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Isolation and characterization of avian reovirus (Egypt/ARV/Giza 2011) associated with arthritis in broiler breeder flocks

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Abstract

Samples of tibiotarsal tendons, tendon sheaths and fluids around ruptured tendons were collected from broiler breeder flock of 28 weeks old in Giza governorate, showed symptoms of arthritis, lameness and swelling in the hock joints. Trial for isolation of avian reovirus (ARV) was done by inoculation in emberyonated chicken eggs (ECE). After propagation and titration of the isolated virus, it had been antigenically characterized by agar gel precipitation test (AGPT) and fluorescent antibody technique (FAT). Pock lesions on the chorioallantoic membrane (CAM) was negatively stained and examined by electron microscope, also histopathological examination was carried out. Molecular characterization was performed by reverse transcriptase polymerase chain reaction (RT.PCR). The results confirm the isolation of avian reovirus from the suspected flock.

Key words: avian reovirus, arthritis, CAM and pock

Introduction

Avian reoviruses belong to genus Orthoreovirus of family Reoviridae **(Mertens, 2004)**. Reoviruses have a double capsid structure comprised of an outer capsid of ~85 nm in diameter and an inner core of ~45 nm in diameter, which accommodates 10-segmented genomic dsRNA **(Zhang et al., 2005)**. The 10 segments encode for at least 12 viral proteins, 8 structural proteins and 4 nonstructural proteins. σC is one of the most important polypeptides among ARV proteins that induces ARV-specific neutralizing antibodies which makes such protein a target and marker in studies on ARV genetic variations as well vaccine development **(Martinez-Costas et al., 1997 and Shapouri et al., 1995)**. The avian reovirus can induce several manifestations in chickens including arthritis, lameness and swelling in the hock joint **(Jones and Guneratne, 1984)**, inclusion body hepatitis **(McFerran et al., 1976)**, enteritis **(Dutta and Pomeroy, 1969)**, hydro-pericardium **(Bains and MacKenzie, 1974)**, myocarditis **(Davis et al., 2012)**, central nervous system disease **(Van de Zande and Kuhn, 2007)** and runting-stunting syndrome or malabsorption syndrome (MAS) **(Goodwin et al., 1993)**. ARVs could be inoculated on CAM as it is a successful route for ARV isolation and the main pathological changes are appearance of pock lesions on CAM **(Schwartz et al., 1976)**. The electron microscope has been used as a powerful tool in the characterization of ARV either by negative stain or thin section electron microscopic examination **(Goldsmith, 2014)**. ARVs were classified into 11 serotypes **(Wood et al., 1980)**, based on antigenic characterization using serological tests like agar gel precipitation test (AGPT), virus neutralization test (VNT), enzyme linked immunosorbent assay (ELISA) and fluorescent antibody technique (FAT). Molecular-based techniques including reverse transcriptase polymerase chain reaction (RT-PCR) **(Bruhn et al., 2005)**, nested PCR **(Liu et al., 1997)**, multiplex PCR **(Caterina et al., 2004)**, real-time PCR **(Ke et al., 2006)**, PCR followed by restriction fragment length polymorphism (RFLP) **(Lee et al., 1998)**, and in situ hybridization (ISH) **(Liu and Giambrone, 1997)**, have been used to detect ARVs. Phylogenetic studies classified isolates of ARVs into various groups and lineages also provided evidences showing frequent reassortments among the circulating lineages which responsible for variation in the ARV genome segments. Previous studies on comparison of sequences of ARVs were focused on σC-encoding gene **(Liu et al., 2003)**. The aim of the present study is to isolate and characterize ARV associated with arthritis in broiler breeder chickens.

Material and methods

Samples of tibiotarsal tendons, tendon sheaths and fluids around ruptured tendons were collected from chickens of broiler breeder farm of 28 weeks old located in Giza governorate, suffered from rupture of Achilles tendon, swelling in joints and lameness, showed 10% morbidity 0.2% mortality. The chickens were vaccinated with reovirus

S1133 live vaccine at 38 days old and inactivated ARV vaccine at 18 weeks and boostered at 22 weeks old. Homogenate of the samples was prepared in phosphate buffer saline (PBS) pH 7.2 with antibiotic (200 IU/ml of penicillin and 100µg/ml of dehydro-streptomycin) followed by three times freezing and thawing then centrifuged at 5000 r.p.m. for 15 minutes at 4°C. The supernatant was further passed through 0.45 µl filter membrane. The filtrate was used for virus isolation by inoculation on the chorioallantoic membrane of specific pathogen-free emberyonated chicken eggs (SPF-ECE) at 11-13 days old. The inoculated eggs were incubated at 37° C and 80% humidity for 5 days with daily candling then the CAMs were harvested **(Tantawy, 1999)**. Propagation of the isolated virus on CAM was carried out for 6 passages in (SPF-ECE) **(Wood et al., 1980).** Virus titration test was performed in SPF-ECE according to **(Neelima et al., 2003)** using filtrate of the harvested CAM from the sixth passage that showing pock lesions and the virus titer was calculated by Karber method **(Finney, 1978)**. For the antigenic characterization of the isolated virus, AGPT **(Tantawi et al., 1984)** and FAT **(Adair et al., 1987)** were conducted using specific monoclonal antibodies against ARV. Transmission electron microscopic examination was applied on filtrate of pock lesion preparations **(Goldsmith, 2014)**. Images were captured by CCD camera model AMT. Histopathological examination of pock lesions on the harvested CAM was carried out according to **Tantawy, (1999)**. RT-PCR was employed for molecular characterization of the isolated virus using primers designed by **Xie et al. (1997)**. These primers were specific to the conserved fragment of sigma C gene and amplify fragment with molecular size of 532 bp. Sequences of the utilized primers were as follows:

Primer 1 (Sense)	5`GGTGCGACTGCTGTATTTGGTAAC 3`.
Primer 2 (Antisense)	5`AATGGAACGATAGCGTGTGGG 3`.

Results

Isolation, propagation and titration of ARV

Infected CAM of the inoculated eggs showed thickening, opaqueness and swelling forming pock lesion in the third passage. Number of pock lesions increased and markedly appeared between fourth to sixth passages figure (1). The titer of the isolated virus was $10^{6.1}$ EID_{50}/ml.

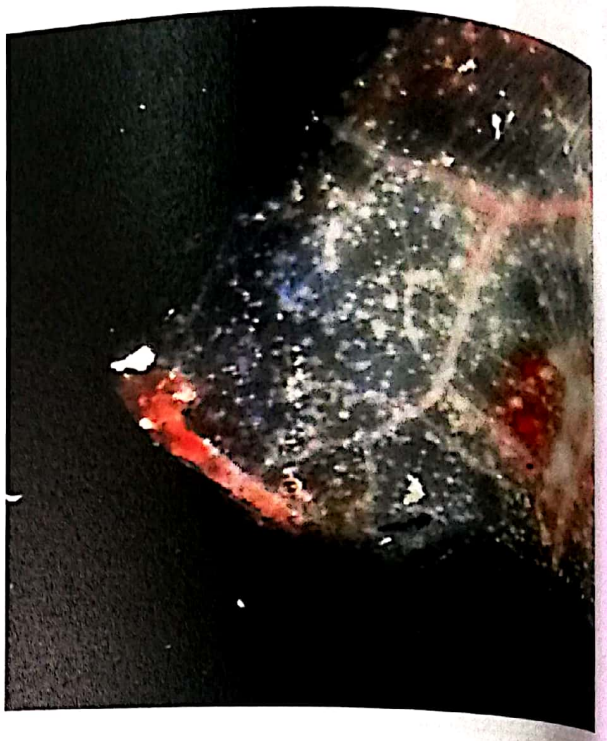

Figure (1) showed marked appearance of pock lesion on CAM of ECE in the fifth passage of the virus.

Antigenic characterization of the isolated virus by AGPT and FAT

AGPT showed positive result using specific monoclonal antibodies figure (2). Examination of frozen sections of pock lesions by fluorescent microscope using specific monoclonal antibodies revealed specific fluorescent foci in the sections confirming the antigenic characterization of the isolated virus figure (3).

Figure (2) AGPT showed line of precipitation.

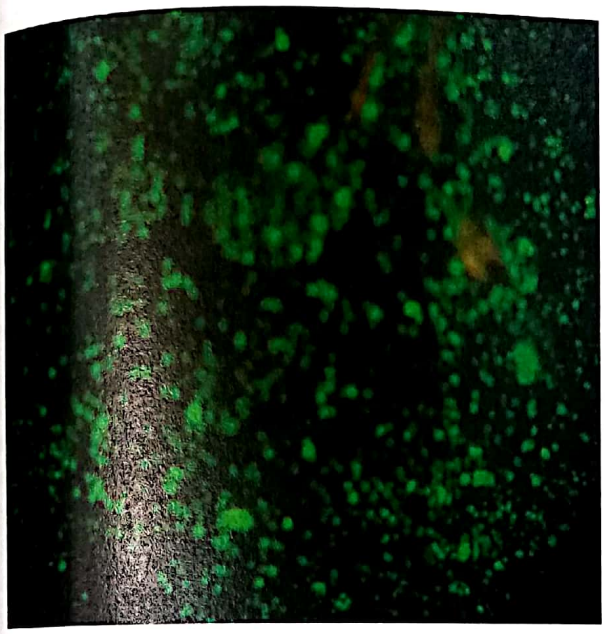

Figure (3) FAT showed positive fluorescent foci on CAM section.

Electron microscopic examination

Appearance of virus particles in aggregates with apparent damage to the outer capsid of the virus with size reached about 45 nm figure (4).

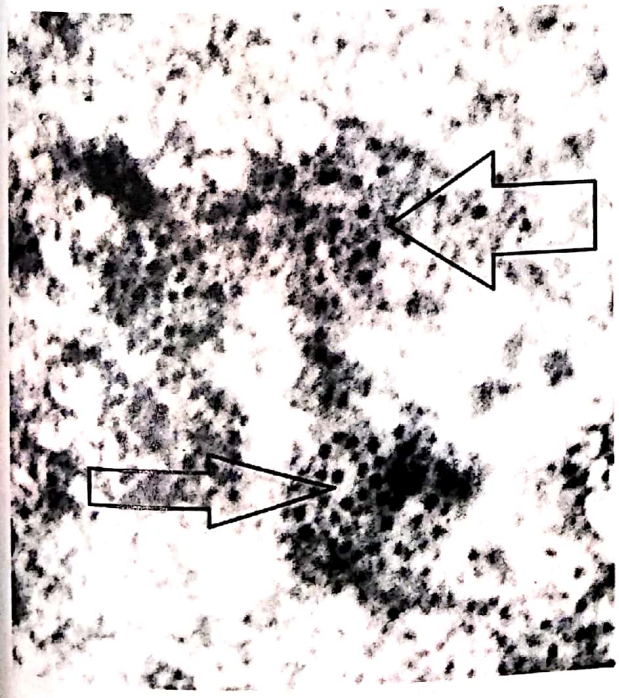

Figure (4) showed aggregates of the virus particles with apparent damage to the outer capsids, by TEM Mode, Direct Mag: 120000 x
Print Mag: 252000x@211 mm

histopathological examination of harvested cams

The ectodermal layer of CAMs showed mild to moderate hyperplasia associated with hypertrophy and vacculation of cells. Very characteristic oesinophilic intracytoplasmic inclusion bodies were also noticed in ectodermal cells figure (5) and (6). Focal necrosis of ectoderm was observed. The mesoderm showed pronounced edema with congested blood vessels, in addition to inflammatory cells and fibroblasts. Few blood vessels occluded with inflammatory cells. The endoderm showed mild hyperplasia with focal necrosis.

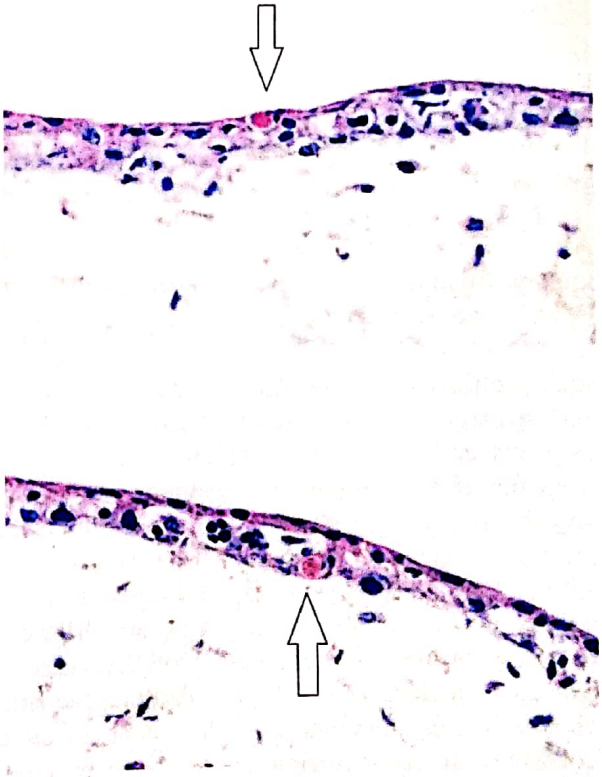

Figure (5) & (6) showed the intracytoplasmic inclusion bodies in sections of pock lesions.

RT-PCR

Electrophoresis of the amplified products in agarose gel revealed the expected band with correct molecular size confirming the molecular characterization of the isolated virus figure (7).

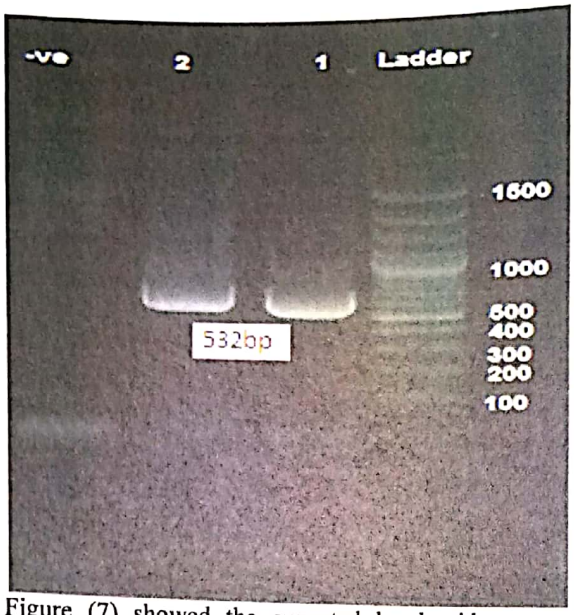

Figure (7) showed the expected band with correct molecular size.
1-sample tested. 2-positive control.

Discussion

ARV could be isolated from different organs like kidney, thymus, ceacal tonsils, spleen, pancrease **(Van Loon et al., 2001)**, liver, intestine, heart, joints and tendons **(Lu et al., 2015)**. In the current study, chickens of broiler breeder farm were suffered from rupture of Achilles tendon, swelling in joints and lameness. Samples were collected from the affected joints and tendons as the virus still for long time in the joints and tendons for at least 285 days post infection **(Kerr and Olson, 1969)**. ARV isolation could be performed in tissue culture or in the SPF-ECE. There are different routes were used for inoculation of the virus in SPF-ECE including yolk sac, allantoic sac and chorioallantoic membranes. The most suitable conditions for propagation of the avian reovirus vaccinal strain were on the emberyonated chicken eggs and the preferable route for inoculation was on the chorioallantoic membrane **(Hassan et al., 1993)**. In this study, ARV was isolated on the CAM of SPF-ECE and after number of passages; characteristic lesions of reovirus including opaque and thick pocks appeared on the CAM figure (1). Performing the infectivity test revealed that titer of the isolated virus reached $10^{6.1}$ EID_{50}/ml after fourth passages. Antigenic characterization of the isolated virus revealed the appearance of line of precipitation in AGPT between the isolated virus and the antibodies specific to ARV confirmed the isolation of ARV figure (2). Similar results were obtained by **Tantawi et al., (1984)** who isolated and characterized two infectious tenosynovitis producing viruses from tendon sheaths and synovial fluids of broiler and broiler breeder flocks in Egypt in 1983. Also, Similar results obtained by **Kheir El-Din and El-Sanoussi (1986)** who isolated reovirus from broiler breeder chickens suffered from retardation of growth, diarrhea and abnormal gate. The isolated viruses were characterized by AGPT. Fluorescent foci in the frozen sections of the obtained pock lesions on the harvested CAM were observed confirming the antigenic characterization of the isolated virus figure (3). Negative staining of the isolated virus and examination under the electron microscope demonstrated the presence of the characteristic aggregates of the virus particles with apparent damage to the outer capsid figure (4). Size of the virus particles reached about 45 nm and that similar to previous studies achieved by others like **Walker et al. (1972); Mustaffa-Babjee et al. (1973) and Tantawy, (1999)**. The collected CAMs of the inoculated SPF-ECE were macroscopically opaque, thickened and oedematous with engorged and tortuous blood vessels. White pock lesions of different sizes appeared on the CAMS, some of them were small minute (pin-headed) and the others looked large prominent specially at sit of inoculation figure (1). Several researchers have been reported these lesions even by inoculation of local isolate of ARV or vaccinal strain as **Tantawi et al., (1984)** and **Tantawy, (1999)**. Histopathological changes of the pock lesions were characteristic to ARV with appearance of oesinophilic intracytoplasmic inclusion bodies in the ectodermal layer which considered pathognomonic to ARV and that ensure the replication and maturation of the virus in the cytoplasm of the affected cells. The virus invaded the CAM leading to appearance of another changes like hypertrophy, hyperplasia, vacculation of the cells and focal necrosis in the ectodermal layer also edema with congested blood vessels and inflammatory cells in the mesoderm. The observed lesions are agree with previous study performed by the reference vaccinal strain of ARV which showed the same picture as

detected by **Tantawy, (1999)**. Different segments were target for amplification in the RT-PCR assay for the detection and molecular characterization of the virus in the clinical samples. RT-PCR could segment **(Lee et al., 1998)** or S4 genome segment **(Roussan et al., 2012)** or L1 genome segment **(Leary et al., 2002)**. The sigma C gene of the S1 segment was selected to be applied in the present study utilizing primers specific to the conserved region to obtain fragment of 532 bp **(Xie et al., 1997)**. The embloyed RT-PCR revealed band of corrective size confirming the molecular characterization of the isolated reovirus. Indeed, be performed depending on the S1 genome segment **(Lu et al., 2015)** or the S2 genome segment **(Zhang et al., 2006)** or S3 genome

the study reports the isolation and characterization of ARV associated with arthritis in broiler breeder chickens and the isolate was designated as Egypt/ARV/Giza 2011 and further studies are needed for sequencing of the amplified gene followed by the phylogenetic analysis to know situation of the current isolate in between the vaccinal strains and/or the field isolates from different countries.

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المستخلص العربى

عزل و توصيف فيروس ريو الدجاج (Egypt/ARV/GIZA 20011) المصاحب لمرض التهاب المفاصل فى قطعان أمهات التسمين

لطيفة بخيت [١]-حسين على حسين [٢]-منير الصفتى [٣]-ليلى طنطاوى [٤]-احمد هلال [٣]

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تم جمع عينات من اوتار التيبيوتارسال و الاغشيه والسوائل حول الاوتار الممزقة من قطيع امهات التسمين عند عمر ٢٨ أسبوع في محافظة الجيزة حيث كانت تظهر أعراض التهاب المفاصل، العرج وتورم في مفاصل العرقوب و و قد تم اجراء محاولة لعزل فيروس ريو الدجاج بحقنه على الغشاء السقائى المشيمائى فى بيض الدجاج المخصب الخالى من المسببات المرضية و بعد تمرير الفيروس و معايرته تم عمل توصيف انتيجينى للفيروس باستخدام اختبارى الترسيب المناعى و التعادل الفلورسنتى الضوئى. وقد اظهرت النتائج وجود بثرات على الغشاء السقائى المشيمائى بالبيض المحقون و قد تم اخذ عينات منها و صبغها صبغ سالب و فحصها تحت الميكروسكوب الالكترونى و أيضا تم عمل فحص التغيرات المرضية فى انسجة الغشاء السقائى المشيمائى للبيض المحقون. كما تم عمل التوصيف الجزيئى بواسطة اختبار تفاعل البلمرة المتسلسل المسبوق بالنسخ العكسى و قد اسفرت النتائج التاكيد على عزل فيروس ريو الدجاج من القطيع المشتبه به.