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VIROLOGICAL AND SEROLOGICAL INVESTIGATIONS ON AVIAN PNEUMOVIRUS IN TURKEYS AND CHICKENS

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ABSTRACT

Avian Pneumovirus (APV), which is termed turkey rhinotracheitis (TRT), is successfully isolated from 5 out of 8 samples of nasal discharge and 10% homogenates of nasal sinuses collected from young turkey poults of unvaccinated flocks by passage on SPF-ECE of 5-7 days old embryos via yolk sac route. Characterization of isolated virus was done by using RT-PCR where the 5 isolates were positive for APV and produced a specific 315 bp products. Serological survey for APV antibodies in serum and egg yolk samples collected from non-vaccinated flocks (608 serum samples were obtained from broiler chickens from El-Fayoum ,Beni suef and Giza, 40 turkey poults serum samples from Al Behira and El Monofia, 278 chicken egg yolk samples from El-Monofia, Dommiat ,Alexandria and Al Behira and 675 turkey egg yolk samples from El-Fayoum and Beni suef Governorates) were tested by using ELISA. The percentages of positive reactors of chicken sera and egg yolk samples were 57.4% and 74.4%, while it were 77.5% and 83.2% respectively in turkey sera and egg yolk. The APV disease was recorded in young chicks and turkey poults showed typical symptoms and APV was isolated regardless the presence of high level of maternal derived antibody in their sera. This denotes the protection against APV independent on the presence of high levels of maternal antibodies.

Keywords: Avian Pneumovirus (APV), APV isolation, Characterization by RT-PCR, Serological survey, turkey poults.

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INTRODUCTION

Avian pneumovirus (APV) causes a serious respiratory disease in chicken and turkeys and heavy economic losses to poultry industry. (**Jones *et al.*, 1988**). The diseases that may result from avian pneumovirus infections of turkeys or chickens have been termed turkey rhinotracheitis (TRT), swollen head syndrome (SHS), and avian rhinotracheitis (ART). APV isolates adapted to embryos or tracheal organ cultures, however, were capable of replication in cultures of chicken embryo cells, turkey embryo cells, VERO cells, with a characteristic cytopathic effect of syncytial formation and relatively high virus titers. Avian pneumoviruses are members of the subfamily *Pneumovirinae*, belonging to the family *Paramyxoviridae*. The subfamily consists of two genera; *Pneumovirus* consisting of mammalian respiratory syncytial viruses and mouse pneumovirus and *Metapneumovirus* in which avian pneumoviruses are placed (**Pringle, 1998**). The virus genome is unsegmented and composed of single-stranded negative sense RNA of approximately 15 kilobases. The virus was shown to have eight structural polypeptides of which two were glycosylated and three were non-structural virus specified proteins (**Collins and Cough, 1988**). These have been identified as nucleoprotein (N), phosphoprotein (P), matrix protein (M), second matrix protein (M2), surface glycoprotein (G), fusion protein (F), a small hydrophobic protein (SH), and a viral RNA-dependent RNA polymerase (L). The pneumoviruses have an F protein that promotes cell fusion, but these viruses do not hemagglutinate and the G attachment proteins of these viruses do not have neuraminidase activity. These are important characteristics distinguishing the pneumoviruses from the other paramyxoviruses (**Collins *et al.*, 1996**). Classification of European aMPV isolates was initially based on physical characterization of the virion (**Collins *et al.*, 1986**), electrophoretic The G gene of aMPV encodes the surface glycoprotein responsible for cell attachment and serves as one of the major antigens of pneumoviruses. The G protein is known to be the most variable protein in TRTV and other MPVs. Early studies using cross neutralization, ELISA techniques,

and polypeptide profiling suggested there were few strain differences between European isolates of avian pneumovirus (**Juhasz and Easton, 1994; Baxter-Jones *et al.*, 1987, Gough and Collins, 1989**). Other studies using monoclonal antibodies (MABs) produced to a variety of isolates demonstrated considerable antigenic differences between strains (**Collins *et al.*, 1993**). In Egypt, Till now no intensive vaccination program was used against APV especially in chicken and turkey and the current situation of the disease is still undefined correctly. Therefore, this work was planed to clarify the situation of APV in chicken and turkey flocks through trials for virus isolation, molecular characterization and serological survey on APV specific antibodies.

MATERIALS & METHODS

Samples for virus isolation:

Nasal discharge and 10% homogenates of nasal sinuses, oropharyngeal swabs were collected from young turkeys located in private turkey farms at Beni-Suef, Giza and Cairo Governorates (un-vaccinated against TRT virus, and suffering from respiratory signs as coughing, ocular and nasal discharge as well as swelling of nasal sinuses). **(Figure 1)** Post-mortem examination of diseased poultes showed sinusitis, tracheitis with accumulation of excess exudates in the sinuses and pharynx. Morbidity rates ranged from 40-60% while mortality rate was 20-50%. Collected samples were stored at -20 °C until tested.

Figure 1. Young turkey poult showing swelling of nasal sinuses.

Serum samples:

a-Chicken sera: a total number of 608 broiler chicken serum samples were obtained from different poultry farms non vaccinated against APV, located at El-Fayoum ,Beni suef and Giza Governorates and used for detection of APV antibodies by using ELISA.

b-Turkey sera: a total number of 40 serum samples were obtained from turkey poults (aged 4-8 weeks) non vaccinated against TRTV, located at Al Behira and El Monofia Governorates and used for detection of TRTV antibodies by using ELISA.

Egg yolk samples:

a-Chicken egg yolk : a total number of 278 chicken egg yolk samples were obtained from different poultry farms non vaccinated against APV located at El-Monfia, Dommiat ,Alexandria and Al Behira Governorates and used for detection of APV antibodies by using ELISA.

b-Turkey egg yolk: a total number of 675 turkey egg yolk samples were obtained from different turkey farms non vaccinated against TRTV located at El-Fayoum and Beni suef Governorates and used for detection of TRT antibodies by using ELISA.

APV-Ab ELISA kits:

Antigen coated kits used for detection of TRT antibodies in collected serum and egg yolk samples , article number 10-1100-02,Svanova Biotecc Ab Uppsala Science Park, Sweden.

Preparation of hyper-immune serum against TRTV was done according to Schmidt (1970).

Virus isolation: It was done on SPF-ECE of 5-7 days old embryos via yolk sac route. The isolation was followed by multiple subsequent cell culture passages for propagation.

Virus characterization: This step had been done on the molecular level by using RT-PCR. Reverse-transcriptase PCR (RT-PCR) is a significantly more sensitive and rapid method for the molecular characterization of aMPV than standard methods because of the fastidious nature of aMPV (**Gough and Collins, 1989 and Cook *et al.*, 1999**). RT-PCR procedures targeted to the F, M, N and G genes were used for the detection of aMPV. However, because of molecular heterogeneity between aMPV strains, most RT-PCR procedures are subtype specific or do not detect all subtypes. Subtype

specific assays are successfully used for the detection and diagnosis of endemic strains.

- RNA extraction and RT-PCR:

RNA was extracted from samples and isolates by using RNA Extraction kit (QIA amp Viral RNA Mini kit, Cat. No. 52904, Qiagen, Valencia, Calif, USA). Reserve transcription (RT) was performed using an RT random hexamer primers and AMV reverse transcriptase (Roche, Cat. No. 13490320), according to the manufacturer's instructions, using 5 µl of purified RNA in a 20 µl reaction volume and this step was carried on thermal cycler profile of 42°C /30min then 85°C for 5 min (Biometra, Germany). The fusion gene (F) of TRTV isolates were amplified by PCR with primers designed from consensus sequences of aMPV (**Shin *et al.*, *2000***) as follow:F1 (sense) from position 3558 to 3576 in strain #8544:5′-CAATTCTCTGAYAGTGCAG, F2 (antisense) from position 3855 to 3872: 5′-GTACCACCCYTGATCTTC. After RT, 5 to 10 µl of the reaction mixture was used for PCR. The cDNA was denaturized at 94°C for 5 m, followed by 35 cycles of denaturation (94°C for 1 m), annealing (54°C for 1 m) and elongation (72°C for 1 m). The expected size of the amplicons is 315 base pair (bp). Each reaction was analyzed by 1.5 % agarose gel electrophoresis and stained with ethidium bromide.

Extraction of antibodies from egg yolk of collected samples was carried out by chloroform method according to Polson (1990).

Detection of TRT antibodies in collected samples by using ELISA kits: Serum and yolk samples were examined for presence of TRT antibodies in collected samples using Avian Pneumovirus (APV-Ab), SVANOVIR kit. The test was performed according to manufacturer description as following ;. All reagent should firstly equilibrate to room temperature then add 1oo ul of positive control and 100 ul of negative control solution to selected wells then add tested serum samples pre diluted 1:2 in PBS (egg yolk samples is pre diluted 1:5 in PBS) and incubate for 30 minutes at room temperature .After incubation the plate was washed 3 times by washing buffer (PBS tween buffer),then 100 ul of

HRP was added to each well and incubate at room temperature for 30 minutes and then wash 3 times using washing buffer .After washing add 100 ul of substrate solution to each well and incubate 10 minutes at room temperature in dark place then stop the reaction by adding 50 ul stopping solution and measure the optical density (OD) at 450 nm using ELISA reader and calculate the percent inhibition for interpretation of results according to manufacturer description.

RESULTS

Virus isolation : TRTV was isolated from collected samples (5 out of 8 samples) on ECE the embryo mortality and stunted growth and congestion of the embryo were clear after 3rd passage. **(Figure 2).**

Results of RT-PCR for aMPV

The 5 isolates were positive by RT-PCR for aMPV and produced a specific 315 bp RT-PCR products **(Figure 3),** Isolates analyzed were: 1) Giza-TRT-1; 2) Giza-TRT-3; 3) Giza-TRT-4; 4) Benisuif-TRT-7; 5) Cairo-TRT-8.

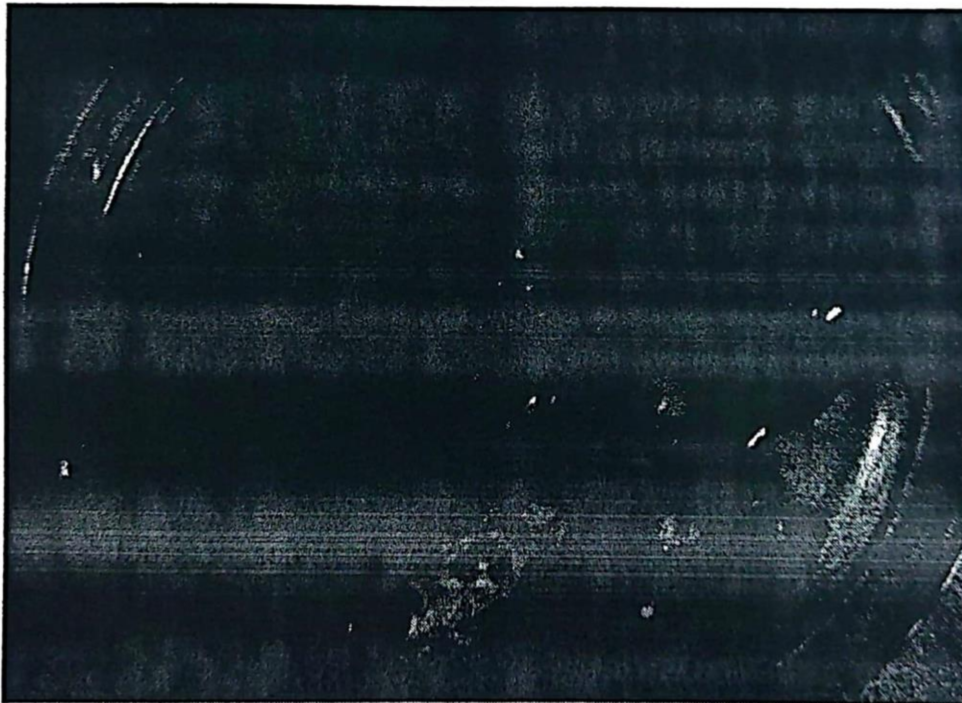

Figure 2. 14 days old embryos inoculated with suspected samples (TRT) showed stunted growth.

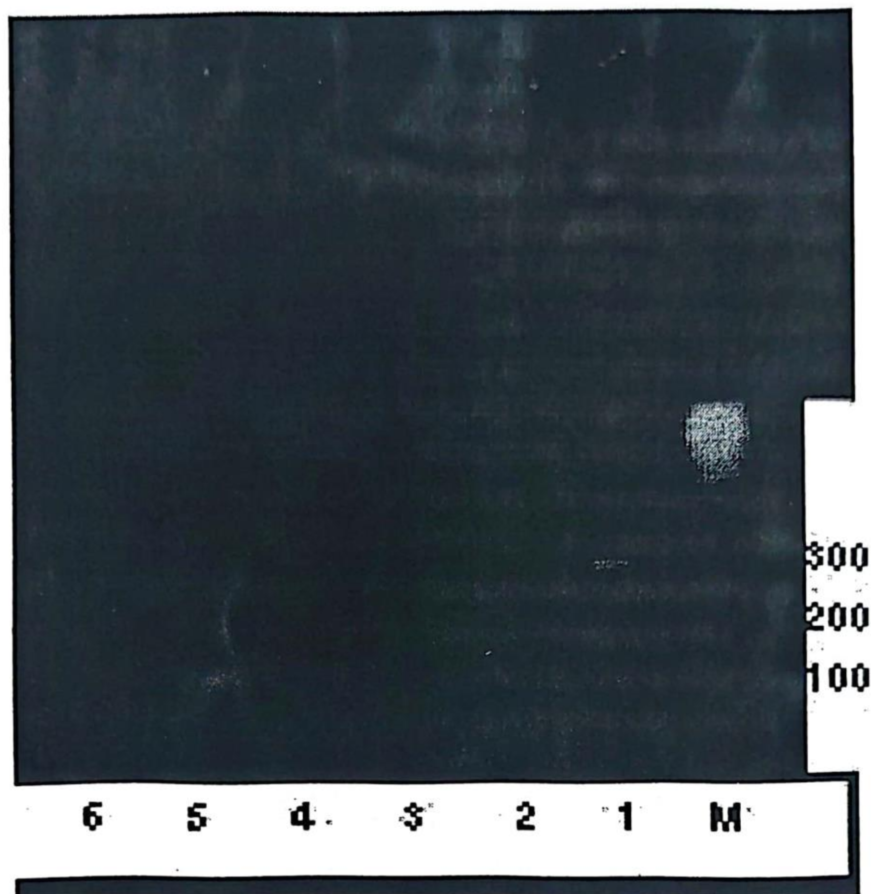

Figure 3. *Results of RT-PCR for detection of F gene of APV in field samples.* M=100 bp DNA size marker, **lanes 1** = positive control at 315 bp, **lanes 2-6** isolates.

Results of serology :

Table 1. Detection of avian pnemovirus specific antibodies in Chicken serum samples by ELISA.

Locality	No of tested samples	NO Positive	NO of negative	% Positive
El-fayoum	400	255	145	63.7
Beni-suef	167	80	87	47.9
Giza	41	14	27	34.1
Total	608	349	259	57.4

Table 2. Detection of avian pnemovirus specific antibodies in Chicken egg yolk samples by ELISA.

Locality	No of tested samples	NO Positive	NO of negative	% Positive
Dommiat	88	69	19	78.4
Alexandria	74	55	19	74.3
El-Monofia	26	14	12	53.8
Al-Behira	90	69	21	76.6
Total	278	207	71	74.4

Table 3. Detection of avian pnemovirus specific antibodies in turkey serum samples by ELISA.

Locality	No of tested samples	NO Positive	NO of negative	% Positive
Al-Behira	20	16	4	80
El-monfia	20	15	5	75
Total	40	31	9	77.5

Table 4. Detection of avian pnemovirus specific antibodies in turkey egg yolk samples by ELISA.

Locality	No of tested samples	NO Positive	NO of negative	% Positive
Beni Suif	455	385	70	84.6
El-Fayoum	220	177	43	80.5
Total	675	562	113	83.2

DISCUSSION

Pneumovirus infections of poultry are associated with serious economic and animal welfare problems, particularly in commercial turkey flocks. Even in countries where vaccination against avian pneumovirus has become routine practice, the disease is still to be the most significant respiratory diseases of turkeys. In Egypt, many viral and bacterial diseases inducing respiratory disorders are confused to each others like Newcastle disease, Infectious bronchitis, laryngeotracheitis, Infectious coryza and APV. Till now no intensive vaccination program was used against APV especially in chicken and turkey. Therefore, this work was planed to clarify the situation of APV in chicken and turkey flocks through trials for virus isolation, molecular characterization and serological survey on APV specific antibodies. For providing these points nasal discharge and 10% homogenates of nasal sinuses, oropharyngeal swabs were collected from young turkey poults located in private turkey farms at Beni-Suef, Giza, and Cairo Governorates (un-vaccinated against TRT virus, and suffering from respiratory signs as coughing, ocular and nasal discharge as well as swelling of nasal sinsuses). TRTV was successfully isolated from collected samples (5 out of 8 samples) on ECE via yolk sac rout and the embryo mortality and stunted growth and congestion of the embryo were clear after 3rd passage **(Figure 2).** This results are in agreement with **Tamam *et al.*, 2004** Who successfully isolated TRTV from naturally infected 10-22 days old turkey poults on ECE via allantoic sac route. Inoculation of infective mucus into the yolk sac of turkey or chicken embryos resulted in embryo mortality after four or five passages, but virus was demonstrated to be at a very low titer (**(Alexander *et al.*, 1986**). The 5 isolates were identified and characterized by RT-PCR for aMPV and produced a specific 315 bp RT-PCR products

following infection of turkeys with APV antibodies were detected as early as seven days post infection by ELISA and NT test and were maintained for 89 days **(Jones *et al.*, 1988**). A variety of commercial kits have been used for detecting APV antibodies , the test can be used for screening large number of samples, but difference in sensitivity and specificity have been reported and this is attributed to variation in the antigenicity and purity of the antigen used in

coating of ELISA plate(**(Cook, 2000)** .

For serological survey for APV antibodies by using ELISA Kit, data presented in **Table (1-4)** showed high percentage of APV antibodies in tested samples (serum and egg yolk samples) collected from non vaccinated flocks of both chicken and turkeys located at different governorates suggesting that these birds are periodically exposed to waves of APV infection and therefore subsequent elevation of these antibodies become more evident . The percentages of positive reactors of chicken sera and egg yolk were 57.4% and 74.4% while it were 77.5% and 83.2% respectively in turkey sera and egg yolk. The percentage of positive reactors in egg yolk in both chicken and turkey were higher than those detected in sera of both species and this may be attributed to the repeated waves of infection by aMPV that elevated the antibody concentration in egg yolk. On the other hand, the positive reactors of chicken sera and egg yolk were lowered than those detected in turkey sera and egg yolk and this can be explained by species variation or lower sensitivity of chicken than turkeys for aMPV. The APV disease was recorded in young chicks and turkey poults showed typical symptoms and aMPV was isolated from them in spite of the presence of high level of maternal derived antibody in young chicks and turkey poults in their serum samples, and this denotes to the protection against aMPV independent on the presence of high levels of maternal antibodies that will not prevent clinical disease following infection with APV **(Naylor *et al.*, 1997).**

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