to collection samples from most popular and major love bird species in Egyptian community.

The molecular biology methods enabled further progress in chlamydial diagnostics and research. DNA based detection is rapid, specific, and reliable. The usual targets include an RNA operon or the outer membrane protein A (*omp A*) gene allowing genus and species differentiation. **(Everett and Andersen, 1999 and Sachse and Hotzel, 2003)**

Depending on this fact, applying of direct molecular identification of collected swabs and fecal matter, revealed that out of 51 examined excreta, 27 samples were positive (52.94%) and 13 out of 24 conjunctival samples were positive (54.17%) as shown in Table (2). The negative samples could not be considered free from *C. psittaci* infection due to there are many factors could be act as PCR inhibitors which, could be reflected on obtained result as false negative **(Vargas *et al*., 2006)**

For the diagnosis of chlamydiosis, isolation is known as the gold standard, even though other methods are also used **(OIE, 2000).**

The embryonated chicken egg inoculation is a traditional method with an established sensitivity, and that the long time requirement is its only disadvantage **(Pearson *et al*., 1989 and Bougiouklis *et al*., 2000).** All negative PCR samples were subjected for inoculation in embryonated chicken eggs for further isolation**.** Chlamydial inclusions were demonstrated in the impression smears of collected yolk sac membranes stained with Gimenez stain.

The cytological examination of inoculated egg revealed that, out of 35 negative PCR samples, chlamydial inclusions were detected in 10 and 6 out of 24 and 11excreta and conjunctival samples respectively from the negative PCR samples.

The recovery rate of identified chlamydiae was going to raise after egg inoculation from 52.94% to 72.55% for excreta samples and from 54.17% to 79.17% for conjunctival swab samples which indicate the effectiveness of isolation although it is considered time consuming. The results revealed that PCR can help in rapid diagnosis and therefore help in effective and rapid treatment. Since isolation takes a long time, requires high-quality samples, and can cause danger for laboratory staff, **(Trevejo *et al*., 1999).**

All over the obtained data of PCR and egg inoculation, the recovery rate of *C. psittaci* was 80.9, 76.4 and 70.2 Red-faced pied lovebird (*Agapornis pullarius*) Australian budgerier (*Melopisittacus andulatus*) and English budgerier (*Melopsittacus andulatus*) respectively. This rate is considered high prevalence when compared with other species of pet birds as the frequency of *C. psittaci* in Paridae family ranged between (58.4 – 50.6 %) **(Holzinger-Umlauf *et al*., 1997),** 34.4 % in Turkey pet birds **(Çelebi and Ak, 2006),** 35·37% in China parrots **(Zhang *et al*., 2015).** On the other hand, many documentation of the infection is highly prevalent in *Psittaciformes* as studies in wild and captive *Psittaciformes* in North and South America, Australia, Europe and Japan have shown wide range of variability in recovery rate 16–81% of the examined birds were positive for *C. psittaci* **(Dovc *et al* ., 2005 & Raso *et al*., 2002).**

These bacteria are transmitted as metabolically inactive particles called elementary bodies (EBs**) (Binet and Maurelli, 2007).** *C. psittaci* has the ability to remain infectious in the environment for months, presenting a variety of public health issues, including economically devastating outbreaks in poultry farms and occasionally severe pneumonia in humans (**Kaltenboeck *et al*., 1991).**

In birds, the course of infection can be rather mild but a mortality rate of 50% or even higher is not unusual. Fecal and nasal excretions of diseased birds are the primary source of human infections (**Harkinezhad *et al*., 2007)**. However, apparent clinically healthy *Psittaciformes* also present a threat to human health, since many cockatoos, parrots, parakeets and lories never get rid of the bacterium once infected and most of them actually become *C. psittaci* carriers, shedding the bacteria again after being stressed **(Schachter *et al*., 1978 & Andersen and Vanrompay, 2003)** .Thus, a considerable number of people are at risk of becoming infected with this bacterium such as people working in pet shops, garden centers, quarantine stations and zoos. But, also visitors of these facilities and people keeping Psittaciformes as pets can become infected.

C. psittaci DNA was detected with nested PCR/enzyme immuno- assay and revealed that 6 (13%) of 146 pet bird owners were infected by *C. psittaci* **Vanrompay *et al.*** (**2007**). Petrovay and Balla reported two fatal cases of psittacosis in two poultry processing plant employees presenting with pneumonia and respiratory failure; the diagnosis was confirmed by serological and PCR methods (**Petrovay and Balla, 2008**). Therefore, psittacine pet birds in urban and rural areas throughout the world should be regarded as the predominant reservoirs of zoonotic psittacosis (**Geigenfeind *et al*., 2012**).

In a previous study in Brazil, it was noticed that 4.7% (17/364) of people who worked in contact with birds presented have anti- C. psittaci antibodies (**Raso *et al*., 2010**).so, authors suggested that prevention and control measures against *C. psittaci* should be implemented in such a work environment.

In a case report of psittacosis involving pet store owners in Japan, **Saito *et al*. (2005)** commented on the risk of occupational infection also **Vanrompay *et al*. (2007)** conducted a study in Belgium using PCR and demonstrated C. psittaci infection in 13% (6/46) of parrot owners. These data emphasize the importance of birds as a source of *C. psittaci* infection in humans, especially when living in close proximity.

Although, Buderigars are one of the most common pet birds species distributed in Egyptian community especially Australian Buderigars (*Melopisittacus andulatus)* followed by English and red-faced pied love birds which, came in 2nd choice for Egyptian's pet birds lover because they are somewhat more expensive in price. However, very little information is available concerning the present incidence of chlamydiosis in pet birds, especially budgerigars sold to the public by wholesalers. The total recovery rate from all collected samples from different love bird species was 74.67% (56 /75). This high frequency mainly attributed to arbitrary ways for importing such type of birds in Egypt. In addition to huge market for these types of birds do not fall under any government control with the absence of any means to prevent and control the transmission of infectious pathogenic diseases for different birds or individual's devotees to pet birds, workers, sellers within such markets.

Stress factors such as, high population density of mixing bird from different species with high temperature and dusty environment could be augmenting factors enhance susceptibility of budgerigars to *C. psittaci* infection which, may be in latent asymptomatic forms. So, that gives the explanation for high recovery rate of examined birds for *C. psittaci.*

The obtained results raise the alarm from exposure to chlamydiosis, which may affect the rights of pet bird lovers from various ages, especially the elderly and young people who were taking such animals as a friend.

Conclusion

Lovebirds are beautiful birds favorite to humans, but the Egyptian authorities with veterinary institutions should give more attention to the examination of such imported birds. Placing the binding government programs to detect and periodic inspection of different bird types that may be the reason for the transfer of epidemic diseases

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