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Investigations on the Prevalence of Chlamydiosis in Turkey Flocks in Egypt with Special Emphasis on Immunopathological Characterization of C*hlamydophila psittaci*

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Abstract: The present study was done to investigate the presence of *Chlamydophila psittaci* in fattening turkey birds from different farms at Giza governorate, Egypt. The antigen of *C. psittaci* was demonstrated in impression smears from turkey internal organs (liver, lung, heart and spleen) by immunofluorescent test whereas, 89.4% of the examined organs were positive. *C. psittaci* was isolated from poolings of internal organs and fecal swabs samples via tissue culture inoculation. The inoculated Vero cells were examined by Giemsa stain for the presence of *C. psittaci* inclusions and cytopathic effect (CPE) and by the transmission electron microscopy for the presence of *C. psittaci* different developmental forms; the elementary, intermediate and reticulate bodies. In conclusion, the high incidence of Chlamydiosis in turkey calls for more efforts to control this zoonotic disease.

Key words:*Chlamydophila psittaci* · Turkey · Immunofluorescent test · Lsolation · Transmission electron microscopy

INTRODUCTION

Worldwide, turkey production suffers from the negative economical impact of respiratory diseases. Nearly all turkey flocks experience one to multiple periods of respiratory disease leading to economic losses due to expensive antibiotic treatment, weight loss, decrease egg production, increased mortality and carcass condemnation at slaughter. *Chlamydophila psittaci* is an important turkey pathogen that causes infections of mucosal epithelial cells and macrophages of the respiratory tract followed by septicemia and localization in epithelial cells and macrophages in various organs. *C. psittaci* can be a primary respiratory pathogen as well as an important complicating agent in any outbreak of respiratory disease in turkeys [1]. Nevertheless, *C. psittaci* is a threat to public health since this zoonotic agent is able to infect poultry workers [2,3].

Efforts to detect and identify *chlamydiae* are important because *chlamydiae* not only cause disease but also interact synergistically with viruses or with other bacteria, increasing the virulence of these organisms [4].

The present study was carried out to investigate the presence of *Chlamydophila psittaci* in fattening turkey birds from different farms at Giza governorate, Egypt with emphasis on the use of immunopathological characterization of the causative organism.

MATERIALS AND METHODS

Samples: Seventy six different turkey internal organs (liver, lung, heart and spleen) were freshly collected from randomly selected birds (Either after death or after slaughter). Each organ was used fresh for making impression smears from the cut surface. Three impression smears from each organ were fixed in cold acetone and used in immunofluorescent test. Tissue samples used in isolation were ground in a sterile mortar using sterile sand, then the volume of diluent [PBS] required to make a 10-20% emulsion was added and the suspension was thoroughly mixed. Suspension was centrifuged at 3000rpm/15min and the supernatant was collected. Antibiotics [Streptomycin 2.5 mg/ml, neomycin 0.5 mg/ml and nystatin 100units/ml] were added to the supernatant and the tubes were held for 1 hour at room temperature, recentrifuged 2 times and the final supernatant was used in isolation.

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Forty fecal swabs were collected, diluted to 20% in Phosphate Buffer Saline (PBS) "pH 7.2-7.4" and clarified by centrifugation at 3.000 rpm for 15 minutes. Samples 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, recentrifuged 2 times and the final supernatant was used in isolation.

Immunofluorescent Test: Used according to Lecomte [5] for detection of *C. psittaci* antigen in organs impression smears samples. Shortly; The organs impression smears were fixed with cold acetone at-20° C for 30-60 minutes, flooded with 100ul reference turkey positive control serum to *Chlamydophila psittaci*, incubated at 37° C for one hour in a moist chamber, washed with PBS 3 times; for 10 minutes each, with agitation, flooded with 100 ul fluorescein labeled anti turkey conjugate (Kirkegard and Perry Laboratories (KPL), Inc. Gaithersburg MD USA] for one hour at 37°C in a moist chamber, washed as mentioned before, air dried and the slides were covered with 10 ul mounting buffer (50% glycerin+50% PBS) and then covered with the coverslips. Each slide was examined under the fluorescence microscope (at magnification of 100-500X). The positive samples appeared as starry sky; fluorescent green spots on a dark background.

Cell Culture Isolation: Pools of organs and fecal swabs were inoculated in Vero cell line cells for isolation of *C. psittaci* according to McElnea and Cross [6]. Shortly; under complete aseptic conditions, the growth media was decanted from each well of the tissue culture plate and 200 ul of each specimen (after vortex three times for 4 minutes each) were added to each well of the tissue culture plate included a positive control specimen. Each sample was inoculated in two wells. The tissue culture plate was put in a shaker for 1 hour (speed: 60 times/minute) at 37° C, the maintenance media was added (5 ml EMEM, pH 7.2, containing 5% fetal calf serum) and plates were incubated for 48-72 hours at 37° C in a humid chamber. After incubation period of 48-72 hours, the tissue culture plates were stained with Giemsa stain and examined microscopically for the presence of intra-cytoplasmic inclusion bodies.

Detection of *C. psittaci* Inclusion Bodies by Giemsa Stain: The media was decanted and the infected monolayer cells were fixed by methyl alcohol for 3-5 minutes. The diluted Giemsa stain was prepared according to Monica [7] and all the tissue culture plates were covered with the fresh diluted stain for 10 minutes. The stain was washed off with buffered water and the tissue culture plates were allowed to dry in air and examined under tissue culture inverted microscope at (200X and 500X) for the presence of *C. psittaci* inclusions and CPE.

Demonstration of *C. psittaci* by Transmission Electron Microscopy (TEM): Negative staining of samples for TEM was done according to Hazelton and Gelderblom [8]. Tissue culture flasks were harvested after 3 times freezing thawing then centrifuged for 15 min at 3000 rpm. The supernatant was collected and centrifuged again for 45 min at 13000 rpm. After discarding the supernatant, the pellet was rinsed carefully with distilled water. A droplet of 3% phosphotungstic acid (PTA) was mixed with a droplet of the sample and a copper grid coated with carbon formvar was dipped into the mixture. After drying, the grid was examined with the TEM.

RESULTS

The present study was done on fattening turkeys reared at different farms at Giza governorate, Egypt. Veterinarians supervising these farms provided information indicating the presence of one or more clinical respiratory symptoms throughout the rearing period.

Immunofluorescent test has been used for the detection of *C. psittaci* antigen in impression smears of liver, heart, lung and spleen from turkeys. *C. psittaci* inclusions were demonstrated in 68 out of 76 (89.4%) samples (14 out of 15 examined livers, 11 out of 12 examined lungs, 10 out of 14 examined heart and 33 out of 35 spleens impression smears) examined by immunofluorescent test. *C. psittaci* inclusion appeared as starry sky; fluorescent green spots on a dark background (Photo 1).

Pools of organs and fecal swabs were inoculated in Vero cell line cells for isolation of *C. psittaci*. All of the pools of organs and fecal swabs inoculated in T.C. were positive proved by induction of the cytopathic effect (consisted of a rounding of the affected cells and the appearance of pyknotic nuclei) with the characteristic intracytoplasmic inclusion of *C. psittaci* that appeared with Giemsa stain as large reddish purple to bluish purple intracytoplasmic vacuoles with the nucleus pushed aside (Photo 2).

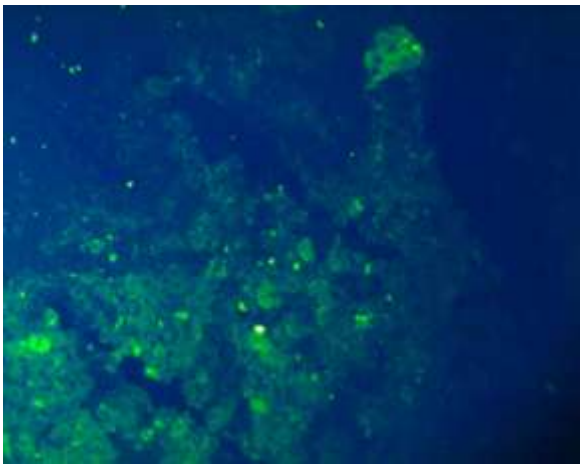

Photo 1: Turkey liver impression smear examined by immunofluorescent test. *C. psittaci* inclusions appeared as starry sky; fluorescent green spots on a dark background

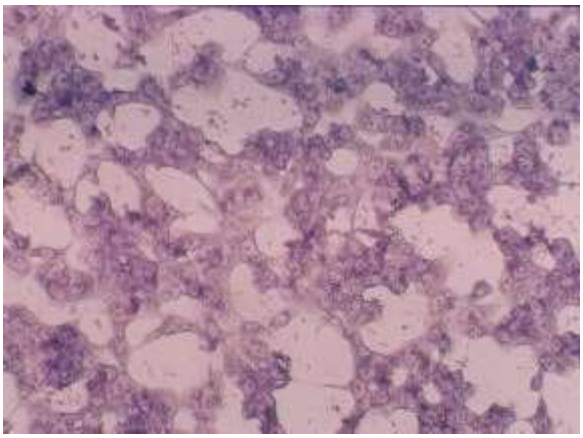

Photo 2: Giemsa stained Vero cells containing CPE and intracytoplasmic inclusion bodies of *C. psittaci* (X 500)

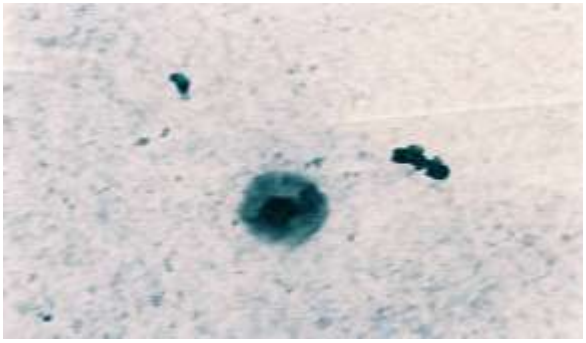

Photo 3: Transmission Electron Microscopy of harvest of Vero cells infected with *C. psittaci* showing the elementary bodies (E) with their dense gene core (X 50000)

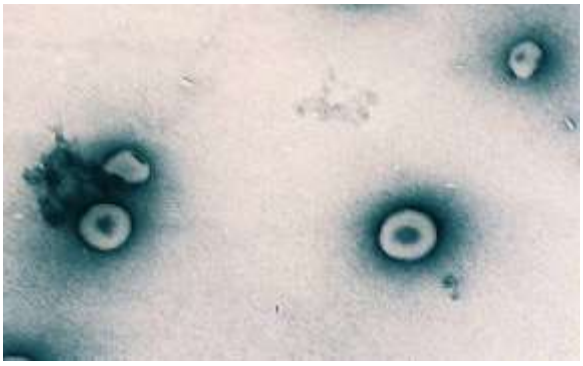

Photo 4: Transmission Electron Microscopy of harvest of Vero cells infected with *C. psittaci* showing the intermediate bodies (I) with their characteristic condensed nucleoids of nucleic acid (X30000)

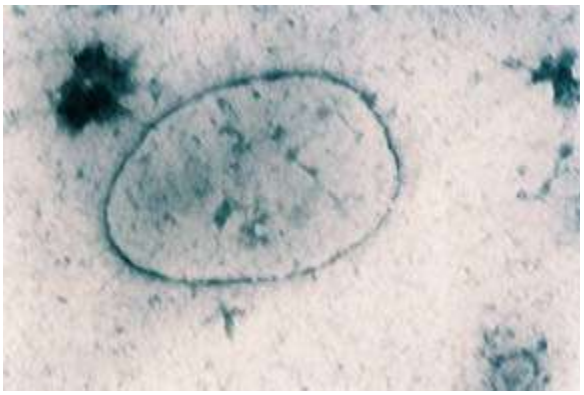

Photo 5: Transmission Electron Microscopy of harvest of Vero cells infected with *C. psittaci* showing the large, fragile, reticulate bodies (R) (X 50000).

Inoculated tissue culture was examined with Transmission Electron Microscope after negative staining of the tissue culture harvest. The different developmental forms of the microorganism have been demonstrated (Photos 3-5). Elementary bodies appeared with their dense gene core, reticulate bodies were large, fragile and the smaller intermediate bodies appeared with their characteristic condensed nucleoids of nucleic acid.

DISCUSSION

Impression smears from turkey organs were examined for the presence of *C. psittaci* antigen by immunofluorescent test. Immunofluorescent test has been used by many researchers for diagnosis of *C. psittaci* infection [9-12]. Sixty eight out of 76 (89.4%) turkey

internal organs were positive with immunofluorescent test. These results are close to that of Vlahovic *et al.* [13] who found *C. psittaci* antigen in 73% of the examined birds using the direct Immunofluorescent (DIF) test. The same method was used to prove the antigen in 88.4% of the examined organs. However the incidence is greater than those reported by Baldelli *et al.* [14], who used the immunofluorescence test to detect Chlamydial antigen in 74 of 252 meat turkeys swabs.

Organs and fecal swabs samples were pooled and inoculated on Vero cell tissue culture for isolation of *C. psittaci*. Pooled visceral organs and fecal swabs are commonly used for isolation of *C. psittaci* in tissue culture [15-17]. Pools of organs and fecal swabs were inoculated on Vero cell line. Vero cell line supported growth of the largest number of most known chlamydial strains as well as supported the rapid growth of the organism [18,19]. All of the pools of organs and fecal swabs were positive after 1 to 3 passages proved by induction of the cytopathic effect with the characteristic inclusion of *C. psittaci* that appeared as large purple intracytoplasmic vacuoles with the nucleus pushed aside. This agrees with what found by Vanrompay *et al.* [10] who isolated *Chlamydiae* from all the examined birds after 1 of 3 passages on cell cultures using samples taken from lung, liver and spleen. These results are in agreement also with that of Tessler [18] who found that most turkey strains induced cytopathic changes rapidly when he grew 6 turkey *C.psittaci* strains on Vero cells.

Tissue culture harvest was examined with Transmission Electron Microscope after negative staining. The different developmental forms were demonstrated. The electron microscopic diagnosis is uniquely suited for rapid identification of infectious agents. A specimen can be ready for examination and an experienced technologist can identify, by electron microscopy, a viral pathogen morphologically within 10 minutes of arrival in the electron microscopy laboratory [8]. After a simple and fast negative stain preparation, the undirected, “open view” of electron microscopy allows rapid morphologic identification and differential diagnosis of different agents contained in the specimen. Electron microscopy can be applied to many body samples and can also hasten routine cell culture diagnosis. However, it should be quality controlled, applied as a frontline method and be coordinated and run in parallel with other diagnostic techniques. As in Photos 3-5 different developmental forms of *C. psittaci* were demonstrated by Transmission Electron Microscopy. The elementary bodies (E) with their dense gene core, the large, fragile, reticulate bodies (R) and the smaller intermediate bodies (I) of *C. psittaci* with their characteristic condensed nucleoids of nucleic acid were demonstrated. These forms were described by Ward and Vanrompay *et al*. [20, 21] who said that chlamydial elementary bodies (EBs) are small, round or occasionally pear shaped, electron-dense rigid structures approximately 0.2 - 0.3 microns in size, has an electron dense "black" core of nucleic acid condensed onto chlamydial histone protein. The elementary bodies begin to differentiate to reticulate bodies with a diameter of 0.5 to 2.0 μm and become fragile and pleomorphic. The nucleic acid core becomes less marked. Reticulate bodies begin binary fission after that differentiate again into elementary bodies (E). The initial sign of this is the re-condensation of chlamydial nucleic acid on to histone protein. This stage is called the intermediate bodies, (I) that are 0.3 to 1.0 microns in size.

In conclusion, the prevalence of chlamydiosis in meat turkey flocks is high. The microorganism was demonstrated directly in internal organs and after isolation from fecal swabs and internal organs and that calls for certain measures to prevent spread of infection to other birds, mammals and human.

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