Chlamydia psittaci infection in canaries heavily infested by *Dermanyssus gallinae*

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Abstract

Dermanyssus gallinae is a haematophagous ectoparasite responsible for anemia, weight loss, dermatitis and a decrease in egg production. *Dermanyssus gallinae* may play a role in the modulation of the host immune system, maybe predisposing the host to some bacterial infections such as chlamydiosis. This is an important zoonosis. Humans are exposed to *Chlamydia psittaci* through inhalation of the agent dispersed from the infected birds. In this study, a syndrome observed in an aviary of canaries was investigated. A heavy infestation by *D. gallinae* was reported. Simultaneously, a *C. psittaci* infection was molecularly confirmed in the canaries. Combined therapy was applied successfully. The association of *C. psittaci* with the examined mites has been confirmed. Therefore, we think that *D. gallinae* have played a role in the spreading of *C. psittaci* infection among the canaries. Moreover, *D. gallinae* could have played an important role predisposing the canaries to the development of chlamydiosis, by inducing anemia and debilitation. The control of mites in the aviaries may represent a crucial step for the prevention of important infection such as chlamydiosis in birds and humans.

Key words: *Dermanyssus gallinae*, *Chlamydia psittaci*, canary, zoonosis.

Introduction

Dermanyssus gallinae, commonly named red mite, is a haematophagous ectoparasite of poultry; it can also infest pet birds although less common. The mite is responsible for anemia, weight loss, dermatitis and a decrease in egg production (Arends 2008). Moreover, *D. gallinae* has been proved to be a vector for a number of pathogens (Valiente Moro et al. 2008). Furthermore, there is preliminary evidence about an inhibition of Th1 response of the host following *D. gallinae* infestation (Harrington et al. 2010). This may lead to hypothesize a predisposing role of mites for bacterial infections as chlamydiosis.

   Chlamydia psittaci is an obligatory intracellular bacterium which mainly affects birds, especially those belonging to the order Psittaciformes, such as parrots, cockatoos, lories and parakeets. However, *C. psittaci* infections have also been reported in other birds belonging to the orders Columbiformes, Galliformes and Passeriformes (Andersen 2005; Magnino et al. 2009). As *C. psittaci* may affect humans, psittacosis is considered as a zoonotic disease. Humans are exposed to the infection through the inhalation of the agent dispersed in dust particles and air from respiratory secretions and dried feces shed by the infected birds. Domestic and companion birds are considered as the main risk for the transmission of psittacosis to humans (Harkinezhad et al. 2009). Canaries are a potential risk for human psittacosis since they are commonly bred as pet birds. However, to our knowledge, there is only one report of an infection from *C. psittaci* in such animals (Ferreri et al. 2007). Instead, there is plenty of data regarding *C. psittaci* infection in Psittaciformes.

   In psittacine species, the incubation period often ranges from a few days to a week or even longer. In latent infections, the clinical signs may occur several months or years after the exposure. Symptomatology includes lethargy, tremor, rough plumage, nasal discharge, conjunctivitis, rales, dyspnea and diarrhea (Andersen 2000; Andersen and Vanrompay 2008). During the chronic phase, the signs may be sub-clinical (Gerlach 1994). Depending of the avian host and strains, *C. psittaci* causes pneumonia, air sacculitis, peritonitis, hepatitis and splenitis (Andersen and Vanrompay 2008). Survived birds may become asymptomatic carriers. In that state, they do not show clinical signs and they intermittently shed the agent through nasal discharges, ocular secretions and feces, mainly following stress-related conditions such as overcrowding, handling, egg laying, breeding or treatments. Due to the lack of commercial vaccines, the control of chlamydial infection is based on the application of a correct management of the aviaries including cleanliness, sanitation and quarantine of pet birds (Smith et al. 2011). In fact, several factors such as other infections, parasite infestations

and overcrowding may predispose the susceptible birds or the asymptomatic carriers to the onset of the disease. In this paper, we described for the first time the role of *D. gallinae* in an outbreak of chlamydiosis in an aviary of canaries.

As there is some controversy in Chlamydiaceae taxonomy, in this study we followed the guidelines of the International Committee on Systematics of Prokaryotes (2010), stating that a single genus, *Chlamydia*, should include both genera *Chlamydia* and *Chlamydophila* previously split by Everett et al. (1999).

Materials and Methods

Case history

Clinical signs were observed on a farm (Marche, Italy) where 150 caged birds were reared. Eighty-two (55%) were canaries (*Serinus canaria*), but other birds belonged to European goldfinches (*Carduelis carduelis*) and bullfinches (*Phyrrula phyrrula*). Moreover, just a single chaffinch (*Fringilla coelebs*) was also present. Canaries were housed indoor whereas the other birds were reared outside in covered aviaries. In particular, 20 couples of canaries were housed in 20 different cages and 42 young canaries were reared in six cages (6-8 birds in a single cage). Only birds belonging to the flock of canaries showed clinical signs, all the other reared birds were healthy. Symptoms were ruffled plumage, weakness, anemia, itching, followed by death in a few days following the onset of illness. After 2 weeks since the beginning of the outbreak, 12% of the canaries died. Mortality drastically decreased soon after the treatment with doxycycline (250 mg/l drinking water), which was continued for 40 days. At the same time, in order to contrast an evident parasitic infestation, each canary was once treated with selamectin (a spot-on product) according to recent studies on the efficacy of various drugs in canaries (Todisco et al. 2008) and permetrin was also used for the treatment of cages and walls weekly for 3 weeks.

Sampling

Five dead canaries were sent for necropsy and laboratory investigations to the Avian Diseases Unit of the Veterinary Medicine Faculty of the University of Bari.

Bacteriological cultures

Bacteriological cultures on nutritious (blood agar; OXOID, Milan, Italy) and selective media (MacConkey agar; OXOID), incubated in either aerobic or anaerobic conditions at 37°C for

24 h, were performed using liver, lung, spleen and cardiac blood samples.

Parasitological investigations

A representative sample of ectoparasites was taken from dead birds. They were washed with lactophenol and identified by stereomicroscopy (ZEISS, Stemi SV11) following the morphology-based keys in Varma (1993) and Baker (1999).

DNA extraction

Liver and lung samples from the five birds were pooled and homogenized. Likewise, three aliquots, each consisting of about 40 *D. gallinae*, mainly adult engorged with blood, all collected from necropsied canary, were homogenized. One hundred µl from each tissue pool or mite homogenate was subjected to DNA extraction by using the Qiamp Blood and Tissue Kit (Qiagen, West Sussex, UK) according to the manufacturer's instructions.

PCR amplification and phylogenetic analysis

The identification of *C. psittaci* was achieved by a PCR approach based on 16S and 23S rRNA genes (rDNA). The primers were chosen among those described in literature as suitable for detection and identification of *Chlamydia* spp. (Table 1). The 16S rDNA first step PCR and the 23S rDNA PCR were carried out in a mixture containing 1 U of Platinum *Taq* polymerase (Invitrogen, Milan, Italy), 20 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl$_2$, 0.2 mM of each dNTP, 25 pmol of each primer and 4 µl DNA solution extracted from the samples. The reactions were performed in a Mastercycler (Eppendorf, Hamburg, Germany) for 1 cycle of 95°C for 5 min, followed by 35 cycles of 94°C for 30 s, 55°C (primer pair G16Sf/G16Sr) or 61°C (primer pair U23F/23SIGR) for 30 s, and 72°C for 1 min. The 16S rDNA second step PCR conditions were the same, except for using 12.5 pmol of primers Cps16Sf and CPS16Sr and 1 µl of the product of the first step PCR. Amplification products for each PCR were analyzed by electrophoresis using a 1.5 % agarose gel.

All PCR products from canary DNA were purified by EuroGOLD Cycle-Pure Kit (EuroClone, Milan, Italy). The amplicon of the appropriate molecular weight from PCR performed using mite DNA as template were eluted from agarose gel by using the EuroGOLD Gel Extraction Kit (EuroClone). The product from the first step 16S rDNA PCR was directly sequenced three times for both strands. The purified 23S rDNA PCR product from canary, and the eluted 23S and 16S rDNA PCR products from mites, were cloned in pGEM®-T Easy Cloning Vector (Promega, Milan, Italy), according to the manufacturer's protocol. For each

4

cloning experiment, no less than three positive clones were chosen and both strands of the cloned products were sequenced. Sequence determination was performed on an Applied Biosystem ABI 3100 at the Bmr Genomics (Padova, Italy). Sequence data were compared to submitted sequences in the GenBank and Ribosomal Database Project-II (RDP-II) release 10 (Cole et al. 2009).

For phylogenetic analysis, the 401 and 553 bp sequences from Chl19 16S and 23S rDNA, respectively, were aligned by the ClustalW algorithm with the corresponding portions of 16S and 23S rRNA genes from the type strains which are listed in Supplementary Material S1. Similarly we aligned 16S and 23S rRNA gene sequences from Chl19 and from mites. Minimum Evolution and Neighbor-Joining trees were both generated and branching order reliability was evaluated by 1,000 replications of bootstrap resampling. Multi-alignments and tree generations were performed by the MEGA v 5.05 software (Tamura et al. 2007).

The nucleotide sequences of the PCR products were submitted to GenBank with the following accession numbers: 16S and 23S rRNA gene fragments from canary (HQ 542857 and JN104392, respectively), 16S and 23S rRNA gene fragments from mites (JN104390 and JN104391, respectively).

Results

Clinical observation and mite identification

The five examined canaries showed severe anemia without weight loss and serious mite infestation levels (Figure 1). Ectoparasites were identified as *D. gallinae*. We counted about 30-40 mites per examined bird. Necropsy evidenced heavy anemia. Moreover, aerosacculitis, pericarditis, lung and spleen congestion were observed with peri-hepatitis and severe hepatitis representing the prevalent lesions (Figure 2).

Detection and identification of *Chlamydia psittaci* from canaries

Bacteriological cultures were all negative. The clinical signs and the necropsy observations were compatible with a *Chlamydia* spp. infection, so we carried out the nested PCR described by Messmer et al. (1997) to detect the pathogen, as it was proven to be sensitive and specific. The first 16S-rDNA PCR, performed using the genus-specific pair of primers, returned the expected amplicon of about 400 bp, while the species-specific nested PCR did not amplify any fragment. The 23S rDNA PCR returned the expected 600 bp amplicon.

To identify the species, we extended the analysis at DNA sequence level. The length of

the PCR products, excluding the primer sequences, was 401 and 553 bp for the 16S and 23S rDNA PCR amplicons, respectively. The BLASTn analysis of the sequence against archived sequences in GenBank revealed that the 553 bp sequence was 100% identical to the corresponding portion of nine *C. psittaci* genes for the 23S rRNA, while the 401 bp sequence was 100% identical to the corresponding portion of 17 *C. psittaci* genes for the 16S rRNA, and to one *C. abortus* gene for the 16S rRNA. However, the comparison of Chl19 16S rDNA sequence with those from type strains in RDP-II database, showed a higher seqmatch (S_ab) score (0.984) when it was aligned with *C. psittaci* 6BC[T], whereas the matching between Chl19 and *C. abortus* B577[T] resulted in a S_ab score of 0.969.

The 401 bp fragment from Chl19 partially overlapped the 16S signature sequence proposed by Everett et al. (1999), so we aligned it with the sequences of the correspondent fragments of 16S rDNA of both the type strains. We found only a single mismatch between the Chl19 amplicon and the corresponding fragment from *C. psittaci* 6BC[T] 16S rDNA (Supplementary Material S2a). The single nucleotide polymorphism (SNP) mapped at position 189 of the type strain 16S rRNA gene (guanine in Chl19 but adenine for 6BC[T]). Four SNPs distinguished Chl19 from B577[T]. The 23S rDNA sequence from Chl19 was 100% identical to the corresponding fragment of *C. psittaci* 6BC[T] 23S rDNA, whereas its similarity with the corresponding fragment from *C. abortus* B577[T] was 98%, as the two sequences differed for nine nucleotides (Supplementary Material S2b). Those findings led us to identify Chl19 as a *C. psittaci*.

Noteworthy, the only SNP which distinguished between Chl19 and 6BC[T] 16S rDNAs, could also account for the negative result of the nested PCR, since the third base from the 5' of Cps16S antisense primer mismatched the corresponding nucleotide, causing instability to the annealed oligonucleotide. Those findings also suggest a possible explanation for the reduced sensitivity of 16S-rRNA based nested PCR when compared to *ompA*-based nested PCR (Van Loock et al. 2005).

The identification was corroborated by the phylogenetic analysis. Minimum Evolution trees (Figure 3) and Neighbor-Joining trees were substantially identical, and they both grouped Chl19 together with *C. psittaci* 6BC[T].

Detection and identification of *Chlamydia psittaci* from mites

Total DNA was extracted from mites and PCRs with primers pairs G16Sf/G16Sr and U23F/23SIGR were performed. They both returned amplicons of the expected molecular weight (data not shown). The amplicons were cloned and sequenced, and the nucleotide

sequences were 100% identical to those from canary, previously identified as belonging to *C. psittaci*.

Treatment

Following molecular confirmation of *C. psittaci* infection, the caged birds had been treated with doxycycline for 40 days. Simultaneously, each canary has been spot-on treated with selamectin. Permetrins were weekly used for 3 weeks to disinfest cages and walls and prevent the recurrence of the infestation. The combined therapy against *Chlamydia* infection and ectoparasitic infestation led to a rapid improvement of bird health and to a strong decrease of mortality among caged birds and to the complete eradication of *D. gallinae* from the aviary.

Discussion

A heavy infestation by *D. gallinae* was observed in the flock of canaries at the same time with an outbreak of chlamydiosis. Several mites were recovered on the examined birds but the actual number of ectoparasites may be higher. In fact, since *D. gallinae* is a nocturnal feeder and spends the daylight hours in the cracks and crevices in the walls, floor and on the cages (Arends 2008), it is probable that many mites escaped from the hosts after the blood meal or, anyway, after the death of canaries before sampling. The infection by *C. psittaci* was confirmed by the analysis of the 16S and 23S rRNA genes. *Chlamydia psittaci* infections are rarely reported in canaries and, to our knowledge, only one case was previously confirmed (Ferreri et al. 2007).

It is interesting to underline that not always there is a close association between chlamydial infection and disease in canaries and others small caged birds. Therefore, infected animals do not always develop an illness status. In particular, following experimental infections with *C. psittaci*, Dovc et al. (2005) evidenced that canaries did not usually show symptoms or increased mortality rates, increasing the risk of the transmission of this infection to pet owners. In fact, although Psittaciformes are considered as the main source of the infection for humans (Harkinezhad et al. 2009), the transmission of *C. psittaci* from canaries to human is possible (Ferreri et al. 2007).

By contrast, the inspected flock was severely affected by the disease with 10% of the canaries dead during the outbreak. It is interesting to notice that the clinical signs were only observed in canaries, while goldfinches and bullfinches, reared in the same farm, appeared healthy. Therefore, factors other than *Chlamydia* infections could have played an important role by predisposing the canaries to the development of the disease. Indeed, environmental

conditions may have contributed to the onset of the outbreak, as the canaries were reared in a location characterized by poor ventilation, dust, overheating and overcrowding. Moreover, the heavy infestation of *D. gallinae* was observed in canaries but not in the other flocks of birds. It is highly possible that mites have found more suitable temperature and environmental conditions for their reproduction and settled in the aviaries inside rather than outside.

We strongly suggest a major role played by the mite infestations in predisposing the canaries to the chlamydial disease by inducing weakness and severe anemia. It is well known that *D. gallinae* is an obligatory blood-sucking mite which causes anemia and sometimes death, as well as dermatitis and decrease of egg production (Arends 2008). The effectiveness of the combined therapy with doxycycline (against chlamydiosis) and selamectin (against *D. gallinae*) confirmed the concurrent impact of both *C. psittaci* and *D. gallinae* in the development of the clinical signs. The detection of *C. psittaci* from the examined mites strongly suggests a major role played by *D. gallinae* also in the spreading of *C. psittaci* among the canaries. The nucleotide sequences from 16S and 23S genes of *C. psittaci* recovered from the mites and canaries were 100% identical.

These findings are relevant since *Chlamydia* spp. has been found in arthropods belonging to other families, such as Menoponidae, Glycyphagidae, Cheyletidae (Eddie et al. 1962) but, to our knowledge, it has never been previously detected in Dermanyssidae. Further efforts should be addressed to assess the interactions between *D. gallinae* and *C. psittaci*. *Dermanyssus gallinae* may have a relevant role of reservoir for *C. psittaci* as known for several viral and bacterial pathogens and parasites (De Luna et al. 2008; Valiente Moro et al. 2008). Therefore, *D. gallinae* could be included among the avian ectoparasites representing potential carrier for *Chlamydia* spp. and in particular for *C. psittaci*.

In conclusion, a concurrent infection with *D. gallinae* and *C. psittaci* was observed in canaries. *Chlamydia psittaci* is the etiologic agent of an important zoonotic disease (Rodolakis and Mohamad 2010). Although Psittaciformes are considered as the major source of *C. psittaci* for humans (Harkinezhad et al. 2009), the transmission of the bacterium from canaries is possible (Ferreri et al. 2007). *Dermanyssus gallinae* seems to be able to predispose the infected canaries to the development of the disease, increasing also the risk of *C. psittaci* infection in pet owners and farmers. Moreover, *D. gallinae* may serve as vector for *C. psittaci*, increasing its diffusion among the canaries, as well as for other pathogens in other birds (De Luna et al. 2008; Valiente Moro et al. 2008). Since *D. gallinae* can also infest mammals and humans (Cafiero et al. 2009), further studies may be useful to assess the possible vector role of *D. gallinae* for *C. psittaci* in humans.

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Figure legends:

Fig. 1 Canary heavily infested by *Dermanyssus gallinae.* Inset: Micrograph of *D. gallinae.*
Scale bar: 100 μm.

Fig. 2 Necropsy of the canary showing severe hepatitis and perihepatitis. The central white
square highlights the strongly anemic and degenerate liver; the white arrow indicates the edge
of the thickened and altered peritoneal capsule of the liver. The upper-left inset shows a
normal liver for comparison.

Fig. 3 Minimum Evolution trees of the strains compared with Chl19 (highlighted by the black
arrows). Bootstrap values are reported at the nodes. The phylogenetic trees have been
constructed using a portion of the 16S rRNA gene (a) and the 23S rRNA gene (b). The trees
are drawn to scale and they are rooted with the corresponding fragments from *Parachlamydia
acanthamoebae* Bn$_9$.