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Conventional and Molecular Detection of Avipoxviruses from Chickens, Pigeons and Turkeys

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ABSTRACT

In the present study, a total of 90 cutaneous lesions samples were collected from chickens, pigeons, and turkeys farms in Dakahlia Governorate, Egypt during summer 2016. These farms suspected to be infected with Avipoxviruses (APVs).Thirty pooled samples were created (10 from chickens, 10 from pigeons and 10 from turkeys). Hyperimmune serum was prepared against standard fowlpox virus in adult white New Zealand rabbits. APV were identified in the collected samples using agar gel precipitation test (AGPT), indirect immunoperoxidase, and polymerase chain reaction (PCR) based on 4b gene of APVs. The results revealed that out of 30 tested samples there were 16 samples (53.3%) tested positive via AGPT including, 6 chicken samples (60%) , 5 pigeon samples (50%) and 5 turkey samples (50%). while using indirect immunoperoxidase, positive results were detected in 23 samples (76.7%) including, 8 chicken samples (80%), 8 pigeon samples (80%) and 7 turkey samples (70%).The 4b gene of APVs was detected using PCR in all tested samples (100%). In conclusion, Indirect immunoperoxidase is superior over AGPT in APVs detection in collected samples from chickens, pigeons and turkeys. PCR could be efficiently used in molecular diagnosis of the virus.

Keywords: Fowl pox; pigeon pox; turkey pox; AGPT; immunoperoxidase; PCR.

1. INTRODUCTION

There were 9000 known avian species in the world; more than 230 in 23 orders infected naturally with Avipoxviruses in the genus Avipoxvirus (Bolte *et al.,* 1999) within the Chordopoxvirinae subfamily of *Poxviridae* family. Poxviruses are oval to brick shaped, large, enveloped DNA viruses. Poxviruses replicate in the host cell cytoplasm and their genome is linear, double stranded DNA about 300Kbp (Murphy *et al.,* 1999; Gubser *et al.,* 2004; Jarmin *et al.,* 2006; Tripathy and Reed, 2013). Avipoxviruses mostly named according to the bird species from which they were isolated. The International Committee on Taxonomy of Viruses reported that the genus *Avipoxvirus* has ten species including fowlpox virus (FWPV), pigeonpox virus (PGPV) and turkeypox virus (TKPV) (www.ictvonline.org/virusTaxonomy.asp) (Weli and Tryland 2011).

There was various antigenic relationship between APV strains, but the degree of antigenic relationship between strains is still not clear (Bolte *et al.,* 1999). Serology has revealed cross-reactivity between several avian poxvirus species. A great difference in pathogenicity is observed among various species infected with different types of avian poxvirus (Pattison *et al.,* 2008)

The disease in chickens occurs in two forms, dry form (cutaneous) and wet form (diphtheritic). The cutaneous form is characterized by proliferative lesions development, ranging from small nodules to wart-like spherical masses on unfeathered areas of the skin especially, comb and wattles. In the diphtheritic form of opaque, white slightly elevated nodules developed on the mucous membranes of the mouth, oesophagus, larynx or trachea. This nodule rapidly increases in size and become a yellowish diphtheritic membrane. In the cutaneous form of the disease, mortality rate is usually low, but it increased up to 50% in generalized diphtheritic form with secondary infection especially in young birds. (Fenner*et al.,* 1993; OIE, 2016).

In turkeys; the two forms of the disease were recorded and the disease is mainly characterized by formation of pox lesions around the eyes which usually cause blindness so the bird becomes unable to see the water and food leading to starvation with subsequent emaciation and death (Forrester and Spalding 2003).

Pigeon pox is a slowly developing disease of pigeons at any age and both sexes can be affected, disease complication as

parasitism and poor body condition leading to high mortality rates in affected pigeons (Mohan and Fernandez 2008).

APVs causing high economic losses from increased bird mortality, reduced growth, emaciation, decrease in egg production and condemnation of affected bird carcasses with multiple nodular skin lesions, cachexia and repulsive appearance (Beytut and Haligur, 2007 and OIE, 2016).

Currently there is no treatment for APVs and the control of the disease depends mainly on vaccination by thigh-stick method with FPV vaccine (Jacob *et al.,* 1998).

The present study aimed to detect the Avipoxvirusesin clinical samples collected different avian species including: chickens, pigeonsand turkeys by AGPT, indirect immunoperoxidase test and polymerase chain reaction.

2. MATERIALS AND METHODS

Samples collection

In the present study, a total of 90 cutaneous lesions samples were collected from chickens, pigeons, and turkeys farms in Dakahlia Governorate, Egypt during summer 2016. These farms suspected to be infected with Avipoxviruses. Thirty pooled samples were created (10 from chickens, 10 from pigeons and 10 from turkeys). Normal chicken skin samples were collected from 3 farms and pooled in one sample to be used as control negative sample.

Preparation of collected sample

Each sample was divided into two parts one part was rapidly frozen in cryostat chamber and sectioned for virus detection by indirect immunoperoxidase, the other part was homogenized using sterile mortar and pestle to produce 10% suspension in phosphate buffered saline (PBS). Homogenates were then centrifuged in cooling centrifuge for 15 minutes at 4000 rpm. Supernatant fluids were then kept at -20^{o}Cuntil use in virus diagnosis by agar gel precipitation test and polymerase chain reaction (Diallo *et al.,* 2010; Fasaei *et al.,* 2014).

Standard Fowl poxvirus was included in this study as lyophilized Diftosec FWPV vaccine supplied from manufacturer (MERIAL, Batch no. 037824851). FWPV was attenuated in tissue culture and supplied in lyophilized vials, each vial contains 1000 doses, and each dose contains 10^3 $TCID_{50}$ FWPV DECP25 strain. Each vial was reconstituted in 1 ml PBS and used in preparation of hyperimmune seum and as control positive in AGPT and PCR

Preparation of hyperimmune serum against fowl pox virus (Mockett*et al.*, 1990)

For preparation of hyperimmune serum against FWPV, 5 adult New Zealand White rabbits about 2 Kg each were used. About 0.25ml of standard Fowl poxvirus was mixed with 0.25ml Freund's adjuvant and inoculated intramuscularly into each of 4 adult New Zealand White rabbits, one rabbit was kept as control negative and inoculated with 0.25ml PBS mixed with 0.25ml Freund's adjuvant. One week later, the inoculation was repeated. Then the virus and PBS (control rabbit) were re-inoculated subcutaneously four times with one week interval. After two weeks from last inoculation, rabbits were bled and their blood was collected and the resulting serum was stored at -20°C until use in AGPT and indirect immunoperoxidase test diagnosis of APVs.

Agar gel precipitation test (OIE, 2016)

APVs can be detected by reacting samples from chicken, pigeon and turkey against FWPV hyperimmune serum. Agarose gel (1%) in PBS was prepared, and then the gel was bunched with gel bunch (a central well and 6 peripheral wells). The hyperimmune serum was placed in the central well and the test samples were placed in the peripheral wells together with a positive control (standard FWPV) and negative control sample collected from normal chickens. The plates were incubated at 37^{o}C in a humid incubator then examined after 48 hours and results were recorded.

Indirect immunoperoxidase test (Beytut and Haligur, 2007)

Collected cutaneous samples from chickens together with control negative samples from normal chicken skin were rapidly frozen in cryostat chamber. These samples were sectioned by cryostat about 5 µm thick each. Then cryostat sections were picked up on glass slides then fixed with acetone for 10 minutes. Rabbit hyperimmune serum raised against standard FWPV was applied to the prepared sections on the slides and the sections were then incubated at 37°Cfor 1 hour with humidity. The slides were then washed in a bath of PBS three times (5 minutes each). The sections were incubated with anti-rabbit IgG horseradish peroxidase (HRP) conjugate at a concentration of 1/200 in PBS for 1 hour at 37°Cwith humidity and the slides were washed as before. OPD indicator was prepared by adding 6 mg of Orthophenylenediamine-2HCl (OPD) powder to 10 ml citrated buffer (0.0654 gm Citric acid, 0.2464 gm and Na_2PO_4 ($12H_2O$) in 10 ml distilled water), Just before use 30 µl of 30% H_2O_2 was added to prepared substrate solution. A substrate-chromogen (OPD solution) was added to the sections and incubated for 30 minutes then the slides were washed thoroughly as before and examined by light microscope for presence of dark brown staining.

DNA extraction

DNA was extracted from supernatant fluid of homogenized samples, control negative sample and FWPV vaccine (control positive) using a commercial kit (QIAamp®MinElut® Virus Spin Kit; QIAGEN GmbH, Grermany), according to the manufacturer's instructions.

Polymerase chain reaction (Masola et al., 2014).

To confirm the existence of APV in the extracted DNA from clinical samples, *Avipoxvirus* specific PCR established with a set of primers that were designed for amplification of 578bp APV 4b gene (virus core protein). The primer sequences were: 4b forward 5′-CAGCAGGTGCTAAACAACAA-3′, and 4b reverse 5′-CGGTAGCTTAACGCCGAATA-3′. The primers used were synthesized by Metabion International AG, Germany. PCR reaction mixture contained 25µl Dream Taq Green PCR Master Mix (2X)(Thermo Scientific), 5µl of the extracted DNA,1µl of each primer and nuclease free water that was added to a final volume of 50µl. A control positive tube (DNA extracted from standard FWPV) and a control negative tube (DNA extracted from control negative sample) were included.

The PCR cycles were, one cycle at 94°C for 2 minutes followed by 25 cycles of 94°C for 1 minute, 61°C for 1 minute and 72°C for 1minute. These cycles were then followed by one cycle at 72 °C for 10 minutes. Then the PCR products were separated by agarose gel electrophoresis using1.5 agarose gel in Tris-Borate EDTA buffer and the gel was stained with 0.5 µg/ml ethidium bromide. Samples were loaded in the gel next to 100 bp DNA ladder (Jena Bioscience, Germany).UV transilluminator was used to visualize the gel and imaged by a digital camera.

3. RESULTS

Clinical signs and postmortem changes

Ninety cutaneous samples were collected and pooled to get 30 working samples (10 from chickens, 10 from pigeons and 10 from turkeys). These samples were collected were collected from chickens, pigeons, and turkeys farms in Dakahlia Governorate, Egypt during summer 2016 from birds suspected to be infected with APVs as wart-like spherical masses were observed on unfeathered areas of the skin including; head, legs and in some birds pox lesions were developed around one eye or both eyes then increase in size till complete involvement of the eye and the bird become blind, unable to see food and water leading to starvation and death Figure(1).

Normal chicken skin samples were collected from 3 farms and pooled in one sample (control negative). Only 3 chicken farms were vaccinated with FWPV vaccine, the other 7 farms were not vaccinated and all pigeons and turkeys were not vaccinated, morbidity rate ranging from 80-100% but mortality rate was 0-45% as shown in Table (1).

Identification of APVs by agar gel precipitation test

Collected samples, standard FWPV and control negative sample were examined against FWPV hyperimmune serum by AGPT. Positive result appear as clear precipitation line 16 samples (53.3%) were positive, 6 chicken samples (60%) , 5 pigeon samples (50%) and 5 turkey samples (50%) and the other14 samples (46.7%) and control negative sample showed no precipitation line, Figure(2), Table (2).

Identification of APVs by indirect immunoperoxidase test.

For identification of APV in collected chicken, pigeon and turkey tissue samples, frozen tissue sections were rapidly sectioned and stained by indirect immunoperoxidase. Positive result was detected as dark brown, deeply stained areas in 23 samples (76.7%) including, 8 chicken samples (80%), 8 pigeon samples (80%) and 7 turkey samples (70%) and control positive sample however the examination of the other 7 samples (23.3%) and control negative samples revealed no dark brown areas, Figure (3), Table (2).

Confirmation of APVs diagnosis by polymerase chain reaction

To confirm the presence of APV in collected samples, conventional PCR was performed for amplification of the APV 4b core protein gene from field samples, control positive (standard FWPV) and control negative sample. The PCR products were then separated by agarose gel electrophoresis, the obtained bands appeared at 578bp. All thirty tested field samples and control positive gave the positive expected band but the control negative sample not show band, Figure (4), Table (2)

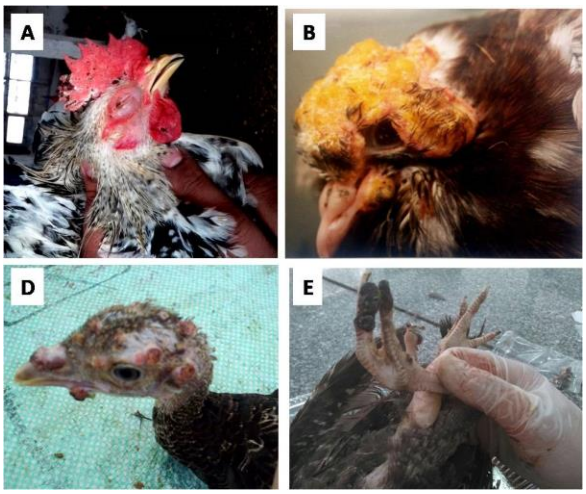
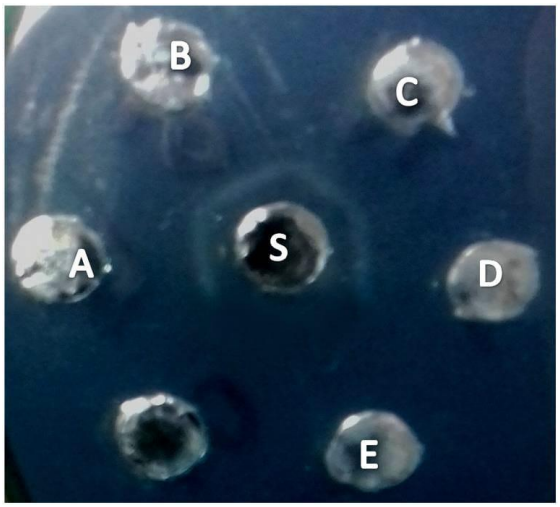


Figure 1. Lesions of APVs in different hosts. **(A)** Chicken with pox lesions on the comb and wattles, the eye was also affected and totally closed. **(B)** pigeon squab with pox lesions which coalesces and involved all the head. **(c)** Turkey with pox lesions distributed all over the head. **(D)** Turkey with pox lesions on the leg.



Figure(2): Result of APVs detection by AGPT, positive samples produce precipitation lines, **(A)** control positive standard FWPV, **(B)** field sample from chicken, **(C)** field sample from pigeon, **(D)** field sample from turkey, **(E)** control negative (non infected chicken skin) and **(S)** hyperimmune serum.

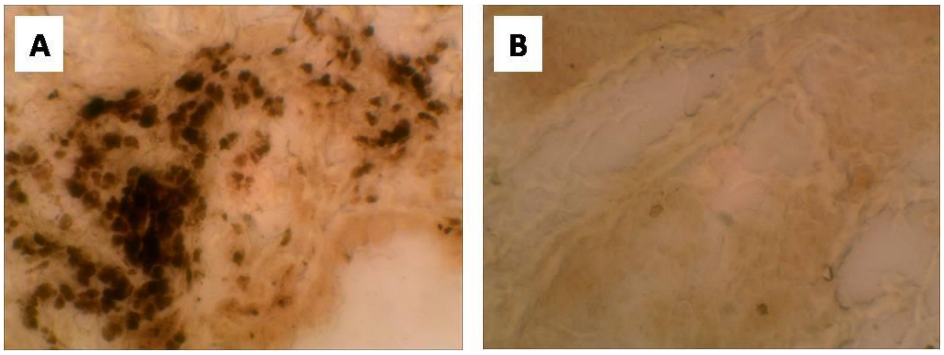


Figure 3. A: deeply stained dark brown areas in cryostat section of skin lesion stained by indirect immunoperoxidase, X40. **B:**Normal cryostat section of chicken skin with no dark brown areas, X40).

Table(1): Details of samples collected from diseased chickens for APVs diagnosis including host, age of bird, form of the disease, morbidity, mortality, sample collected and vaccination history.

Sample no	Sample code	Host	Age (Week)	Morbidity %	Mortality %	Vaccination History
1	F1	Chickens	27	98%	2%	Vaccinated
2	F2	Chickens	12	100%	4%	Non-vaccinated
3	F3	Chickens	35	95%	0%	Vaccinated
4	F4	Chickens	13	100%	40%	Non-vaccinated
5	F5	Chickens	12	100%	1%	Non-vaccinated
6	F6	Chickens	20	80%	1%	Vaccinated
7	F7	Chickens	17	90%	1%	Non-vaccinated
8	F8	Chickens	3	95%	30%	Non-vaccinated
9	F9	chickens	5	90%	45%	Non-vaccinated
10	F10	chickens	12	93%	35%	Non-vaccinated
11	P1	pigeons	4	90%	7%	Non-vaccinated
12	P2	Pigeons	7	96%	9%	Non-vaccinated
13	P3	Pigeons	6	100%	3%	Non-vaccinated
14	P4	Pigeons	5	97%	4%	Non-vaccinated
15	P5	Pigeons	19	95%	1%	Non-vaccinated
16	P6	Pigeons	12	90%	0%	Non-vaccinated
17	P7	Pigeons	17	85%	1%	Non-vaccinated
18	P8	Pigeons	20	90%	1%	Non-vaccinated
19	P9	Pigeons	27	94%	0%	Non-vaccinated
20	P10	Pigeons	18	85%	2%	Non-vaccinated
21	T1	Turkeys	25	94%	0%	Non-vaccinated
22	T2	Turkeys	13	100%	2%	Non-vaccinated
23	T3	Turkeys	4	90%	3%	Non-vaccinated
24	T4	Turkeys	29	93%	1%	Non-vaccinated
25	T5	Turkeys	37	88%	2%	Non-vaccinated
26	T6	Turkeys	12	96%	4%	Non-vaccinated
27	T7	Turkeys	6	90%	9%	Non-vaccinated
28	T8	Turkeys	28	96%	2%	Non-vaccinated
29	T9	Turkeys	20	100%	1%	Non-vaccinated
30	T10	Turkeys	35	100%	0%	Non-vaccinated

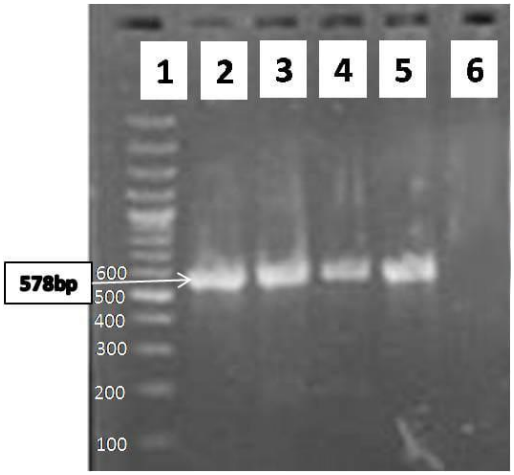


Figure 4. PCR products of the APV 4b gene (578bp) in agarose gel electrophoresis, along 100bp ladder. Lane 1: 100 bpladder, Lane 2: control positive sample (standard FWPV), Lane 3: field sample from chicken. Lane 4: field sample from pigeon, Lane 5: field sample from turkey, Lane 6: negative control (DNA from normal chicken skin)

Table 2. Comparative results of APVs identification using AGPT, IP test and PCR.

Number of tested samples	Number of positive samples (%)		
	AGPT	IP test	PCR
10 chicken samples	6 (60%)	8 (80%)	10 (100%)
10 pigeon samples	5 (50%)	8 (80%)	10 (100%)
10 turkey samples	5 (50%)	7(70%)	10 (100%)
Total (30 samples)	16 (53.3%)	23 (76.7%)	30 (100%)

4. DISCUSSION

Fowl pox, pigeon pox and turkey pox are important viral diseases of chickens, pigeons and turkeys caused by FWPV, PGPV and TKPV, respectively. All of them are members in the genus *Avipoxvirus*, subfamily *Chordopoxvirinae* of the family *Poxviridae*.(Weli and Tryland 2011 , Tripathy and Reed, 2013)

Clinical samples were collected from chickens, pigeons and turkeys with pox lesions on unfeathered areas of skin, some bird's loss one or both eyes and mortality rate increased up to 45% of infected birds due to secondary infections. These finding were in agreement with that mentioned in OIE report, that fowl pox virus characterized by proliferative lesions formation on the skin, (OIE 2016). Also, Kabir*et al. (*2015), Masola*et al*. (2015) and Masola*et al.* (2016) observed cutaneous nodular lesions in featherless parts of chickens and pigeons.

Thirty pooled clinical samples (10 chicken, 10 pigeons and 10 turkeys) were collected from pox virus characteristic skin lesions. Identification of APV in collected samples was carried out by AGPT and indirect immunoperoxidase test, and then obtained results were confirmed by PCR. The results of AGPT appear as clear line of precipitation, out of 30 tested samples there were 16 samples (53.3%) gave positive results by AGPT. These results were in concurrence with Babiker (1992) who compared a reference strain (Beaudette strain) with field isolates by the AGPT and he found that all the isolates were identical with vaccine strain. Tamador*et al.,* (2001) used AGPT to confirmed the identity of the virus with known fowl pox antiserum. In this study only one precipitation line was observed between hyperimmune serum and each of 6 chicken samples, 5 pigeon samples and 5 turkey samples but Uppal and Nilakantan (1970) studied serological relationships between fowl pox and pigeon pox viruses by agar gel precipitation tests and observed that when antisera against FWPV were diffused against homologous antigen, two lines of precipitation were formed and when antisera prepared against pigeon pox virus were diffused against either homologous or heterologous antigen, only one line of precipitation was formed. Frozen tissue sections from chickens, pigeons and turkeys were stained with indirect immunoperoxidase using hyperimmune serum prepared against FWPV vaccine and positive results appear as deeply stained dark brown areas; out of 30 tested samples 23 samples (76.7%) were positive. These results were coincided with Tripathy *et al.,* (1973) and Beytut and Haligur (2007) who identified FWPV by an immunoperoxidase method and observed that when FWPV inclusions reacted with FWPV antibody labeled with peroxidase enzyme that react with its substrate leading to staining of tissue with dark brown color then detected by ordinary microscope. The avipoxvirus 4b core protein gene encodes a protein of 75.2kDa, this gene usually amplified for comparative genetic analysis and used as a molecular tool for avian pox virus detection Manarolla*et al.,* (2010). In this study the 4b gene PCR product of expected size (578bp) was detected in all tested samples and standard FWPV (positive control). These results were in accordance with Masola *et al.,* 2016; Masola *et al.,* 2015; Puro *et al.,* (2015); Kabir *et al.,* (2015); Fasaei *et al.,* (2014); Masola *et al.,* (2014); Manarolla *et al.,* (2010) who described that 578bp PCR product was successfully amplified from different avian species.

From these results, it can be concluded that the PCR is the most sensitive and specific method for diagnosis of APVs followed by immunoperoxidase test which is an easy, not expensive and rapid test but it is less sensitive than RCR as it detected APV in 76.7% while PCR amplified the 4b gene from all samples (100%). AGPT is the cheapest and easiest test but its sensitivity in APV detection is low (53.3%) and precipitation lines takes long time (about 48 hours) to be formed, also high concentrations of both virus and antibody are needed.

FWPV, PGPV and TKPV give the same result when tested with hyperimmune serum prepared against standard FWPV, so there is an antigenic relationship between the three viruses and the FWPV vaccine can be used for vaccination of pigeons and turkeys. Further studies are crucial to differentiate between FWPV, PGPV and TKPV at molecular level.

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