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COMPARATIVE GENETIC CHARACTERIZATION BETWEEN ISOLATED POX VIRUS FROM DOVE AND SOME AVIAN POX STRAINS

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SUMMARY

Avipoxviruses from different geographic regions of the world have been characterized to study their genetic and biological properties, but so far, no such work has been performed on Egyptian isolates. Lesions suggestive of avian pox; found on Egyptian wild dove; were used for isolation of pox virus in a previous study. The resulting virus was propagated in chorio-allantoic membrane (CAM) of specific pathogen free (SPF) Embryonated chicken eggs (ECE).

PCR was carried out on the DNA of the dove poxvirus (DPV), pigeon poxvirus (PPV) and a vaccinal strain of fowl pox virus (FPV), then restriction fragment length polymorphism (RFLP) assay was carried out on the resulting amplicons of 578 bp length; using EcoRV and NlaIII restriction enzymes. The restriction profile revealed that the dove pox virus is identical to the PPV and both are different from FPV.

The results of immunoblotting analysis of the 3 pox viruses against chicken anti FPV revealed that in spite of the minor antigenic differences observed between them the DPV is closely related to the PPV. In conclusion the Egyptian wild doves are found to play a serious role in the epidemiology of PPV among pigeon flocks.

INTRODUCTION

Natural pox virus infections have been reported in more than 200 species of wild and domestic birds (Bolte et al., 1999). The pox viruses which infect birds belong to the genus Avipoxvirus of the Poxviridae family. Although a few members

(fowl pox, turkey pox, pigeon pox, canary pox, and quail pox) of the genus have been characterized.

Vaccination was found to be an effective method for control of this disease and thus, commercial fowl pox and pigeon pox virus vaccines were developed (Tripathy and Hanson, 1975).

The disease produces lesions on the skin, mouth, esophagus and trachea, although viruses may be found in other organs of the affected birds, resulting in substantial economic losses in commercial poultry (Tripathy et al,2003). However, there is no unified view point yet ,as to the exact number of species ,strains, or variants that exist within the genus Avipoxvirus (Bolte et al,1999)further more, information on genomic characterization as well as pathogenicity and effect of avipox virus isolates from wild birds on commercial chickens is limited (Weli et al 2004). So commercial poultry may be susceptible to various members of the genus Avipoxvirus (Tripathy and cunningham ,1984).

In Egypt the role of the local wild birds such as doves, sparrows and others in the epidemiology of the disease in Egypt is unknown.

In the present study we undergo to clarify the genomic characterization of an avipox virus isolated from an Egyptian dove compared with a fowl pox vaccinal strain and pigeon pox viruses , to investigate the role of doves in the epidemiology of the disease in Egypt.

MATERIALS AND METHODS

Virus propagation and purification:

In the present study the following viruses we obtained from the department of Pox virus, Se um and Vaccine Research Institute (SVRI): FP vaccinal strain of the SVRI; PPV of the SVRI a DPV which was isolated in a previous work (Na dia et al. 2005).

Each virus was propagated by inoculation c dropped CAM of 12 day old embryos (Schnitzlei et al 1988), the CAMs were collected 5 days po inoculation, homogenized in 1/10 (wt / vol) pb containing 10µg / ml gentamicin , 10 mg / m streptomycin and 1000 IU / ml penicillin G sod um.

The homogenate was centrifuged at 551 xg for min. and finally the supernates were collect The viruses were pelleted at 21,525 xg for 60 m at 4°C the suspended pellet was sonicated to 6 cles, for 15 sec, and placed on ice for 40 sec. tween each cycle (Tadese and Reed 2003). T sonicated resuspension was centrifuged throug 9 ml cushion of 36% (wt/vol) sucrose at 31,(xg for 90 min at 4°C. The pellet was resuspen in TE (10 mM Tris-1 mM EDTA) of pH 9.0 sonicated again to 2 cycles. The resulting re pended pellet was layered on a 20%-50% (wt/

continuous sucrose gradient and centrifuged at 31,000 xg for 60 min at 4°C. The band containing the purified virus particles was collected and pelleted by centrifuging at 86,100 xg for 60 min at 4°C. The pellet was then resuspended in TE and stored at -80°C. Virus suspensions were also purified by sodium diatrizoate gradient centrifugation (Esposito et al 1978).

Viral DNA Isolation:

Viral DNA was isolated from the infected and homogenized CAMs by a modification of the technique Schnitzlein et al (1988). In brief the homogenized CAMs were clarified by centrifugation at 551 xg for 10 min. the supernatants were centrifuged at 21,525 xg for 60 min, and the pellets were resuspended in TE. The resuspended pellets were further suspended in o.5% 2-mercaptoethanol (2-ME) and 10% Triton X-100. The suspension was agitated on ice for 10 min and centrifuged at 1,240 xg for 5 min; the resulting supernatant was centrifuged at 31,000 xg for 60min at 4°C. The pellet was reconstituted in TE buffer containing; 0.25% 2-ME, Proteinase K (10 mg/ml) and 20% N-lauryl sarcosinate. After 30 min of agitation on ice, 54% of sucrose in water and 20% sodium dodecyl sulfate (SDS) were added and incubated at 55°C for 2 hr. The resulting mixtures were extracted twice with 25:24:1 (vol/vol) phenol:chloroform:isoamyl alcohol and once with 24:1 (vol/vol) chloroform: isoamyl alcohol and concentrated by ethanol precipitation.

PCR Amplification:

The oligonucleotide primers were designed according to (Hofmann, 2006). The primers were designed on the basis of published DNA sequences of the 4 bp core protein gene sequence of FPV (HP444) (Binns et al 1989; Ghildyhal et al 1989). The primers sequence for the forward and reverse primers were illustrated in table (1). PCR was carried out in a final volume of 50µl containing 10 X PCR buffer, 2.5 µM Mgcl2, 200 µM of each of deoxynucleotide ,1 µM of each primer , 2.5 units Taq DNA polymerase and 0.2µg template DNA. The reaction mixture was subjected to 94°C for 7 min. followed by 35 cycles of 94°C for 1 min., 50°C for 1 min. and 72°C for 2 min. and final extension at 72°C for 7 min. The PCR products were separated on 1% agarose gel in 1X TAE .The PCR products are purified using PCR product purification kit of Qiagen Company.

Table(1): The sequences of the primer set

Primer	Sequence	Nucleotide position	Amplicons
Forward	CAG CAG GTG CTA AAC AAC AA	458 – 477	578
Reverse	CGG TAG CTT AAC GCC GAA TA	1016 - 1035	

RFLP assay:

RFLP assay was carried out on the 578- bp amplicons of FPV, DPV and PPV using EcoR V and Nla III restriction enzymes; according to the manufacturer instructions and Hoffmann, 2006. the resulted fragments for each virus were separated on 2% agarose gel in 1 x TAE.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting: This technique was carried out according to Tadese and Reed, 2003. Purified virus suspensions were solubilized in equal volumes of lysis buffer (50 mM Tris-HCl, pH 6.8; 5% 2-ME; 10% glycerol; 1% SDS; 0.001%bromophenol blue) and heated in a microwave oven (power output 725 W) for 60 sec. Supernatant extract from uninfected cells was also prepared and used as control. The protein concentrations of purified virus particles were determined photometrically using a protein assay kit of Bio-Rad Company, and samples of equal protein concentrations were loaded in stacking gel of 4% polyacrylamide and a resolving gel of 12% polyacrylamide. After 1 hr of electrophoresis at 120 V and 4 hr at 200 V, protein on the gel were transferred to nitro cellulose filters. The filters were blocked with 5% nonfat dry milk in phosphate-buffered saline (PBS), pH 7.4 washed 4 times with PBS, and made to react with 1:200 chicken anti-FPV serums for 24 hr at room temperature. After washing 4 times with 0.02% Tween-20 in PBS, peroxidase-labeled goat anti chicken IgG (1:2,000) was added, and incubation was performed for 2 hr at room temperature. Following 4 washes with PBS, the filters were exposed to 4- chloro-1-naphtol, as substrate, containing 0.02% hydrogen peroxide. The relative molecular mass (rmm) of the reacted proteins was determined by comparison with180 KD protein maker of Sigma Aldrich Company.

Table (2): The restriction fragment profile of DPV, PPV and FPV with either EcoR V or Nla III restriction enzymes.

EcoR V			Nla III		
DPV	PPV	FPV	DPV	PPV	FPV
252	252	252	446	446	185
177	177	177	132	132	132
149	149	149			112
					96
					53

RESULTS

PCR and RFLP analysis of the FP, PP and DP viruses:

The primer set designed from the 4b core protein gene of FPV amplified a 578 bp fragment for the 3 viruses. There was no difference in size of the amplified products. There was no amplification in the control sample from non infected CAM. (Fig.1).

PCR fragments of 578 bp of the FPV, PPV and DPV were digested with the EcoR V & NlaIII restriction enzymes (Table 2, Fig 2,3). The RFLP profile of the DPV was identical to that of the PPV and FPV after EcoRV digestion which yields 3 fragments of 252, 177 and 149 bp lengths for each virus. On the other hand the FPV showed a different RFLP profile after NlaIII digestion which yield 5 fragments of 185, 132, 112, 96 and 53 pb lengths, while the RFLP profile of both DPV and PPV were still identical and yield 2 fragments of 446 and 132 bp lengths.

Immunoblotting analysis of the FPV, PPV and DPV antigens:

In order to determine if FPV, PPV and DPV could also be differentiated antigenically i.e. on the level of immunogenic proteins, purified virus suspensions were first standardized and after electrophoreses on polyacrylamide and transfer to nitrocellulose filters, immunoblotted using chicken anti FPV serum. When antiserum against FPV was used (Fig. 4 and Table 3), antigens with approximate molecular weights of 65, 60, 36, 35.5 and 25 KD were detected in the 3 Pox viruses. Two other antigens of relative molecular mass (rmm) of 73 and 37 KD were unique to both PPV and DPV. On the other hand a 38 KD antigen were detected in the FPV only, while an antigen of rmm of 34 KD was detected in both FPV and DPV.

Table (3): Antigen profile of DPV compared with PPV and FPV using antiserum against FPV.

Strain	Relative molecular mass (KD)								
	73	65	60	38	37	36	35.5	34	26
DPV	+	+	+	-	+	+	+	+	+
PPV	+	+	+	-	+	+	+	-	+
FPV	-	+	+	+	-	+	+	+	+

DPV = Dove pox virus; PPV = Pigeonpox virus; FPV = Fowlpox virs
KD = Kilo dalton; + = Presence of the specific antigen;
- = Absence of the specific antigen.

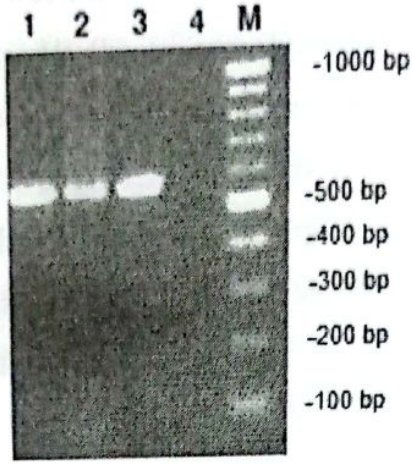

Fig. (1) Amplification of DNA from FPV (lane 1) DPV (lane 2) and PPV (lane 3). lane M, 100 bp DNA ladder (of Fermentas company).

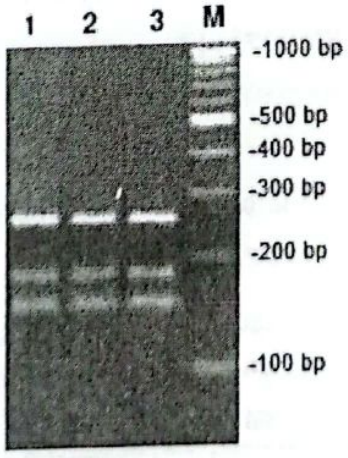

Fig. (2) Restriction profile of the 578-bp amplicons of the 3 avipox viruses using EcoR V. lane M, 100 bp DNA ladder; lane 1, FPV; lane 2, DPV and lane 3, PPV.

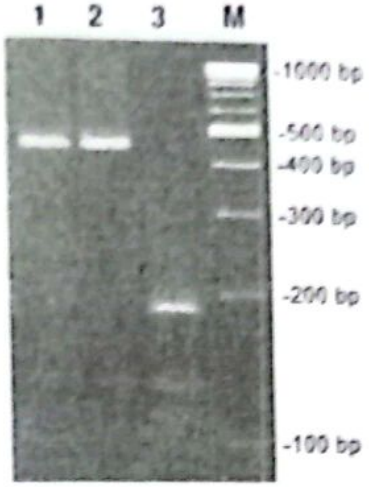

Fig. (3): Restriction profile of the 578-bp amplicons of the 3 avipox viruses using NlaIII. lane M, 100 bp DNA ladder; lane 1, DPV; lane 2, PPV and lane 3, FPV.

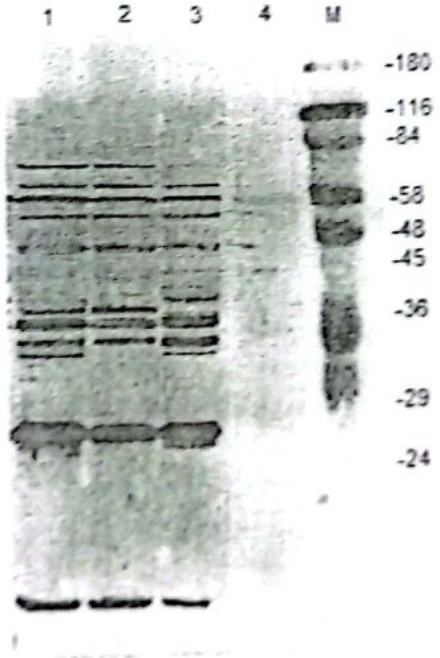

Fig. (4) *Immunoblotting of purified lysates from non-infected CAM or infected with* avipoxviruses with antiserum against FPV. Lane 1, DPV; lane 2, PPV; lane 3, FPV; lane 4, lysate of non infected CAM; lane M, pre-stained molecular weight marker.

DISCUSSION

This study was designed to characterize an avipoxvirus isolated from local wild dove in Egypt, and to compare it with the fowl pox virus and PPV. Since such local wild birds like doves may play an important role in the epidemiology of avian pathogens. Via transmission of such pathogens either from wild migratory birds to the domestic birds (commercial chickens, Turkey, Pigeons or in between the domestic birds.

A trial to characterize the isolated DPV in comparison with the available avipoxviruses (FPV and PPV) to determine if such DPV is either related to any of them or even represent another species of avipox viruses. The result of the PCR/RFLP analysis for species differentiation in the genus avipox revealed that the DPV is closely related and identical to the PPV, and both of them are different from the FPV.

The 4 b core protein gene amplifying a 578-bp fragment provided the target for PCR/RFLP analysis for species differentiations in the genus avipoxvirus (Hoffman 2006).

When the FPV, PPV and DPV are compared by immunoblotting analysis, The PPV and DPV showed a similar immunogenic protein profile which was distinct from that of FPV.

Inspite of the obtained results, there are many common antigens which were present in the FPV, PPV and DPV. However, through the use of SDS-PAGE, the immunogenic viral proteins were resolved and minor antigenic differences between the 3 viruses were observed. Since the uncommon viral proteins are detected by antiserum against FPV, at least some epitops or portion of these proteins have been conserved. In this regard the genetically similar DPV and PPV contained antigens common to FPV. The antigenic relatedness of FPV and PPV has previously been indicated by their ability to cross-immunize chicken (Winterfield and Hitchner, 1965).

The results of both PCR/RFLP assay and immunoblotting analysis revealed that DPV is closely related to PPV and could not be considered a new species.

From the epidemiological point of view, the DPV might have been acquired from infected pigeon, so doves may be considered as a biological vector for PPV and could play a serious role in the spread of the disease among non vaccinated or even vaccinated pigeon flocks.

So application of biosecurity measures in the pigeon farms and chicken farms will prevent entrance and contact of such wild birds with the commercial flocks and subsequently prevent spread of FPV, PPV or any avian pathogen among our poultry flocks.

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مقارنة التوصيف الجيني لفيروس الجدري المعزول من اليمام وبعض عترات جدري الطيور

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لقد تم دراسة الخصاءص الجينية والبيولوجية لمجموعات فيروسات جدرى الطيور وذلك فى مختلف بقاع العالم، لكن والى حد بعيد لم تتم هذه الدراسات بالشكل الكافى على المعزولات المصرية من هذه الفيروسات. فى هذه الدراسة تم لأول مرة محاولة توصيف وتعريف لاحدى معزولات فيروس الجدرى_والتى تم عزلها فى دراسة سابقة من يمام مصرى مصاب بمرض الجدرى،

وفى هذه الدراسة تم عمل اختبارتفاعل البلمرة المتسلسل لفيروس جدرى اليمام وفيروس جدرى الحمام والعترة المستخدمة فى لقاح جدرى الطيور بمعهد الأمصال واللقاحات البيطرية. ثم عمل اختبار RFLP على ناتج تفاعل البلمرة المتسلسل لكل فيروس بإستخدام انزيم الفطع EcoRV وانزيم القطع NlaIII حيث افادت النتائج ان فيروس جدرى اليمام متشابة تماما مع فيروس جدرى الحمام كما ان الأثنين مختلفين عن فيروس جدرى الطيور. وبالرغم من أن نتائج الـ Immunoblotting افادت أنه يوجد فروق ضئيلة جداً بين الفيروسات الثلاث إلا أن فيروس جدرى اليمام متشابه إلى حد اكبر مع فيروس جدرى الحمام. وخلصت الدراسة إلى اهمية الدور الوبائى الذى يلعبه اليمام البرى فى إنتقال فيروس الجدرى بين قطعان الحمام فى مصر.