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DETECTION AND IDENTIFICATION OF *CHLAMYDOPHILA PSITTACI* FROM WILD SPOTTED DOVES IN THE KLANG VALLEY IN MALAYSIA

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SUMMARY

A total of fifty-six wild spotted doves was captured for the isolation and identification of *Chlamydophila psittaci* from the Klang Valley in Malaysia. Cloaca swab samples were taken from these doves for Clearview® chlamydia test and found that fourteen (25%) birds tested positive for chlamydiosis. From these fourteen positively infected birds, five showed clinical signs of poor feathering, chilling and diarrhoea. The less positively infected birds showed no clinical signs. Several methods were used for the identification of Chlamydia from the positively infected spotted doves, including indirect immunofluorescent and peroxidase techniques, Giemsa and Gimenez staining, and transmission electron microscopy (TEM). From all these methods, the chlamydia appeared as intracytoplasmic inclusion in the McCoy cell. In the TEM, two morphologically distinct forms of chlamydia, termed elementary body (EB) and reticulate body (RB) were seen in the infected cell.

Keywords: *Chlamydophila psittaci,* wild spotted doves

INTRODUCTION

Wild spotted doves (*Streptopelia chinensis*) are increasingly popular as pet birds in Malaysia. They are commonly kept by individual owners or placed in aviaries in recreational parks. Spotted doves are most commonly seen in open areas. They are not shy of humans and when approached, often keep pecking on the ground and fly off only at the last moment. Besides being kept as pets, doves are often trained in bird-singing contests and useful as decoy to trap the wild ones. Wild spotted doves are often heavily trapped for sale as cage birds.

The important disease commonly encountered in birds is avian chlamydiosis. Avian chlamydiosis is known to be zoonotic in nature. Therefore the disease is of public health significance because of the popularity of birds as pets and increased placement of birds at home for a long period. Many of the birds may be chronically infected but show no clinical signs until they are stressed. These birds often shed chlamydia organism intermittently and serve as a source of human infection and other healthy birds.

Chlamydiosis is caused by *Chlamydophila psittaci*, formerly known as *Chlamydia psittaci*. *Chlamydia psittaci* has been renamed as a separate species in the genus Chlamydophila as *Chlamydophila psittaci* (Everett *et al.*, 1999). The reclassification was based on a recent description of several novel chlamydia-related bacteria (Fritsche *et al.*, 2000, Horn *et al.*, 2000) and genetic analysis (Bush and Everett, 2001; Hermann *et al.*, 2000). *Chlamydophila psittaci* is an obligate intracellular coccoid gram-negative organism that can induce disease in most species of wild and domestic birds as well as in human beings. Chlamydia infection in birds, mammals or other animals is generally referred to as chlamydiosis. The disease in human beings caused by chlamydia contracted from psittacie birds was called psittacosis (Cullen, 1993; Salisch *et al.*, 1996). Ornithosis, a term first used in 1941, was introduced to describe chlamydia infection in human beings contracted from non-psittacine birds (Meyer, 1941). Chlamydia has been isolated from many avian species and is most commonly identified in psittacine birds (Fudge, 1989). Among non-psittacine caged birds, infection occurs most frequently in pigeons, doves, and mynah birds (Andersen and Tappe, 1989).

Detection and identification of chlamydia can be attempted by various methods, including culture, histochemical stains, antigen antibody detection, transmission electron microscopy and nucleic acid amplification. Chlamydiosis status in wild spotted doves in Malaysia is virtually unknown. Therefore, this study was done to determine the presence of *Chlamydophila psittaci* in wild spotted doves in Malaysia.

MATERIALS AND METHODS

Wild spotted doves

A total of 56 wild spotted doves was captured from Klang Valley for the research. Cloaca swab samples were taken using cotton wool swabs for Clearview® chlamydia test (Clearview Chlamydia, Unipath limited). A total of five birds showed clinical signs of poor feathering, chilling and diarrhoea. These clinically infected doves were kept for further research and were later found to have chronic diarrhoea.

Propagation of Chlamydia

The cloaca swab samples were collected from the clinically infected doves and were transfered into 1 ml of sucrose phosphate glutamine (SPG) transport medium (Mukhopadhyay *et al.*, 2004). The SPG samples were centrifuged at 1000 rpm for 15 min and 0.1 ml of the supernatant was collected and inoculated onto the confluence McCoy cells in 24-well cell culture plates. The McCoy cell was grown on sterile glass coverslip in each well of a 24-well cell culture plate. Negative and positive controls were prepared in the cell culture plate. After inoculation, the plates were centrifuged at 3000 rpm for 1 h at 29°C and followed by incubation at 37°C for 2 to 3 h in a CO_2 incubator. The inoculum was then discarded and replaced with 1 ml of growth medium containing cycloheximide (1ug/ml) in each well. The plates were then incubated in 5% CO_2 at 37°C for 48 to 72 h.

Indirect immunofluorescent technique (IIF)

After 48 to 72 h incubation, the growth medium of the infected cell culture was discarded. The glass coverslips cell culture plate were washed with phosphate buffered saline (PBS) and drained dry. Cold acetone methanol (50/50; v/v) was added to each glass coverslip in the well to fix the cells for about 10 min. The glass coverslip was then drained dry and 35μl of unconjugated specific monoclonal antibody (Whittaker Bioproducts, MD) was added to the glass coverslip to cover the cells, followed by incubation for 25-30 min at room temperature in a moist chamber. It was then washed with PBS and drained dry. A drop of fluorescein isothiocyanate (FITC) conjugated antibody was added, followed by incubation for 30 min at room temperature. It was then washed and mounted in buffered glycerol and was examined under a fluorescence microscope.

Indirect immunoperoxidase technique (IIP)

Glass coverslips that contained confluent monolayer of McCoy cells were prepared, inoculated with the sample as mentioned above and observed as in indirect immunofluorescent (IIF) technique. To process the infected cell culture for indirect immunoperoxidase (IIP) technique, a combination method described by Bancroft and Cook (1988) and the Harlow and Lane (1989) was used. Briefly, the fixed McCoy cell was immersed in 0.3% H_2O_2 in absolute methanol for 30 min. It was then incubated in blocking buffer (3% BSA in 0.05M Tris buffer pH7.6) for 15 min, followed by covering it with unconjugated specific monoclonal antibody (Whittaker Bioproducts Inc, MD). The glass coverslip was incubated for 1 h, followed by 3 washes in PBS. After the three washings, 1: 250 dilution of goat anti-mouse peroxidase labeled IgG was added on the glass coverslip and incubated for 1 h in room temperature. The glass coverslip was then washed. The substrate of 4-chloro 1-naphthol was added and it was then incubated until blue-black reaction was developed.

Giemsa and Gimenez stain

The infected cell culture on the glass coverslips were fixed with acetone methanol (50/50; v/v) and stained with Giemsa (Campbell, 1986) and Gimenez (Andersen and Tappe, 1989) stain. The glass coverslips were rinsed with water and then mounted in the buffered glycerol for examination under a light microscope.

Transmission electron microscopy (TEM)

Following the method described by Kluge *et al.* (1972), the infected McCoy cell culture was rinsed free of medium with PBS and scrapped off to collect the cells. The cells were pelleted by 2000 rpm centrifugation for 20 min at 4°C. The pelleted cell was cut into a number of 1mm^3 pieces and put into separate vials, then followed by fixing in 4% buffered glutaraldehyde solution for 12 to 24 h at 4°C. The pellets were then washed with 0.1M sodium cacodylate buffered 3 times for 10 min each. The pellets were then subjected to post-fixation with osmium tetroxide for 2 h at 4°C. The post-fixed blocks were rinsed 3 times for 10 min each in 0.1M sodium cacodylate buffer and dehydrated in a series of acetone (35%, 50%, 75% and 95% in distilled water) for 10 min each and in 100% acetone, 3 times for 15 min each. After removal from the 100% acetone, the blocks were infiltrated with resin and acetone mixture (50/50; v/v) for 1 h and (1/3;v/v) for 2 h, followed by 100% resin overnight. The next day, the blocks were infiltrated with 100% resin for 2 h, embedded into beam capsules and filled up with the resin. The resin was polymerised in an oven at 60°C for 24 to 48 h. After each step, the blocks were subjected to thick sectioning and placed onto glass slides and stained with toluidine blue. The stained glass slide was examined under a microscope to select the best area of the block for ultrathin sectioning. The ultrathin sections were cut and mounted onto electron microscope copper grids to be subjected to uranyle acetate and lead citrated staining.

RESULTS

A total number of 56 cloaca swab samples from wild spotted doves was tested for Clearview® chlamydia test and found that 14 (25%) birds tested positive for chlamydiosis. From the 14 positively infected birds, five showed clinical signs of poor feathering, chilling and diarrhoea. The less positively infected birds showed no clinical signs.

The chlamydia can be seen as apple green inclusions in the cytoplasm of the McCoy cells (Figure 1) in the IIF technique. Sometimes multiple inclusions can be seen in

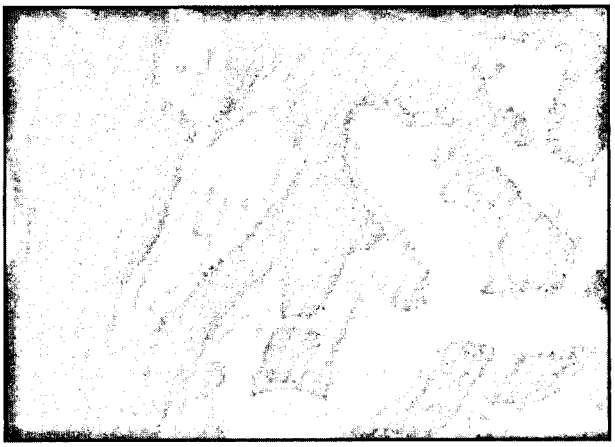

Figure 1: The chlamydia can be seen as apple green inclusion in the cytoplasm of the McCoy cells.

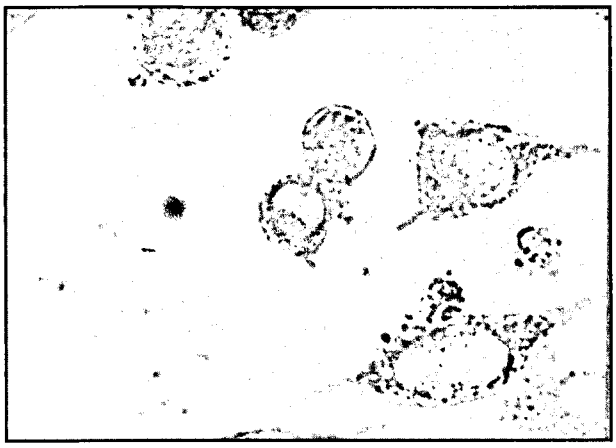

Figure 2: The inclusion of chlamydia can be seen as bluish black in colour in the McCoy cell.

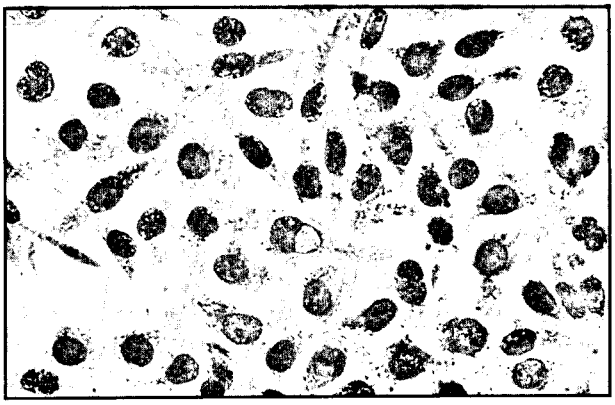

Figure 3: McCoy cells showed the presence of bright white-green inclusions in the cytoplasm.

Figure 4: Staining of infected McCoy cells with Gimenez stain showed the presence of bluish red intracytoplasmic inclusions.

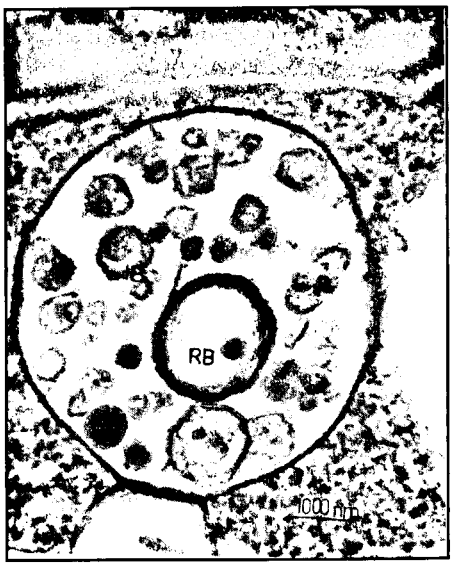

Figure 5: The various morphological forms of chlamydia, the EB, RB and IB under TEM (x 70 000 magnification).

Figure 6: The EBs were released extra-cellularly when the McCoy cell lysed (x 40 000 magnification).

the cytoplasm of the McCoy cells. The apple green intracytoplasm inclusion was at the final stage of the development cycle (48h), and mostly contained elementary body (EB). The apple green granule can sometimes be seen extra-cellularly indicating lysis of the McCoy cells and release of EB from the cytoplasm. In the IIP technique, inclusion of *Chlamydophila psittaci* can be seen as bluish black colour (Figure 2). In the Giemsa stain, McCoy cells that were infected with chlamydia revealed presence of intracytoplasmic inclusions. These inclusions appeared as granulated bodies, which stained dark purple within the purple-blue cytoplasm. The nuclei of the same infected McCoy cells were stained dark purple-red. Some other infected McCoy cells showed the presence of bright white-green inclusions in their cytoplasm (Figure 3). Such inclusions was not observed in McCoy control cells.

Staining of infected McCoy cells with Gimenez stain showed the presence of bluish red intracytoplasmic inclusions within a greenish background (Figure 4). However, the control cells were stained green but with no inclusions when they were examined under a light microscope. In the TEM, two morphologically distinct form of chlamydia, termed elementary body (EB) and reticular body (RB) were seen in the infected cell TEM. Figure 5 shows the various morphological forms of chlamydia, the EB, RB and intermediate body (IB). The chlamydia inclusion contained predominantly reticular bodies (RB). Some of them divided to IB and RBs from EB in the process of condensation into EBs. Multiple inclusions were also observed. In Figure 6, the EBs were released extra-cellularly when the McCoy cell lysed.

DISCUSSION

In the study, the chlamydia organism has been successfully isolated from the wild spotted doves captured from Klang Valley and 25% were found positive for Chlamydophila psittaci. From the 14 (25%) positively infected birds, five of them showed clinical signs of poor feathering, chilling and chronic diarrhoea. While the others nine positive birds showed no clinical signs but by continuously shedding the organism, they might serve as latent carriers. Fudge (1990) reported that while the prevalence rate of chlamydial infection might range from 10 to 40%, the carrier rate might exceed 90%. In a 1976 to 1984 survey, he found 13% of the samples tested positive for chlamydiosis and that pigeons, collared doves and psittacies were mostly affected (Bevan and Bracewell, 1986). Chlamydiosis can occur as a chronic inapparent, subacute, acute or peracute infections (Arizmendi and Grimes, 1995). Common reported clinical signs are diarrhoea, anorexia, depression, biliverdinuria, sneezing, mucopurulent nasal discharge, dyspnoea, sinusitis and conjunctivitis. Clinical signs associated with chronic infections include poor feathering, wasting, diarrhoea and sometimes conjunctivitis (Ruplesy, 1997).

The chlamydia was successfully detected in the infected McCoy cell as demonstrated by staining with Gimenez and Giemsa stains. It was confirmed to be *Chlamydophila psittaci* using IIF, IIP and TEM techniques. In IIF, the inclusions appeared as bright apple green granular intracytoplasmic bodies. Schachter and Dawson (1979) revealed that the chlamydia inclusions in IIF appeared as discrete bright green granular intracytoplasmic bodies. The same types of inclusions have also been observed by other researchers (Quinn *et al.*, 1994; Grimes and Wyrick, 1991). In Giemsa stain, two types of inclusion were observed under the light microscope. The first type of inclusion appeared as bright white-green granular bodies and the second inclusion appeared as dark purple granular bodies. The inclusion of chlamydia stained with Giemsa appeared as basophilic intracytoplasmic inclusion in McCoy cells (Quinn *et al.*, 1994). In the Gimenez method, Grimes and Wyrick (1991) found that the chlamydial elementary bodies (EBs) appeared bluish red in colour. Only the EBs inclusion is more meaningful in diagnostics because reticular bodies (RBs) are easily mistaken for normal cellular structures and are not readily differentiated from the background colouration (Grimes and Wyrick, 1991). In this study, the inclusions appeared as bluish red using Gimenez stain as described by Grimes and Wyrick (1991). In TEM, the major morphological features of intracellular chlamydial development in 7-day-old cell culture are EB, intermediate body (IB) and RB. The elementary body is a small dense, spherical body, about 200-300 nm in diameter. The RB is the intracellular, metabolically active form, which divides by binary fission and is larger than EB, about 600-1500 nm in diameter.

In conclusion, the results showed the presence of chlamydia infection in wild spotted doves. The widespread occurrence of chlamydia infection in doves, especially pet birds, is dangerous to human beings as this infection is zoonotic. Any human being that comes in contact with the infected birds will in turn be infected by the organism. However, according to Fudge (1989), many people have been exposed to chlamydia shedders but experience no clinical episode. Chlamydiosis in human beings is dependent on strains and pathogenicity of the chlamydia organism.

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