Isolation and Identification of Turkey pox virus in Turkey Flocks in Egypt

Safaa M. Mohammed*, Shahira A. Abdelwahab,Fetaih,H**,Neveen,R*,Abdel satar ,A* and Mohamed S. Elshahidy****

Animal health research insititue,Dokki,Cairo.*

Virology Department, Faculty of Vetrinary Medicine, Suez Canal University, Ismailia, Egypt**

ABSTRACT

In this study, turkey pox virus (TKPV) was recorded in 6 commercial turkey flocks aged 48-65days old. The disease was examined clinically ,histopathologically and immunohistochemically. Turkey pox virus was isolated on CAM of embryonated chicken eggs and identified by agar gel precipitation test and polymerase chain reaction .Two main types of TKPV infection were observed in examined flocks ,cutaneous and diphtheritic forms. Morbidity rate ranged from 5-15%with no mortalities .75% of examined serum reacted positive to TKPV .All TKPV isolates were amplified using specific P4b primers and visualized by gel electrophoresis at 578bp.

INTRODUCTION

The pox infection in turkey was first reported as Turkey pox virus (TPV) by Brunett in 1934 in a turkey flock in New York ,Veterinary college. This virus is an enveloped, brick shaped dsDNA virus of the family Poxviridae, Subfamily Chordopoxvirinae and Genus Avipoxvirus (Fenner, 1989). The viral genome are single molecule and linear approximately 300 Kbp (Couper et al. 1990). It consists of centrally placed biconcave core and two lateral bodies.

Heating of the virus at 50C for 30 minutes or 60C for 8 minutes inactivate the virus (Andrews et al. 1978). Differences in sensitivity to PH and heat were observed among pigeon pox virus. When the virus desiccated, it shows marked resistance, it can survive in dried scabs for months or years (Tantawi et al. 1979).

Avipoxvirus genus includes fowl pox (FP), turkey pox (TP), quail pox (QP), pigeon pox (PP) and canary pox (CP) as major viral strains. Fowl poxvirus represents the prototype species of the genus avipoxvirus (Mathews, 1982). The viruses causing avian pox are antigenically similar but distinct in genome sequences and host specificity (Jarmin et al. 2006).

Host specificity is considered to be one of the most important criteria for differentiation of avian poxviruses. Vaccines of fowl pox virus origin have been routinely used for vaccination of turkeys to prevent turkey pox in commercial turkeys in endemic areas. However, outbreaks of turkey pox have occurred in previously vaccinated flocks due to the emergence of variant strains with enhanced virulence (Singh et al., 2000). Turkey pox virus infection is manifested by two forms of the disease, cutaneous (dry pox) and diphtheric (wet pox). The infection may display different signs and various degrees of severity.

Interestingly, turkey production in Egypt becomes increased and turkey pox virus has a direct economic impact on meat and egg production in turkey farms. With the inspection of the literature, there is no information

available in relation to the characterization of turkey poxvirus and/or epidemiology of the virus in Egypt so the present study will bring the new avenues for effective management of turkey pox disease in Egypt. To achieve this aim, epidemiology of turkey pox disease and characterization of the disease and its causative virus are studied to design preventive and control strategies for the future.

MATERIALS AND METHODS

1-Chicken flocks

six commercial turkey flocks (white Holland breed) were examined ; the flock localities, age, morbidity and pox vaccine status were detailed in (table 1). From each flock, five turkey poults were assayed; they kept for clinical check up, post mortem examination and sample collection.

2-Samples

A part of crusts, scabs and nodular lesions from each bird were collected aseptically and pooled for virus isolation and antigen detection. Birds are sacrificed for postmortem inspection and internal organs were collected. Larynx trachea and lung were kept in 10% formalin for immunohistochemistry and histopathological examination in addition to another parts of crusts, scabs and nodules were kept for molecular identification of pox virus DNA by PCR. Blood were collected for screening of pox antibodies in serum.

3-Agar gel precipitation test

Agar gel precipitation test (AGPT) are carried out according to the method described by verma and Malik ,1968. Using reference precipitating antigen for avian pox (Charles River, Spafas).

4-Virus isolation and titration in embryonated chicken eggs

Nodular lesions from infected birds were prepared for virus isolation and titration. About 0.1 ml of suspension was inoculated onto the chorioallantoic membranes (CAMs) according to the standard procedures of OIE 2012. Inoculated embryos were incubated at 370 C, observed and candled for 5 days. Five non inoculated eggs were kept as negative controls. Five days post inoculation; CAMs were examined for white pock lesions. Infected CAM s and crusts were pooled and used as agar gel precipitating antigens.

5-Histopathological technique

CAMs, crusts, scabs, larynx and trachea are fixed in 10 % buffered formalin and stained with conventional haematoxylin and eosin; then the slides were examined and photographed under a light microscope according to the method described by Bancroft and Gamble ,2007).

6-Immunohistochemistry

The tissue specimens were stained using an indirect immunoperoxidase technique (Hsu et al. 1981), The primary antibodies were omitted and replaced by PBS for negative controls.

7- DNA extraction, amplification and sequencing

Genomic DNA from each bird flock was extracted from crusts, scabs samples and of CAM containing virus cultures after the 2nd passage.Conventional PCR was conducted in Takara PCR Thermal Cycler (Takara Bio Inc., Japan) using a set of primers designed for targeting P4b gene at 578bp as described by Lee et al. 1997. Primer sequences used are, Forward primer (P1) 5'-CAGCAGGTGCTAAACAACAA-3' and Reverse primer (P2) 5'-CGGTAGCTTAACGCCGAATA-3'. The PCR product was visualized by gel

electrophoresis under UV light using UVI tec transilluminator and photographed using a digital camera.

Table(1): Breeds, localities , age and mortality percentage of turkeys

Flock No.	Localities	No.of birds	Age/day	Morbidity %	Pox vaccine status/age
1	Hefna	1000	48	10	Not vaccinated
2	Hefna	1000	60	10	Vaccinated/7 day
3	Elnahas	650	65	5	Vaccinated/38 day
4	Hefna	1000	48	12	Not vaccinated
5	Shobralenb	5000	59	15	Vaccinated/7 day
6	Belbis	2000	68	15	Vaccinated/14 day

RESULTS

During the late of 2013, six commercial turkey flocks in Sharkia governorate showed signs and lesions of turkey pox. Birds showed rise in body temperature, anorexia, emaciation and cutaneous lesions in the peak, face, eyes and feet (Table 2). Some birds exhibits locomotion difficulties, diarrhea, dehydration and recumbancy. Some birds in two flocks showed gasping and respiratory distress. Post mortum examination of these birds showed nodular lesions in pharynx and larynx. All flocks are treated with antibiotics and multivitamin therapy and vaccinated emergency with avian pox vaccine. After 15 days post vaccination and treatments, the birds begin to improved and completely recovered after 30 days post infection. Two main types of turkey pox are observed in this study, cutaneous and diphtheritic form. Skin form is found in all examined turkey flocks (1-6) and diphtheritic form found in flock 1 and 4 (Table 2). Mild cutaneous form noticed as small focal lesions in the skin and showed in birds of flock 2 and 5. Generalized skin lesions showed in flock 1, 3, 4 and 6, appears as multiple blisters in feathered and unfeathered area of skin, initially small in size and may enlarge due to coalescence of adjoining foci becoming grayish, elevated and wart like.

Six isolates of the diseased turkeys were inoculated in embryonated chicken eggs for 3 successive passages. They showed edematous thickening and diffused pock lesions in CAM at higher dilutions of second passage level, while clear and distinct pock lesions were observed at the lower dilutions of the same passage level. Small sized diffused pocks having diameter of about 2-3 mm were observed (Fig. 1). Different phenotypes of pock lesions were observed in CAM of inoculated eggs as white, grey and glistening pocks.Titers of the virus isolated from 6 infected flocks are measured in the virus suspension of the three passages. Virus titer are expressed as ID50/ml. as shown in table (3).

All examined lesions in all necropsied turkey revealed similar histopathological feature, mainly characterized by the presence of epidermal hypertrophy and hyperplasia in cutaneous lesions and marked epithelial hyperplasia and ballooning of crusts and scabs section of naturally infected turkey. Cytoplasm of the hyperplastic epithelial cells contained characteristic large eosinophilic inclusions (identified as Bollinger bodies) and vacuoles (Fig.2). The

diphtheritic lesions are characterized by edema, hydropic degeneration and metaplasia of the epithelium of larynx and trachea and by the presence of cytoplasmic inclusions , Bollinger,1873. Immunohistochemical examination showed the dermal and epidermal cells of the skin and the hyperplastic epithelial cells reacted strongly with antibodies against avipoxvirus. The immunoreaction was granular and occurred mainly in the cytoplasm of the infected cells, in the inclusion bodies and in the necrotic and desquamated cells in the stratum corneum of the skin (Fig.3).

Table 2: Skin lesions and post mortem findings of TPV in infected flocks

Flock No.	Skin form	Diphtheritic form	Description of symptoms and lesions
1	++	++	Generalized skin lesions in whole body specially comb, wattles, wings, foot, legs, vent, axilla, eyes, mouth and peak. Proliferative yellowish diphtheritic membrane in larynx, trachea, mouth and tongue
2	+	-	Mild cutaneous scabs and crusts in face, comb and eyes . Trachea, pharynx, larynx, tongue, palate, bronchi and lung not involved
3	++	-	Generalized skin lesions in unfeathered area specially comb, wattles, wings, axilla, groins foot and peak.
4	++	+	Multiple skin lesions distributed in most unfeatherd area in face, foot, axilla, comb and peak. Larynx, pharynx, mouth, nose, and palate are involved
5	+	-	Mild cutaneous lesions in skin of face, neck, leg and hock joint
6	++	-	Multiple skin lesions in comb, wattles, wings, foot, vent, face, axilla and peak.

Table 3: Titers of three passages of turkey pox virus isolates

Virus s isolates	Titer of first passage (log 10/ml)	Titer of second passage (log 10/ml)	Titer of third passage (log 10/ml)
1	2.3	3.7	6.3
2	3.2	3.8	4.5
3	2.8	3	3.3
4	1.9	3	4.2
5	2	3.5	5.1
6	3.1	3.3	3.4

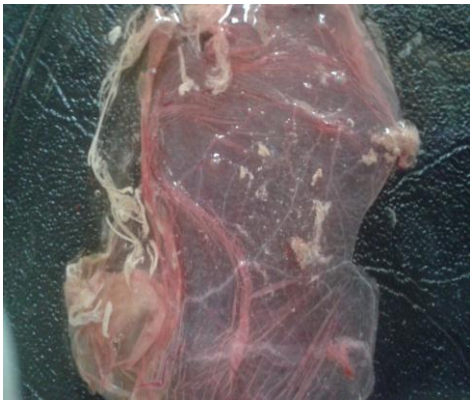

Fig.(1): small sized white pock lesions with thickening and hemorrhagic CAM

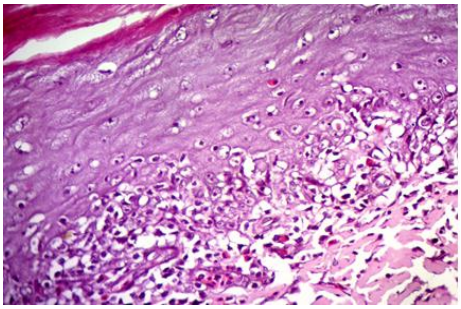

Fig.(2): Histopathological picture of skin lesion of turkey pox showing necrosis and vacuolar degeneration of epidermal cells with loss of nuclei and exocytosis and inflammatory cell infilteration in underlying dermis with intracytoplasmic inclusion bodies. H&E.120x.

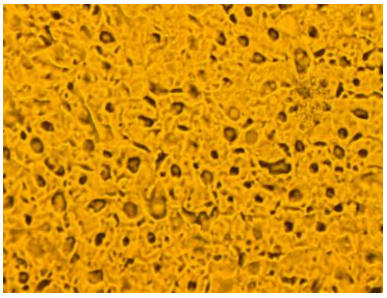

Fig.(3): Positive immune reaction of necrotic and desquamated cells in the stratum corneum of the skin

Pooled crusts and scabs of turkeys collected from all flocks and prepared in both supernatant and cell lysate form showed negative results when examined by agar gel precipitation test against reference anti avian pox immunoglobulins, however when pooled scabs and crusts of flock no.1 were propagated two serial passages in embryonated chicken eggs, AGP antigen of both supernatant and cell lysate form reacted positive with different degree of precipitation. CAM antigen of pooled crusts and scabs of flock 2, 3 and 6

reacted positive only in the second passage and shown negative results in the 1st passage. AGP antigen prepared from flock 4 and 5 either from crusts and scabs or from CAM revealed negative in both supernatant and cell lysate preparations.

Screening of pox antibodies in serum of infected turkeys showed that fifteen serum samples out of twenty with overall seroprevalence of 75% of turkey pox antibody level reacted positive when used turkey pox AGP antigen but when reference avian pox AGP antigen and avian pox vaccine antigen used, the prevalence percentage decrease to 50 and 55% respectively (Table 4).

The highest number of positive samples was shown in flock no. 1 in which 8 samples reacted positive with a percentage of 100%. Only two serum samples from flock 3 and 4 showed line of precipitation similar to positive controls. Turkey pox AGP antigen recorded the highest prevalence when compared to reference and vaccine avian pox virus.

Polymerase chain reaction (PCR) of turkey pox virus was carried out by applying the primer set targeting the p4b core gene from DNA extracted from crusts and scabs of 18 samples represented 6 turkey flocks before and after propagation in embryonated chicken eggs. Cell lysate of CAM of inoculated eggs with pooled samples from 5 turkey flocks reacted positive to PCR on contrary cell free supernatant of CAM does not show any visible band when examined with UV table (5).

Table (4):Seroprevalence of turkey pox in infected flocks using AGP antigen of avian and turkey pox virus

Flock No.	No. of serum samples	Turkey pox Ag +ve/total	avian pox Ag +ve/total	Avian pox vaccine Ag +ve/total
1	8	8/8 (100%)	4/8(50%)	5/8(62.5%)
2	2	1/2 (50%)	0/2(0%)	0/2(0%)
3	3	2/3(33.33%)	2/3(33.33%)	2/3(33.33%)
4	3	2/3(33.33%)	2/3(33.33%)	2/3(33.33%)
5	2	1/2(50%)	1/2(50%)	1/2(50%)
6	2	1/2(50%)	1/2(50%)	1/2(50%)
Total	20	15/20(75%)	10/20(50%)	11/20(55%)

Table (5): PCR results of turkey pox virus in clinical samples and CAM of inoculated eggs

Flock No.	Crusts & scabs	supernatant CAM 1st passage	Cell lysate CAM 1st passage
1	+	-	+
2	-	-	-
3	+	-	+
4	+	-	+
5	+	-	+
6	+	-	+
FP vaccin	-	+	+

DISCUSSION

Turkey pox is a slow spreading viral disease causing severe economic losses in terms of meat condemnation, weight loss and drop in egg production in Egyptian turkey flocks. The disease has an emerging status in Egypt and a little information is available. Turkey pox virus was considered more or less similar to fowl pox virus and the available literature reveals the characteristics of fowl pox virus in detail but now turkey pox virus is different from other avipox viruses at the molecular level (Triapathy et al., 1991 and 2000). Turkey Pox virus infection in most birds is mild and rarely results in deaths, particularly with cutaneous pox. However, when pox virus infection spreads to the mucous membranes of the oral cavity and upper respiratory tract, or when the flock is affected with a secondary infection mostly in poor environmental conditions, mortality rates are usually higher (Yoshikkawa et al., 2002 and Medinaet al., 2004).

In our study, two main forms of turkey pox, cutaneous and diphtheritic form have been described in table (2). Both forms are clearly shown in birds of flock 1 and more severe than birds of flock 4. Mild cutaneous form may be noticed in birds of flock 2 and 5. The variability of signs and lesions of turkey pox in different flocks depending upon susceptibility of the host, virulence of virus, mode of transmission, presence of stress factors and distribution of lesions (McFeran and McNlty, 1993).

The data obtained in table (1) revealed that morbidity rate of six turkey flocks due to turkey pox infection was very low ranged from 5-15% with no mortalities. Several factors may contribute to this finding as differences in virulence of pox virus strains, injuries of skin due to fights and host specificity. These results more or less described before by (Davidson et al., 1980) who stated that, mortality rates in wild turkeys are probably similar to chickens with regard to the severity and course of avian pox infections.

One can noticed that from results presented in table(1), that turkey pox virus spread in turkey flocks located at the boundaries of Belbis, villages Egypt during the time of fall and early winter, these results coincide with wide spread of mosquito vectors and change of weather conditions in Egypt. The same results obtained by (Akey et al., 1981; La Pointe, 2000)whose stated that, in warmer regions of the world, avian pox is reported throughout the entire year, but most often during fall and winter months. Moreover the diphtheritic form of turkey pox was prevalent in flock 1 and 4 with no mortalities, these results may correlate to the aerosol transmission of pox virus complicated with secondary bacterial invasion. Similar data obtained and supported our explanation by (Cunningham, 1972), who stated that diphtheritic lesions are infrequently detected in wild bird avian pox infections and described lesions on the mucous membranes of chickens

To isolate and characterize turkey pox virus, crust and scab filtrates were inoculated on CAM of embryonated chicken eggs. Turkey pox virus showed edematous thickening and diffused pock lesions in CAM at higher dilutions, while clear and distinct pock lesions were observed at the lower dilutions of the same passage. Small sized diffused pocks having diameter of about 2-3 mm. These results agreed with (Manarolla et al., 2010) whose reported variable levels of thickening, ranging from mild to severe, in CAMs infected with APV

isolates. In an Egyptian study, pox virus isolates from chickens and turkeys produced compact, greyish-white pocks and marked thickening of the infected CAM tissue (Abdallah and Hassain, 2013).

Concerning to histopathological examination of crusts and scabs of naturally infected turkeys with pox virus data is confirmed by the results obtained by (Metz et al.,1985). Inclusion bodies resemble Bollinger bodies which are described in avian poxvirus infections by (Eaves and Flewett, 1955; Purcell et al., 1972).

immunostaining of turkey pox viral antigen, in both the skin and respiratory tract, occurred mainly within the cytoplasm of hyperplastic epithelial cells Fig (3) and with cytoplasmic inclusion bodies, these results consistent with previous observations in quails (Gubahar et al., 2005) and strongly confirm that viral replication occurs in the cytoplasm of the virus-infected epithelial cells and the immature virions penetrate the inclusion body vacuoles, where they mature (Tripathy et al.,1991 and Tanizaki et al.,1989). The results showed that the most reliable and best AGP antigen of turkey pox virus is the concentrated supernatant antigen of CAM second passage and supernatant of avian pox antigen prepared from avian pox vaccine after 2 passage in CAM of embryonated chicken eggs. This data coincide with the fact that turkey pox antigen is a homologus antigen. Alao seroprevalence of 75% pox antibody level in infected turkeys in our study listed in table (4), using AGID test is less than 89% observed by (Ohore et al., 2007) in unvaccinated indigenous chickens. In contrast an estimated 5% seroprevalence were observed in North West Nigeria by (Saidu et al., 1994) using AGID, which is much lower than our observation. In other way conventional agar-gel immunodiffusion are still globally used for surveillance and diagnosis of viral infections in poultry (Baxi et al., 1999 ; Tadese et al., 2003 and Saidu et al., 1994). Sensitivity of AGID appears to be low but highly specific in diagnosis of pox virus infections when compared with other detection method (Smits et al., 2005, Buscaglia et al., 1985 and Ohore et al., 2007). It is also faster and easier method to detect antibodies against fowl pox, particularly when large numbers of sera are involved. AGID is still a useful test because of its simplicity in terms of test reagents, equipment and analysis that can readily be performed in standard laboratories with low budget (Mockett et al., 1987). Identification of DNA extracted from 6 turkey samples naturally infected with turkey pox virus or in CAM of infected eggs using PCR based methods targeting p4b core gene can amplify a DNA fragment of expected size at 578 bp. These results was proven before as recorded by (Lee and Lee, 1997; Jarmin et al., 2006 and Manarolla et al., 2010). PCR is rapid and sensitive test for diagnosis of turkey pox infection in clinical samples and in CAM of eggs inoculated with crusts and scabs of turkey pox. Our results are in confirmation with previous studies of (Siddique et al., 2011 and Luschow et al., 2004).

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