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Isolation and Identification of Chlamydophila in poultry species in Egypt

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Abstract : *Chlamydia psittaci* infects many domestic birds poultry,large animals and mammals. *Chlamydia psittaci* is of great economic importance and cause sporadic but sometimes devastating disease in humans. Avian chlamydiosis (AC) is caused by the bacterium *Chlamydophila psittaci* that causes a severe loss in meat and egg production. In this study we collected 466 cloacal swabs,311ocular swabs and 205 nasal swabs from diseased and apparently healthy poultry species (turkey, pigeons, duck and chickens) from farms and Souq Al-Qalaah in Sayedah Aisha, these samples were isolated in specific pathogen-free embryonated chicken eggs (SPF-ECE) and stained with specific stain (Giemenez stain). *Chlamydia psittaci* were demonstrated in 370 samples from 497(74.5%)in turkeys, 282 samples from 356(79.2%) in pigeons, 5 samples only from 89 (5.6%) in ducks and just 7 samples from 40 were positive (17.5%) regarding chicken. Isolation of *Chlamudia psittaci* in SPF-ECE and identification by Giemenez stain confirmed to be the gold standard method.
Keywords : Chlamydia psittaci, isolation, identification, poultry and Gimenez stain.

Introduction

Avian chlamydiosis is caused by *Chlamydia psittaci* and is systemic, infectious, zoonotic and occasionally fatal disease in domestic and wild birds[1].*Chlamydia psittaci*, a Gram-negative obligate intracellular bacterium, is an important turkey pathogen causing infections of mucosal epithelial cells and macrophages of the respiratory tract, followed by septicaemia and localization in epithelial cells and macrophages in various organs [2].Infection can be clinically in apparent or result in disease of respiratory tract, conjunctiva, serous membranes, joints, central nervous system (CNS), reproductive organs, and gastrointestinal tract. Reservoirs of the organism include the respiratory, intestinal and genital tracts of birds and mammals with transmission occurring via inhalation or ingestion [3]. *Chlamydia psittaci* infects many domestic birds and poultry. Moreover, birds in psittacidae, pigeon, dove, canary, turkey, duck, water birds [1]avian chlamydiosis (AC) is caused by the bacterium *Chlamydophila psittaci*. The disease in birds was originally called psittacosis, but the term ornithosis was introduced later to differentiate the disease in domestic and wild fowl from the disease in psittacine birds. The two syndromes are currently considered to be the same[1]. Their earlier separation was based on the assumption that in humans, ornithosis was a milder disease than psittacosis. However, it should be noted that the disease in humans contracted from turkeys and ducks is often as severe as at contracted from psittacine birds Infection of birds with *Chlamydophila psittaci*is common all over the world and has been found in about 465 avian species [2]. Outbreaks of AC in psittacine birds and domestic poultry farms cause considerable economic damage [4].The infection can lead to systemic and occasionally fatal disease in birds. The clinical signs are

generally nonspecific and vary greatly in severity depending on the species and age of the bird and the strain of chlamydia. AC can produce lethargy hyperthermia, abnormal excretions, nasal and eye discharges, and reduced egg production. Mortality rates will vary greatly. In pet birds the most frequent clinical signs are conjunctivitis, anorexia and weight loss, diarrhea yellowish droppings, sinusitis, biliverdinuria, nasal discharge, sneezing, lachrymation and respiratory distress [5]. Many birds, especially older psittacine birds, may show no clinical signs; nevertheless, they may often shed the agent for extended periods. Necropsy of affected birds will often reveal multifocal hepatic necrosis spleen and liver enlargement, fibrinous airsacculitis, pericarditis and peritonitis [1,6]. Histological lesions are suggestive of infection but are non-pathognomonic unless there are identifiable chlamydiae present avian chlamydiosis (AC)is caused by the bacterium *Chlamydophila psittaci*., the genus Chlamydiawas divided into two, Chlamydia and Chlamydophila. A proposal to re-combine them into the single genus Chlamydia is under consideration but has not been adopted for this chapter. The avian strains of *Chlamydophila psittaci* include at least fifteen genotypes, some of which correlate with the avian species from which theyare usually isolated. Chlamydiosisas it occurs naturally in mammalian species and not contracted from avian species is caused by different species of the organism. Depending on the virulence of the chlamydial strain and the avian host defense, Special laboratory handling (biosafety level 3)is recommended because avian chlamydial strains can cause serious illness and possibly death in humans. Whilethe disease in psittacine birds is best known, the infection in ducks and turkeys is of particular concern as transmission to humans is common during handling and slaughter of the birds. The diagnosis of AC requires the isolation and identification of the organism, the demonstration of chlamydiae in tissues, or the demonstration of a four-fold increase in specific humoral antibody, aswell as typical Clinical signs[7]. Identification of the agent: Isolation of chlamydiae requires the inoculation of embryonated eggs or cell cultures and testing for chlamydiae by cytochemical stains or immunohistochemical methods. The direct inoculation of samples into cell cultures is preferable asthey are as sensitive for the isolation of most avian strains of chlamydiae as are chicken embryos. The cell cultures are then stained by immunofluorescence or by other appropriate stains at appropriate times to demonstrate the presence of inclusion[1]. Chlamydial infections in turkeys present significant economical losses, but public health is also a consideration, since veterinary surgeons and poultry workers are at risk of becoming infected by this zoonotic agent. Chlamydia vaccines are non-existent, although considered efforts have been made [8,9,10,11].Isolation of *Chlamydia psittaci* in (ECG-SPF)Chicken embryos are still used for the primary isolation of chlamydiae. The standard procedure is to inject up to 0.5 ml of inoculum into the yolksac of a specific pathogen free 6–7-day-old embryo. The eggs are then incubated in a humid atmosphere at 39°C, rather than at 37°C, as multiplication of chlamydiae is greatly increased at the higher temperature. Replication of the organism usually causes the death of the embryo within 3–10 days. If no deaths occur, two additional blind passages are usually made before designating any sample as negative. Chlamydial infections will give rise to a typical vascular congestion of the yolk sac membranes. These are harvested and homogenised as a 20% (w/v) suspension in SPG buffer and can befrozen to preserve the strain, or inoculated into eggs or on to cell cultures. The organism can be identified by preparing an antigen from an infected yolk sac and testing it by direct staining of smears using appropriate stains (Giemenez stain) or by using the antigen in a serological test. Cell culture monolayers can be inoculated with the yolk sac suspension and examined by direct immunofluorescence48–72 hours later for the presence of chlamydial inclusions. Typical inclusions are intracytoplasmic round or hat-shaped bodies. With some virulent strains, the inclusions rapidly break up and the chlamydial antigen is dispersed throughout the cytoplasm[9].

2. Materials and methods:

2.1. Sampling:

Four hundred and sixty six cloacal swabs,three hundred and eleven ocular swabs and Two hundered and five nasal swabs from farms and souq al-Qalaah in Sayedah Aisha were collected using sterile swabs in clean labeled tubes containing PBS "7.2 pH" from living diseased and asymptomatic animals (turkey, pigeons, duck and chicken) **(Table1)**. The samples were transferred to the laboratory as soon as possible. And in **(Table 2)** record the types of fecal, ocular and nasal swabs collected from asymptomatic or sub clinical and diseased animals**.**

Table1. Records the sources, types and number of the examined samples

types of samples tested	Turkey	Pigeon	Duck	Chicken	Total
Ocular swabs	100	175	36	-	311
Cloacal swabs	285	125	36	20	466
Nasal and pharangyl swabs	112	56	17	20	205
	497	356	89	40	982

Table2. Records the types of fecal, ocular and nasal swabs collected from asymptomatic and diseased animals

Type of swab samples tested		Number and types of swab samples		
		Cloacal swabs	Ocular swabs	Nasal swabs
Turkey	Asymptomatic	57	17	33
	Diseased	228	83	79
Pigeon	Asymptomatic	25	44	15
	Diseased	100	131	41
Duck	Asymptomatic	10	10	7
	Diseased	26	26	10
Chicken	Asymptomatic	5	-	4
	Diseased	15	-	16
Total		466	311	205

Methods of Chlamydialisolation

Chicken embryo inoculation via the yolk sac method[13]

The fertile eggs (7-8 days) were cited in a carton, large end up. The eggs were examined just before inoculation to determine embryo activity. Eggs were labeled with pencil, 3 embryonated chicken eggs (ECE) were used for each sample. The top of the eggs were swabbed with Betadine.18-gauge needle was used to puncture a hole through the shell at the top of the egg.200µl of the sample (previously grinded by PBS and treated with antibiotics [streptomycin and gentamicin] was inoculated into each of the eggs being used per sample. The hole in the top of the egg was then covered with sterile adhesive tap and incubated at 37˚C in a humidified incubator. Non inoculated control eggs were labeled and incubated with the inoculated eggs and subjected to daily observation. The eggs were candled daily. The eggs that died within 3 days of inoculation were discarded while those that died after day 3 to day 10 are opened and the yolk sac membranes were harvested.

Chicken egg harvesting

1. Preparation of the bench

Eggs were candled daily to determine the dead. Dead eggs were removed and the live were returned to the 37˚C incubator. Biological safety hood was set up for harvesting by placing absorbent paper where eggs are to be opened. Double thick plastic bag in a wire basket.Using sterile scissors and forceps. Using disposable Petri dishes.5 ml snap-cap test tubes for yolk sac storage. A piece of tape long enough to seal the plastic bag once it is full of waste. Glass slides were used. Gloves and mask were used. The gloves and mask were weared. The top of the egg was punctured with the scissors and a circular hole was being cut large enough to extract the inner contents. The embryo, yolk sac and all egg contents were poured into the deep side of the Petri dish.The embryo and yolk sac were then transferred to the shallow side of the Petri dish. The contents in the deep side of

the Petri dish were discarded into a plastic bag. The embryo was observed and noted if it was hemorrhagic. From the hemorrhagic embryo, a smear from the yolk sacs inside layer was made, using one pre cleaned slide to remove as much yolk as possible and another to make an impression smear. The yolk sac was then stored in a snap-caped test tube and the tube was labeled; Egg ID and date of collection. The slide was then air dried. The slides were then heat fixed and stained with Gimenez stain. The yolk sac that indicated *Chlamydiae* was grinded using a Tenbroek grinder.10 ml (to dilute 1:10) sterile sucrose phosphate glutamine (SPG) were added with continuous grinding. The sample was then transferred to a 15 ml centrifuge tube, and centrifuged at 3000 rpm for 15 minutes. The supernatant fluid was collected and kept in 1.5 ml labeled freezer vials and stored at -40˚C until needed.When needed, the sample was thawed in a 37˚C water bath.

2. Egg passage of the isolated strains

Each sample showing suspected embryonic lesion or suspected chlamydial inclusions in the impression smear was passed in yolk sac of a 7-9 days old ECE 3 times, 3 eggs each. After 72 hours, embryos of specific deaths were examined for pathological changes and lesions specific for chlamydial infection, in addition to reisolation of the organism and preparation of impression smears for detection of suspected chlamydial inclusions.

3. Staining of embryo yolk sac using Gimenez stain (**Gimenez**, **[16]).**

Fresh working solution of basic carbol fuchsin was prepared by adding 4 ml stock solution to 10 ml 0.1 M. phosphate buffer (pH 7.5). The solution was then filtered through Whatman filter paper No 2. The dried impression smears from infected yolk sac were fixed gently with heat then covered with filtered working solution of basic carbol fuchsin for 1-3 minutes. The smears were then rinsed with tap water till the water ran clear. This was followed by flooding of the smears with 0.8% malachite green for 9-20 seconds or till the smears appeared "sky blue to slight green color". The smears were then rinsed with tap water, slipped and read under a light microscope using the oil immersion within 2 hours to 2 days after mounting.

3. Results and Discussion:

Avian chlamydiosis is caused by *Chlamydia psittaci* and is systemic, infectious, zoonotic and occasionally fatal disease in domestic and wild birds [1].Infection can be clinically in apparent or result in disease of respiratory tract, conjunctiva, serous membranes, joints, central nervous system (CNS), reproductive organs, and gastrointestinal tract. Reservoirs of the organism include the respiratory, intestinal and genital tracts of birds and mammals with transmission occurring via inhalation or ingestion [2]. *Chlamydia psittaci* infects many domestic birds and poultry. Moreover, birds in psittacidae, pigeon, dove, canary, turkey, duck, and waterbirds [1].The collected conjuncival, nasal and cloacal swabs from each domestic birds (turkey, pigeons, duck and chickens)via intra yolk route. Positive cases were confirmed by pathological lesions encountered in 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 as shown in**(fig1)**.Diagnosis of chlamydiosis can be problematic, isolation and identification is the gold standard for most infectious diseases, but these techniques may be difficult to use for chlamydiosis[16]. First, many birds can be carriers and may harbor subclinical infectious. Second, culturing requires the use of specialized cell lines or specific pathogen free embryonated chicken eggs and inoculation techniques that are not available in all laboratories. Third, because chlamydial organisms are shed intermittently in the feces, a single culture can lead to a false-negative result. Culture of chlamydia is difficult and infrequent because of obligate intracellular nature of the bacteria and the hazard exposed to microbiologists doing the work [17]. Isolation is known as the gold standard, even though other methods are alsoused [18]. So far the isolation of viable chlamydia organisms requires infection of embryonic egg [18]. In this study, the cytological examination of the inoculated egg revealed the typical vascular congestion of yolk sac membranes. Embryonated egg inoculation considered the gold standard for diagnosis of chlamydial infections and that the long time requirement is its only disadvantage [19].Available isolation techniques require special equipment and a supply of specific pathogen-free embryonated egg which are not routinely available in many diagnostic laboratories[20]. Moreover, the isolation of some species poses a zoonotic risk to classified as a biosafety level 3 organism [21]. Results of cytological examination of impression smears of the collected yolk sac membranes using Gimenez stain.Impression smears of the collected yolk sac membranes were subjected to staining. Chlamydial inclusions appeared as small, rounded red dots against a bluish green background as

shown in **(Fig2)** .As shown in **(table 3)**out of 497 samples from turkey (ocular, Nasal and cloacal swabs), chlamydial inclusions were demonstrated in 370 samples (74.5%),and this agree with Enany et al.,[23]. From356 samples from pigeons, chlamydial inclusions were in 282 samples (79.2%) , from 89 samples from duck , chlamydial inclusions were in 5 samples (5.6%) and from 40 chicken samples, chlamydial inclusions were 7 samples (17.5%).The total recovery rate of identified chlamydiae after egg inoculation was (664/982) 67.6%.and **(table 4)** show the result of diseased and apparently healthy birds and this agree with Osman et al., [24].

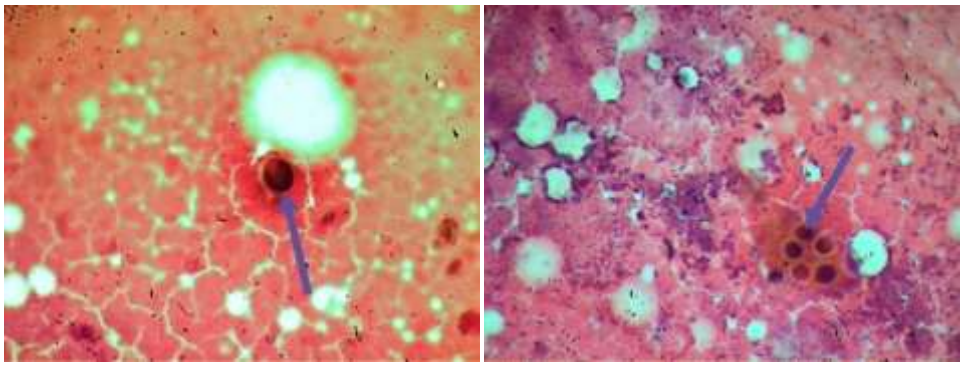
Table (3): Results of cytological examination of impression smears of the collected yolk sac membranes using Gimenez stain.

Poultry	**Positive C.psittaci**			**Total positive**	**Percentage** %
	Ocular swab	**Cloacal swab**	**Nasal and pharyngeal swab**		
Turkey	83	230	57	370	74.5%
Pigeon	170	90	22	282	79.2%
Duck	-	4	1	5	5.6%
Chicken	-	5	2	7	17.5
total	253	329	112	664	67.6%
Recovery rate	38.1%	49.5	16.9		

Table (4):Results of cytological examination of impression smears of the collected yolk sac membranes using Gimenez stain(Diseased and Asymptomatic).

		Positive *C. psittaci*				
Poultry		**occular**	**Cloacal**	**Nasal and pharyngeal swab**	**Total**	**percentage %**
Turkey	**Diseased**	78	202	50	330	89.1%
	Asymptomatic	5	28	7	40	10.1%
Pigeon	**Diseased**	147	85	19	251	89%
	Asymptomatic	23	5	3	31	10.9%
Duck	**Diseased**	-	4	-	4	80%
	Asymptomatic	-	1	-	1	20%
Chicken	**Diseased**	-	4	2	6	85.7%
	Asymptomatic	-	1	-	1	14.3%

(Fig 1) Embryos appeared dwarfed with normal embryo presence of hemorrhagic spots In the head and toes(Positive chlamydia psittaci)



(Fig2) : yolk sac membrane stained by Gimenez stain shows inclusion bodies of C. psittaci

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