Isolation of *Chlamydophila psittaci* from pet birds in Iran

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Abstract

Avian chlamydiosis is one of the most important infectious diseases of birds. Despite the rapid growth of exotic bird populations in Iran, there is little or no information on the specific infections that these types of birds carry. In this study, conventional isolation methods were used in cell culture to study occurrence of infection in pet birds. Samples from the conjunctiva, choana, and cloaca and/or droppings were provided from 17 birds of different species. The samples were used to infect McCoy cell culture to isolate *Chlamydophila psittaci*. The inoculated cells were fixed, stained by Giemsa, mounted on slides using Entellan® and observed by light microscope for the presence of typical chlamydial inclusion bodies. *Chlamydophila psittaci* was isolated from four birds including a ring-necked parakeet (*Psittacula krameri*), an Alexandrine parakeet (*Psittacula eupatria*), an African grey parrot (*Psittacus erithacus*), and a Timneh grey parrot (*Psittacus erithacus timneh*). All negative cultures were passaged a further two times. To the best of our knowledge; the report represents the first isolation of chlamydia from birds in Iran.

Introduction

Avian chlamydiosis, caused by the bacterium *Chlamydophila psittaci*, is usually a systemic and occasionally fatal disease of avian species (Andersen and Vanrompay, 2008a). The disease was first recognized in parrots and other psittacine birds, and in humans associated with psittacine birds, owing to the original name of psittacosis. (Woldehiwet, 2008). Although only occasionally fatal, the severity of gross lesions caused by the disease has been found to vary due to the host susceptibility, severity, and duration of the disease (Woldehiwet, 2008). Chlamydiosis appearance varies and in most cases it is a latent infection without any clinical or pathognomonic signs (Gerlach, 1994). Infection can be transmitted from pet birds to humans, causing an influenza-like disease that can progress and lead to severe pneumonia (Weese *et al*., 2002).

Chlamydophila psittaci is an obligate intracellular bacterial parasite (Andersen and Vanrompay, 2008a). The family *Chlamydiaceae*, which formerly had only one genus, *Chlamydia*, was recently divided into two genera, *Chlamydia* and *Chlamydophila* (Everett, 1999). As the chlamydial organisms are highly dependent on the host cell for energy (Gerlach, 1994; Andersen and Vanrompay, 2008a), isolation attempts should be made by inoculation onto cell culture monolayers, into the yolk sac of embryonated chicken eggs, or into mice (Andersen and Vanrompay, 2008b). Cell culture has been identified as the most convenient method for the isolation of *C. Psittaci* (Andersen and Franson, 2007; Andersen and Vanrompay, 2008b).

In recent years, exotic cage bird populations in Iran have grown quickly. According to Iranian government regulations, the import of exotic birds is prohibited without the supervision of Iranian Veterinary Organization. However, despite attempts made to restrict the illegal import and capture of exotic psittacine birds such as African grey parrots (*Psittacus erithacus*), ring-necked parakeet (*Psittacula krameri*), and Alexandrine parakeet (*Psittacula eupatria*), these types of birds are frequently traded in live exotic bird markets and consequently impose a severe health threat to both pet bird owners and the birds.

Due to the lack of routine sampling and monitoring of cage and aviary birds for *C. Psittaci* in Iran, we attempted to isolate *C. Psittaci* from clinically suspected cases in order to elucidate the current state of *C. Psittaci* infection in exotic cage birds.

Materials and Methods

Sample collection

Seventeen birds of varying species were chosen based on clinical presentations (Table 1). All birds had recently been bought or transported. Clinical signs of depression, urate discoloration or upper respiratory signs such as nasal discharge, sinusitis and/or keratoconjunctivitis were noted. Sucrose-phosphate-glutamate (SPG) buffer consisting of 74.6 g/L sucrose,

0.512 g/L KH_2PO_4, 1.237 g/L K_2HPO_4, 0.721 g/L L-glutamic acid (all chemical agents of SPG buffer were provided from Merck KGaA, Germany), 10% fetal calf serum (Jahad Daneshgahi, Iran), 100 mg/L from each of vancomycin and streptomycin (Jaber Ebne Hayyan, Iran), 50 mg/L gentamicin (Alborz Darou, Iran), and 50mg/L amphotericin B (Bristol-Myers Squibb, France) was used as transport media (Andersen and Vanrompay, 2008b). In live birds with sizes larger than canaries, samples were obtained using triple swabbing technique. In this method, a single sterile swab was used to take samples from conjunctiva, choana and cloaca. When it was not possible to take triple samples, droppings were taken as clinical samples. One sample was taken from air sac exudates of a canary whilst postmortem examination was performed. Each swab sample was immersed in 1 ml SPG buffer and refrigerated at 4°C overnight, before being centrifuged at 500 x *g* for 20 min. The supernatant was harvested, re-centrifuged, and the final supernatant used for inoculation (Andersen, 2008).

Cell culture inoculation

McCoy cell line (National Cell Bank of Iran, Pasteur Institute of Iran, Iran) was used for sample inoculation and *C. Psittaci* isolation (Schachter, 1995; Scidmore, 2005; Andersen, 2008; Andersen and Vanrompay, 2008a; Andersen and Vanrompay, 2008b). Freshly trypsinized McCoy cells were passaged into 1 dram shell vial containing a 2 cm diameter circular coverslip at the bottom and incubated for 24-48 hours at 37°C to achieve a sufficient growth of McCoy cells on the circular coverslip. Then, each prepared sample was inoculated into one shell vial containing a cover slip rich in McCoy cells. To enhance the attachment of chlamydial elementary bodies to host cells, vials were then centrifuged at 1000 x g for 30 min (Andersen, 2008; Andersen and Vanrompay, 2008b). The remaining fluid on the inoculated cell culture was then discarded and replaced by cell culture media containing 1 mg/L cyclolohexamide (Sigma Chemical Co., St. Louis, MO).

Staining of cover slip and inclusion body identification

Cultures were examined at 2-day intervals. At day 4 or 5, the first monolayer culture medium of each sample was removed after inoculation and washed once with sterile PBS. The inoculated cell on the coverslips was fixed with aceton-methanol (Merck KGaA) (50:50, v:v) for 5 min and stained with Giemsa as previously described (Munday *et al*., 1980). The cover slips were then removed and mounted on a slide using Entellan® (Merck KGaA), and examined microscopically for chlamydial inclusion bodies under a high power field. Cultures that appeared negative at 7 days post-inoculation were re-passaged with the same method as described above.

Results

Four samples (23.5%) were found positive for chlamydial inclusion bodies (Figure 1). The positive samples were from a ring-necked parakeet (*Psittacula krameri*), an Alexandrine parakeet (*Psittacula eupatria*), an African grey parrot (*Psittacus erithacus*), and a Timneh grey parrot (*Psittacus erithacus timneh*) (Table 1). All birds had been recently bought or transferred. Depression, anorexia, urate discoloration, and biliverdinuria were the only clinical signs in these affected birds.

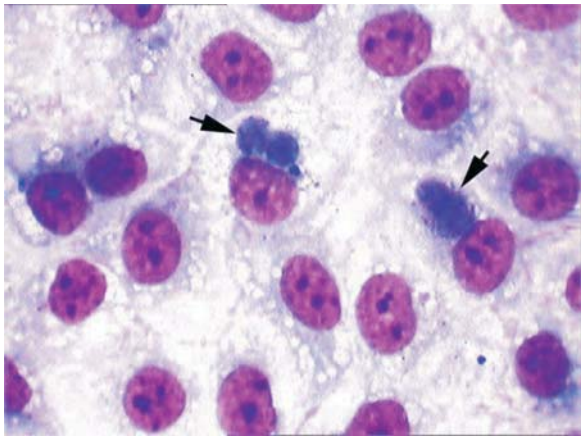

Figure 1: Characteristic perinuclear chlamydial inclusions (arrows) were isolated from an Alexandrine parakeet, case 16 in Table 1, x1,000, Giemsa.

Table 1: Bird species, sampling type, and isolation results of birds involved in the study.

No.	Bird species	Scientific name	Clinical sample	Isolation result
1	Ring-necked parakeet	*Psittacula krameri*	Triple	Negative
2	White-eared Bulbul	*Pycnonotus leucotis*	Triple	Negative
3	Canary	*Serinus canaria*	Air Sac	Negative
4	African grey parrot	*Psittacus erithacus*	Triple	Negative
5	Java finch	*Lonchura oryzivora*	Triple	Negative
6	Rock pigeon	*Columba livia*	Triple	Negative
7	Ring-necked parakeet	*Psittacula krameri*	Triple	Negative
8	Common myna	*Acridotheres tristis*	Triple	Negative
9	Common myna	*Acridotheres tristis*	Triple	Negative
10	Ring-necked parakeet	*Psittacula krameri*	Triple	Positive
11	Timneh grey parrot	*Psittacus erithacus timneh*	Triple	Positive
12	Alexandrine parakeet	*Psittacula eupatria*	Triple	Negative
13	African grey parrot	*Psittacus erithacus*	Triple	Negative
14	African grey parrot	*Psittacus erithacus*	Triple	Positive
15	Rock pigeon	*Columba livia*	Triple	Negative
16	Alexandrine parakeet	*Psittacula eupatria*	Triple	Positive
17	Budgerigar	*Melopsittacus undulatus*	Droppings	Negative

Discussion

Chlamydophila psittaci infection has already been confirmed in more than 460 avian species in 30 orders (Kaleta and Taday, 2003). Currently, all known avian chlamydial strains belong to species, *C. Psittaci* (Andersen, 2008) and to the best of our knowledge, this is the first report of the isolation of this species in Iran. Isolation of *Chlamydophila* spp was first reported in

Iran in 1981, obtained from a domestic cat and named *Chlamydia psittaci* by the authors (Tabatabayi and Rad, 1981). However, based on new classification guidelines of chlamydial microorganisms, the isolated microorganism would now be recognized as *Chlamydophila felis,* due to the use of a cat in isolation of the sample. Unfortunately, the isolated chlamydia is no longer available; hence a more accurate identification is not possible at present time. All isolated bacteria in our study were found in psittacine birds. The order Psittaciformes contains by far the most chlamydia-positive bird species (Andersen and Franson, 2007). The three species found positive for Chlamydial inclusion bodies in this study are popular cage bird species in Iran. The presence of *C. Psittaci* infection in these species (African grey parrot, ring-necked parakeet, and Alexandrine parakeet date) has already been reported (first reported in 1952, 1989, and 1994, respectively) (Kaleta and Taday, 2003).

This finding is supported by research that has identified a high prevalence of *C. Psittaci* in other bird species. A survey for the chlamydial antigen in 95 apparently healthy, captive Amazon parrots from three breeder collections in Brazil, determined a prevalence of 16.7-56.1% detected by direct immunofluorescence, and a seroprevalence of 60-100% by ELISA (Raso *et al*., 2002). PCR detection of *C. Psittaci* in endemic free-ranging Galapagos doves (*Zenaida galapagoensis*) and introduced rock doves (*Columba livia*) in Spain found a prevalence of 24% (Padilla *et al*., 2004). In Amsterdam, The Netherlands, the prevalence of *C. Psittaci* was 7.9% among the feral rock dove as determined by PCR performed with fresh fecal samples from feral pigeons (Heddema *et al*., 2006). Two main approaches can be used to diagnose infections caused by *Chlamydia* and *Chlamydophila* spp. in mammals and birds (Sachse *et al*., 2009). The first approach involves the direct detection of the agent in tissue or swab samples, and the second involves the serological screening of blood samples for the presence of anti-chlamydial antibodies (Sachse *et al*., 2009). Cell culture is the most convenient method for isolating avian strains of *C. Psittaci* (Andersen and Vanrompay, 2008b) with cell lines such as McCoy, HeLa, Vero, L929, and BGM, most commonly used for this purpose (Andersen and Vanrompay, 2008b). With the exception of lymphogranuloma venereum (LGV) serovars; most *Chlamydia trachomatis* isolates do not readily infect tissue culture cells but infectivity can be enhanced through chemical or mechanical assistance (Scidmore, 2005). Other *Chlamydia* species can be isolated from cells that are replicating normally, however most diagnosticians, prefer to use nonreplicating cells to provide increased nutrients for the replication of chlamydiae, and because nonreplicating cells can be maintained for longer periods for observation (Andersen and Vanrompay, 2008b). Cell line replication can be suppressed by cytotoxic substances like cyclohexamide, as used in the cell culture media in this study. Other staining methods such as Gimenez, modified Gimenez (PVK stain), and Machiavello could be considered for this study but based on our experience in the laboratory, Giemsa staining was found to be more convenient for observation of inclusions in cell culture (Andersen and Vanrompay, 2008b). Visualizations of the characteristic perinuclear chlamydial inclusions have also been made using Giemsa staining (Schachter *et al*., 1995). Although not employed in this study, the modified Gimenez technique, is routinely used by several laboratories for detection of chlamydial inclusions in smears and paraffin-embedded tissue sections (Andersen and Vanrompay, 2008b). Comparison of different methods of chlamydiae detection has revealed polymerase chain reaction to be a more sensitive technique than either cell culture isolation (McElena and Cross, 1999) or embryonated chicken egg inoculation procedures (Çelebi and Ak, 2006). However, isolation is known as the gold standard in the diagnosis of Chlamydiosis, even though other methods are popular (Çelebi and Ak, 2006).

Psittacosis is the name used for human infection with *C. Psittaci*. In most cases, infection is caused by direct exposure to infected birds or environmental contamination from droppings of infected birds (Weese *et al*., 2002). From a zoonotic point of view, the study suggests the need for greater awareness of *C. Psittaci* in pet bird populations by avian clinicians in Iran. No pathognomonic clinical signs were observed in the four infected birds in this study but all had recently experienced stressful conditions (owner change and transportation). Shedding of Chlamydophila has previously been attributed to stress (National Association of State public Health Veterinarians, 2002). Therefore, it is sensible to suggest that birds experiencing stress should be tested for chlamydiosis to prevent further spreading of the disease. Further comprehensive molecular works are underway using samples from exotic cage birds for the detection and characterization of *C. Psittaci.*

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