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Phenotypic and genetic characterization of fowl pox and turkey pox viruses

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Fowl and turkey pox viruses were analyzed for their heterogeneity on the basis of protein profile, western blotting, PCR analysis and restriction endonuclease analysis. On the protein level, only a single band with a WM of 115KDa was seen with turkey pox virus but not with the fowl strain, and a band reacted at 10KDa with Turkey but not fowl pox virus in western blotting analysis other protein profiles were nearly the similar. PCR amplification of the lateral terminal repeat (LTR) region of both viruses confirmed a single band migrating about 900 bp with both strains and restriction digestion proved the homology between both strains.

Fowl pox is a viral disease of poultry due to infection with avipoxvirus from the family *Poxviridae* .There is a number of pox viruses which affect different species of birds. Fowls and turkeys are particularly susceptible to fowl pox virus while pigeons suffer mostly from pigeon pox virus.

Turkeys are also frequently affected with poxvirus infection. The pox disease in turkeys have attracted attention as this disease has been reported worldwide, resulted into severe economic losses in spite of proper management and health care (Dash *et al.,* 2003; Tripathy and Reed, 1989). This disease causes severe diptheritic and cutaneous lesions leading to high mortality in case of diptheritic form and appreciable economic losses in case of cutaneous form of disease due to meet condemnation (Tripathy and Reed, 1997). Pox infection in turkeys tends to be more chronic in nature with longer duration than fowl pox virus (Wakenell *et al.,* 2001).

Virions consist of an envelope, a surface membrane, a core, and lateral bodies. During their life cycle, virions produce extracellular and intracellular particles that can occur in two phenotypes; may be enveloped during their extracellular phase. The infection is initiated by extracellular virions. Virus may be sequestered within inclusion bodies that are not occluded and typically contain one nucleocapsid (Boulanger *et al.,* 2000). Virus capsid is enveloped and Virions mature naturally by budding through the membrane of the host cell. Virions are generally brick-shaped, or pleomorphic and measure200 nm in diameter, or 260-280 nm in diameter; 330-350 nm in length displaying tubular units. The core is biconcave with two lateral bodies. Lateral bodies are lens-shaped, nested between the core membranes, or between the surface membranes that contains a folded nucleoprotein thread.

Consistent with their size and complex structure, poxvirus virions contain numerous polypeptides. About 30 prominent bands are readily resolved by one-dimensional polyacrylamide gel electrophoresis of the membrane fraction of the virus which have been purified from infected cells and disrupted with sodium dodecyl sulfate and a reducing agent, but 75 or more are detected on two-dimensional gels (Jensen *et al.,* 1996) or by mass spectrometry (Chung *et al.,* 200). About 25 polypeptides have been localized near the exterior of purified virus. The surface proteins can be divided structurally into those with or without transmembrane domains and functionally into those required for morphogenesis and those with known or presumed roles in entry. None of the virus membrane proteins are glycosylated, which has important implications for the derivation of the viral membrane.

Virus cores can be produced in vitro by treatment of virus with a nonionic detergent and reducing agent. About 50 polypeptides, distinct from those in the membrane fraction, were identified in such cores. Of these, about 30 are enzymes of which at least half are directly involved in early mRNA biosynthesis the nonenzymatic proteins may be primarily involved in morphogenesis and structure.

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Fowl pox virus genome is not segmented and contains a single molecule of linear double-stranded DNA that encode for DNA ligase, ATP-GTP binding protein, uracil DNA glycosylase, DNA polymerase, DNA topoisomerase, DNA processing factor and replication essential protein kinase (Alfonso *et al.,* 2000). The genome has a GC content of 35 %. The genome sequence has termini with cross-linked hairpin ends (i.e. single- stranded loops thus forming one continuous polynucleotide chain). The genome has terminally redundant sequences. The terminally redundant sequences have reiterated inverted terminal sequences which are tandemly repeated; are reiterated internally. The genome sequence is repeated at both ends (Srinivasan *et al.,* 2006).

The current study aimed to recognize the differences between FPV and TPV phenotypically and genetically.

Materials and methods

Viruses. Fowl pox (FPV) was obtained from Pox department, Veterinary serum and vaccine research institute, Abassia, Cairo, EGYPT. Turkey pox (TPV) virus was isolated and characterized from natural outbreak of pox in turkey in EGYPT (Nakhla and Daoud 2004).The viruses were first propagated onto the chorio-allantoic membrane of 9-11 days SPF embryonated chicken eggs, after 5 days of incubation at 37°C, the chorioalantoic membrane was collected, homogenized in 5 mL Tris EDTA (TE) buffer (10mM Tris-HCl, 1mM EDTA pH 8) and centrifuged briefly at 900 rpm/5min/4°C the supernatant was then collected and stored at -20 °C till used.

Virus purification. Both FP and TP viral suspensions were subjected to purification by sucrose density gradient ultr till used.

Dual extraction of the viral DNA and protein. Both viral DNA and viral proteins were extracted from a 0.5 mL sample of the purified viruses, using Trizol reagent (Life technology cat # 15596) according to the instruction of the manufacture. First, 0.5 ml of Trizol reagent was added to 0.5 ml of the virus, and incubated at 20°C/30min, and then 200µl of chloroform was added and incubated for 3 min before centrifugation at 14000rpm /15min at 4°C. The upper aqueous phase was completely removed; the DNA in the interphas was precipitated by adding 0.5ml of absolute ethanol and centrifuged as before.

Extraction and purification of viral DNA. The precipitated DNA from the interphas washed twice with 0.1M sod. citrate in absolute ethanol and finally redissolved in 50ul of 8 mM NaOH. The pH was then adjusted at 8 by adding 115 µl/ml HEPES (0.1 M). Two µl of RNAase were then added and incubated at 37°C for 1 hour. Purification of the genomic DNA was done using Wizard DNA clean up system (Promega). The DNA was then analysed by agarose gel electrophoresis on 1% agarose and visualised using the UV. transilluminatore. The size of the genomic DNA was determined using Lambda DNA \Hind III ladder (sibEnzyme cat# M01) which has bands with molecular size of 23, 9.4, 6.5, 4.3, 2.3, 2.0, 0.5 and 0.12 Kpb

Extraction and purification of viral protein. The protein rich supernatant was transferred to another 1.5 microfuge tube and the protein was precipitated with double volume isopropyl alcohol and sedimented by centrifugation as before. Protein was then washed twice with 0.3 M guanidine hydrochloride in 95% ethanol, centrifuged as before. The protein pellet was then redissolved in 100µl of absolute ethanol and stored at -20°C till used.

Determination of protein concentration. The protein concentration was estimated using modified lowery method (Ohnishi and Barr, 1978).

Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE). Extracted proteins of the purified virus preparations were resolved on discontinuous buffer system composed of 12% (w/v) acrylamide separating gel and 4% stacking gel (Laemmli, 1970). Electrophoresis was carried out at a constant voltage (100 V) until the bromophenol blue dye moved to the bottom of the gel. The gels were stained with coomassie brilliant blue for 2 hours and destained overnight. Molecular weight of each protein band was calculated with reference to a standard curve derived from the migration pattern of standard unstained molecular weight markers (Page Ruler, Fermentas cat# 00018067).

Western blotting analysis. The electrophoretic transfer of polyacrylamide gel resolved proteins to the nitrocellulose membrane was carried out by electroblotting as described (Towbin et al., 1979) using BioRad Electro Transfere unit. The unoccupied sites on the nitrocellulose membrane were blocked with blocking buffer (Tris buffered saline TBS, pH 7.2 containing 0.1% Tween-20, 1% (w/v) western blot grade gelatin and 0.05% Triton X100). The nitrocellulose membrane was then incubated with Anti fowl pox virus antibodies (pox vaccine department VSVRI)

(1:100 in blocking buffer) at 37°C for 1 hour followed by washing three times with TBS-Tween 20. The membrane was then incubated at 37°C for 1 hour in anti-chicken peroxidase labeled dilution of 1:5000 in TBS- tween 20. The membrane was then washed as above and incubated in freshly prepared substrate solution (10 mg aminoethyle carbazone in 50 ml PBS containing 50 µl of 30 % H_2O_2) for 3-4 min for colour development and visible bands were developed, then the reaction was stopped by washing the membrane with running distilled water. Page Ruler prestained protein ladder (Fermentas cat#SM0671) was used in this experiment.

PCR amplification. A primer was designed to amplify the lateral terminal repeat gene (Afonso et al., 2000) using NCBI/ Primer-BLAST. The PCR was performed in 50-µl reaction mixtures containing 50 mM KCl, 10mM Tris-HCl (pH8.8), 3 mM MgCl2, 200 mM (each) deoxynucleoside triphosphate, 10µl Q solution (Qiagene) and 2.5 U of thermostable recombinant Taq DNA polymerase and 50 pmol of the forward primer (5'- TTA-ACC-AGT-CTT-ATT-ATT-AA–3') and the reverse primer(5'- TAT-AAA-GGT-GTA-AAT-CCT-AAT-TAC-3'). Thermal cycling was performed using T gradiant , thermal cycler (Biometra , Germany), the parameters for amplification were denaturation at 95 ^{o}C for 3 min and 40 cycles at 95^{o}C for 1 min, 54^{o}C for 45 sec, and 74^{o}C for 1 min. A final extension at 72^{o}C for 10 min was also included. The amplicon was electrophoresed on 1% agarose. 100pb DNA ladder (100 – 1000 pb ladder, SibEnzyme Cat# M15) was used to calculate the exact amplicon size

Restriction digestion of the genomic DNA. The genomic DNA of both viruses was digested with both Sau3A (promega cat# R6191) and Hid III (Fermentas cat #ER0501), the reaction mixture consists of 10µg of DNA, 1µl of 10Xrestriction buffer and 5u of either enzyme. The reaction volume was adjusted to 10µl using nuclease free water and incubated at 37°C /1h followed by inactivation of the enzymes by further incubation at 70°C/10min. the reaction was then analyzed by electrophoreses on 1% agarose.

Results and 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 Indian turkey flocks. This disease has an emerging status and it was found that the past literature reveals no information with regards to characterization of this virus. TPV was considered more or less similar to FPV and the available literature reveals the physicochemical characteristics of only FPV and QPV in detail. But now it has been established using cross protection studies that TPV differ from other avipox viruses (Villegas, 1998) and this warrants for the characterization of turkey pox virus isolated from natural outbreak of pox in turkeys. Therefore, in the current study comparison between a turkey poxvirus which was isolated from a natural outbreak of pox in turkeys and the FPV was done on the level of DNA and protein profile.

In order to analyses the phenotypic relatedness of both fowl and turkey pox viruses, 2 step viral purification was done 1st on sucrose gradient then ultra-speed centrifugation followed by total purification of viral DNA and protein using Trizol reagent. The advantage of such method is that this method preserve the integrity of the protein so that the proteins are not subjected to extreme denaturation during the process of separation; in addition to this, it offers complete separation of the protein from the nucleic acids so even the nuclear protein, which is common in pox viruses will be separated and can be analyzed with SDS-PAGE (Chomczynski and Sacchi, 1987).

Avian pox viruses are antigenically and immunologically distinguishable from each other, but varying degree of cross relationship does exist. Attempts have been made to differentiate strains by immunological methods (Singh et al., 2003), antigenic and genomic characterization (Tadese and Reed, 2003) to detect the minor differences among the strains. In the current, study nearly the same protein profile was seen with both FPV and TPV except a single band migrates about 115KDa in TPV which completely absent in FPV (Table 1, Fig. 1) there were about 7 and 8 clearly visible bands with FPV and TPV respectively. On the other hand, some protein bands although present in both viruses yet the concentration of the band seems different which indicates different in the level of protein expression or different in the amount of mRNA expressed. Western blotting analysis (Fig. 2) revealed a difference in a single band reacted at about 11 KDa with the TPV, although this band was invisible in the SDS-PAGE profile. It worth mention that other studies on the fowl pox virus revealed differences in the number of the poly peptide fractionated by SDS-PAGE for example Obijeski

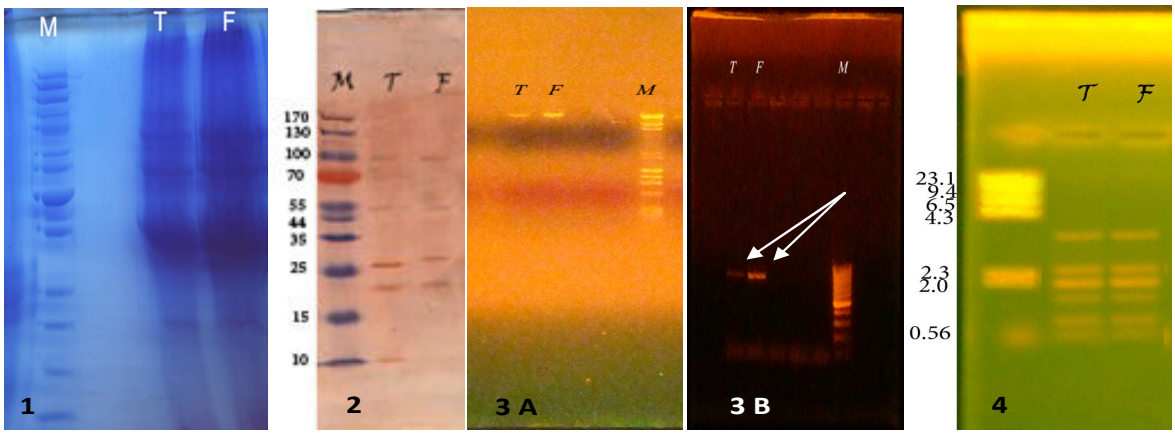

Fig. (1): SDS-PAGE analysis A and western blotting analysis (B) of turkey pox (T) and fowl pox (F) viral proteins. M: Page Ruler protein marker.

Fig. (2): Western blotting analysis of turkey pox (T) and fowl pox (F) viral proteins. M: prestained Page Ruler protein marker. Note the absence of the 10KDa band from the FPV.

Fig. (3): The genomic DNA (A) and The PCR amplification of the gene (B) of turkey pox (T) and fowl pox (F) Note the amplification of a 900bp band in both turkey and fowl pox viruses. M: DNA marker [lambda Hind III DNA maker (A) or 100 pb ladder (B)]

Fig. (4): Restriction endonuclease analysis of both turkey and Fowl pox virus. Both TPV and FPV gave the same restriction pattern.

Table (1): The analysis results of the protein profile of both fowl and turkey pox viruses.

Molecular weight marker			FPV		TPV	
Bands	**Band #**	**MW**	**Band #**	**MW**	**Band #**	**MW**
1			1	210	1	210
2	1	200				
3	2	150				
4	3	120				
5	4	100			2	115
6	5	85	2	94	3	94
7	6	70	3	82	4	82
8	7	60	4	68	5	68
9	8	50	5	55	6	55
10	9	40				
11	10	30	6	26	7	26
12	11	25	7	22	8	22
13	12	20				
14	13	15				
15	14	10	8	10	9	10

Table (2): The estimated MW bands and the expected protein nomenclature and function.

Bands molecular weight (KDa)	**Expected protein**	**references**
94	Early promoter specificity factor associated with RNA polymerase, packing of transcription complex	Ahn *et al.,* (1994)
82	Large subunit , heterodimer ATPase enzyme	Baldick *et al.,* (1994)
68	Putative metalloprotease	Ansarah and Moss, (2004)
55	Poly A polymerase catalytic subunit	Hodel *et al.,* (1996); Latner *et al.,* (2002)
26	Myristylated , intra S-S factore	Su *et al.,* (2005)
22	Phosphorylated, interacts with A20, transcription, morphogenesis	DeMasi and Traktman, (2000)
10	phosphorylated (F10-dep), myristylated, complex	Mercer and Traktman, (2003)

et al., (1973) detect about 28 polypeptides in purified FPV while Mockett et al., (1987) observed about 30 structural polypeptides other studies revealed the presence of 21FPV-coated polypeptides when resolved by ^{35}S methionine pulse labeling and 57 major structural (Prideaux and Boyle, 1987) such differences in the number of bands may due to difference in the expression profile of both viruses and the purification procedures undertaken.

Some of the protein antigens resolved by SDS were characterized (Table 2), for example, the 94 KDa protein antigen found in both FPV and TPV was found to be Pro-associated protein (RAP94) coded by H4L gene and it is one of the core-associated enzymes and transcription factor (Ahn *et al.,* 1994). The 82 KDa protein was found to be large subunit, heterodimer, DNA binding ATPase enzyme needed for the transcription and coded by the A7L gene (Baldick *et al.,* 1994). The 68KDa protein antigen is a protease 2 enzyme called putative metalloprotease coded by G1L gene of Poxviridae (Ansarah and Moss, 2004). Other factors that regulate the viral transcription were characterized such as the 55KDa protein was found to be a large subunit, poly (A) polymerase stimulatory and transcription factor coded by the J3R gene (Hodel *et al.,* 1996; Latner *et al.,* 2002). The 26KDa protein is a myristylared intra S-S F9 related factor required for viral morphogenesis and coded by L1R gene (Su *et al.,* 2005).

Like other pox viruses both FPV and TPV genomes are composed of a single linear dsDNA molecule with a hairpin loop at each end. Although the overall genomic organization of the FPV appears to be similar to that of the other members of the family *Poxviridae*, some genomic rearrangements may occur (Schnitzlein *et al.,* 1988). As seen in (Fig. 3A), both viruses gave genomic DNA of about 130kb (Fig 3-A) which is different than the size of the vaccine like strain of FPV which was determined as 288kb (Alfonso *et al.,* 2000) or the tissue culture propagated FPV strain FP9 which had a genomic size of 260kb. These differences in the genomic size may due to the integration of variable length long terminal repeats (LTR) of the reticuloendotheliosis virus (Singh *et al.,* 2005; Tadse and Reed 2003)

Concerning the PCR, Amplification of the conservative sequence of the LTR region of fowl and turkey pox virus gave nearly the same result. A single amplicon migrates about 900bp (Fig3-B). Based upon restriction endonuclease analysis, both FPV and TPV genome gave the same pattern when digested with Hind III enzyme were using Sau3A no cutting was observed. In this regard, the genomic profile the canary pox, goose pox and quail pox viruses are markedly different from those of the FPV (Kim and tripathy, 2006; Ghildyal *et al.,* 1989; Schnitzlein *et al.,* 1988).

In conclusion, based on the antigenic analysis there were a degree of hetrology between FPV and TPV which could not be proved by the genomic analysis conducted in this study. Further genetic analysis including gene sequencing is needed to examine wither these differences due to genetic alteration or different in gene expression.

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التوصيف المظهري و الوراثي لفيروسات جدري الطيور وجدري الرومي

تمت في هذه الدراسة تحليل الأنتيجينات من فيروس جدري الطيور و جدري الرومي بعد تنقيته بواسطة التحليل الكهربي للبروتينات و وجد ثماني بروتينات متماثلة بكل الفيروسين مع وجود اختلاف وحيد متمثل في بروتين ذو كتلة ١١٥ كيلو دالتون في جدري الرومي فقط و غير متواجد بجدري الطيور. في حالة النقل الكهربي للبروتين وجد تشابه في كلا الفيروسين ما عدا بروتين واحد ذو طول ١٠ كيلو دالتون موجود بجدري الرومي و ليس جدري الطيور. التحليل الجيني لكلا الفيروسين باستخدام تفاعل البلمرة المتسلسل و كذلك التقطيع بواسطة إنزيمات القطع لم يبرز أي اختلافات بين الفيروسين . و الخلاصة إن كلا الفيروسين علي مستوي الأنتيجيني يبدوعلي درجة من الاختلاف.