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VIROLOGICAL STUDIES ON FOWL POX VIRUS (FPV) ISOLATED FROM CHICKENS IN DAKAHLIA AND DAMIETTA GOVERNORATES, EGYPT

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ABSTRACT

In the present study, 10 pooled samples including nodules and diphtheritic membranes were collected front vaccinated and non-vaccinated chicken flocks at Dakahlia and Damietta governorates, Egypt during the period from June 2016 to August 2016. Trials for isolation of suspected virus from the collected samples were carried out via choriallantoic membranes (CAMs) of 10 days old embryonated chicken eggs (ECEs), collected from hens free from Fowl pox virus (FPV). Three serial egg passages were carried out for each sample. The isolated virus from field and 3rd egg passaged samples was identified by agar gel precipitation test (AGPT), indirect fluorescent antibody test (IFAT) and histopathological examination. Hyperimmune serum was prepared against standard FPV vaccine in rabbit. The identified virus was confirmed by polymerase chain reaction (PCR). The results of virus isolation revealed that out of 10 pooled samples, 7 samples were positive results after 1st passage, 8 samples were positive results after 2nd passage and 10 samples were positive results after the 3rd passage. The percentage of the positive results for field samples identified by AGPT and IFAT were 90% (9 out of 10 samples) and 100% (all 10 samples) respectively, while after 3rd egg passage was 50% (5 out of 10 samples) by AGPT and 100%) (all samples) by IFAT. The results indicate that IFAT and AGPT could be used for virus identification., but IFAT is more sensitive. Histopathological examination revealed specific eosinophilic intracytoplasmic inclusion bodies of FPV. PCR for field samples and the 3rd egg passages gave positive results in all samples targeting P4b gene at 578 bp, so PCR technique is more accurate and sensitive.

Keywords: Fowl pox virus (FPV), Virus isolation, agar gel precipitation test (AGPT), indirect fluorescence antibody test (IFAT), PCR (Polymerase Chain Reaction).

INTRODUCTION

Fowl pox, a common viral disease of chickens and turkeys, is responsible for substantial economic losses to poultry farming. It has a worldwide distribution and is caused by fowl poxvirus (FPV), the prototypical member of the genus Avipoxvirus, family Poxviridae **(Moss 1996).** FPV produces lesions on the skin (cutaneous form) and/or in the mouth, pharynx, larynx, oesophagus and trachea (diphtheritic form) of affected birds. Both forms of the disease can occur in a single bird. The morphology of the FPV virus is like that of other viruses of the Poxviridae family. The mature virus is brick shaped and measures about 330 x 280 x 200 nm. The outer coat is composed of random arrangements of surface tubules. The virion consists of an electron-

dense centrally located biconcave core or nucleoid with two lateral bodies in each concavity and surrounded by an envelope, the 288 kbp FPV virus genome encodes for over 250 genes **(OIE 2016).** The virus contains approximately 300 kbp of double stranded DNA genome **(Muller et al. 1977).** PCR based on the amplification of a 578-bp region of the highly conserved P4b gene of Avipoxvirus had been increasingly used for FPV diagnosis in the last few years **(Lu'schow et al. 2004).** In this study, a trial for isolation and identification by different serological tests (AGPT and IF AT) in both field samples and 3rd egg passaged samples followed by histopathological examination and molecular confirmation by PCR.

MATERIAL AND METHODS

Collection of clinical specimens:

A total of 10 pooled samples including nodules and diphtheritic membranes were collected from vaccinated (7 samples) and non-vaccinated (3 samples) chicken flocks from Dakahlia and Damietta Govemorates. (each sample was pooled randomly from five infected birds from each farm) for 30 to 60 days old. The first six samples were collected from Dakahlia governorate while the last four samples were from Damietta. Diseased chickens exhibited proliferative nodular skin lesion on the non-feathered parts and diphtheritic membranes in the upper respiratory tract mucus membrane, mouth and esophagus.

Each collected sample was divided into two parts; the first part was taken rapidly to the freezing chamber of a cryostat for IF AT and the second part was put in a sterile plastic bottle containing phosphate buffer saline (PBS) with antibiotics (1000 U/ml penicillin, 1000ug/ml streptomycin and 500 pg/ml gentamycin) then transported to laboratory on ice box and stored at - 20 °C till using for virus isolation and identification.

Preparation of the collected samples (first part):

Field samples were prepared for virus isolation according to **Tripathy (1989)** as following: Each Sample was minced using sterile scissors and forceps then homogenized using sterile mortar and pestle. A volume of 1 g samples was added to 9 ml of sterile PBS (pH 7.2) containing 1000 U/ml penicillin, l000pg/ml streptomycin and 500 pg/ml gentamycin to be a suspension of 10% w/v. The sample suspension was followed by three cycles of slow freezing and rapid thawing and centrifuged at 3000 rpm for 10 minutes. The supernatant was collected and stored at - 20 °C till using for virus isolation.

Isolation of the prepared samples on CAM of ECEs:

It was done according to **OIE (2016).** 0.2 ml from prepared field tissues (nodules and diphtheritic membranes) was inoculated by dropped membrane method onto the CAM of 10 days old ECEs obtained from hens free from FPV (3 ECEs for each sample). The inoculated eggs were incubated horizontally in egg incubator at 37°C and humidity 60% and candled daily for 7 days. The died inoculated eggs within first 24 hours were discarded. CAMs showing pock lesions were harvested and pooled for each sample till the seventh day CAMs harvested aseptically into petri dish,

washed with PBS and examined for the characteristic lesions. At each passage, CAMs minced in sterile PBS containing antibiotics till forming a 10% w/v suspension. The homogenates were centrifuged at 3000 rpm for 10 minutes at room temperature and the virus-containing supernatant was collected, aliquoted, and stored at - 20°C until using for further passages. Three egg serial passages were performed for each sample. The CAM of the 3rd passage used for histopathological examination.

Standard FPV vaccine:

Standard FPV vaccine (Abbasia strain):

Fowl pox vaccine obtained from Veterinary Serum and Vaccine Research Institute, Abbasia, Cairo. Each vial contains 106 EID50 /ml fowl pox virus in attenuated form. It was used in preparation of hyperimmune serum and used as a positive control in the serological and molecular studies.

Preparation of hyperimmune rabbit sera:

It was done according to **El-Hossiny (2002)** in which 5 rabbits were used as following: Four New Zealand white rabbits were inoculated by three intramuscular injections of 0.5 ml reconstituted attenuated FPV vaccine emulsified in 0.5 ml Freund's complete adjuvant (first and second injection) while the third injection contains 0.5 ml reconstituted vaccine only, the rabbits injected weekly. The rabbits were observed daily for two months after injection. The rabbits were bled after 21 days from the last injection. The blood was left for clotting then the hyperimmune serum was separated by centrifugation at 3000 rpm for 10 minutes and kept at -20°C till using serologically. The fifth rabbit was injected with saline and housed separately from injected rabbits for preparation of a negative control serum sample. The concentration of immunoglobulins in these sera was detected by spectrophotometer using readymade kits (provided from Stanbio laboratory- USA) according to **Young (2001)** then compared with negative control serum.

Serological identification of the isolated suspected FPV by using AGPT and IF AT:

1- Agar gel precipitation test (AGPT):

The detection of the isolated suspected FPV in field tissues and 3rd egg passaged samples using AGPT was done according to **OIE (2016)** as following: 10 ml of 1.5% agarose dissolve in PBS (pH 7.2) was poured in petri dish. In each dish ,6 peripheral wells and one central well were performed. The hyperimmune serum was put into the central well, while the peripheral surrounding wells were filled with the prepared field samples (nodules and diphtheritic membranes) and 3rd egg passage samples (CAMs). Control positive FPV (standard vaccine) and control negative FPV (normal tissues and CAM) were included.
Dishes are incubated at 37 C in a humidified chamber for 2-5 days and examined daily for observation of precipitation lines which indicate the positive results.

2- Indirect fluorescence antibody test (IFAT):

It was carried out according to OIE **(2016)** as following: Prepared slides containing cryostat sections of the selected samples were fixed with cold acetone for 10 minutes then

incubated for lhour at 37 °C in incubator with humidity 60% with a few drops of the prepared rabbit hyperimmune serum were added to the section. The slides were washed with PBS of pH 7.2 for 30 minutes for 3 times (10 minutes for each). The slides were incubated for 30 minutes at 37 °C in the dark humidified chamber with few drops of 1:200 dilution of antirabbit FITC conjugate (Sigma Aldrich Company), the slides were thoroughly washed with PBS three times (5 minutes for each), followed by mounting with glycerol, then covered with a cover slip and examined under a fluorescent microscope to show yellowish green color which indicate the positive result.

Titration of isolated virus in ECEs:

Field sample and 3rd egg passage samples (sample No. 6) that gave severe lesions on CAMs of ECEs were selected for FPV titration, serial ten-fold dilutions (10^{-1} to 10^{-7}) of field samples and 3 egg passage of the selected sample prepared in sterile PBS containing antibiotics, 1 ml of each dilution was inoculated onto CAM of ECEs using five ECEs for each dilution. The inoculated eggs were incubated at 37 C and candled daily. After the incubation period (7 days) the CAMs were harvested aseptically into a petri dish and examined and the observed pocks were counted for each dilution. The virus was titrated according to the formula of **Reed and Muench (1938).** The titer was expressed as logio EIDso/lml.

Histopathologicat examination

Histopathological examination was performed according to **Drury and Wallington (1980).** 3rd egg passage were cut by microtome, were fixed in 10% neutral buffered formalin, dehydrated and embedded in paraffin wax. These sections were stained with H&E for histopathological examination.

Molecular confirmation of the identified virus:

1-DNA extraction of the isolated virus:-

The viral DNA extracted from the field samples and the 3rd egg passage using a commercial kit (RTA Viral Nucleic Acid Isolation Kit) according to the procedure in the kit handbook and **Boulanger et al., (1998).**

2-PCR amplification of the extracted viral DNA:-

It was performed by using a commercial kit My Taq PCR kit according to **Lee et al., (1997).** Primers were designed for amplification of P4b gene of fowl pox virus and synthesized by Sigma Chemical Company, USA. The primers sequences for PCR amplification were as follows: forward primer,5'-CAGCAGGTGCTAAACAACAA-3' and reverse primer, 5'-CGGTAGCTT AACGC CGAATA-. The reaction mixture contained 12.5 pM Master Mix, 1.5 pM for each primer, 7 µL of extracted target DNA and 2.5 pL of nuclease free water added to make volume 25 pL. The PCR had an initial cycle of 94 °C for 5 min, 95 °C for 10 s followed by 40 cycles of 61°C for 10 s, 72 °C for 30 s, and a final elongation step of72 °C for 7 min.

Amplified product analysis

Briefly 10 pL of the amplified PCR product for each sample loaded to the

individual wells of a 1.5% agarose gel. In addition, 2 ℓℓ of a 100 bp DNA molecular weight marker was loaded with 2|µL loading buffer in a single outside well to be used as a DNA ladder. The gel was run at 10 Volt/cm of gel. The power supply was turned off when the gel loading dye (bromophenol blue) had migrated about two-thirds the gel length. The bands of the amplified DNA product bands detected in comparison with DNA ladder using the U.V. transilluminator. The bands were photographed.

RESULTS

As illustrated in **Table (1),** the virus was successfully isolated on CAM of ECEs, 7 Out of the 10 pooled samples samples showed positive results after the 1st passage, 8 out of the 10 pooled samples samples showed positive results after the 2nd passage and all the samples showed positive results after the 3rd passage. In positive cases, the harvested egg CAMs showed pock lesions and thickening **(Fig.l).** In case of inoculation of ECEs with positive control (FPV vaccine vims) resulted in pock lesions on CAMs, subcutaneous hemorrhage, stunted growth and dwarfing of embryo **(Fig.3)** while no lesions were observed on CAM inoculated by saline (negative control) **(Fig.2)** These lesions became more pronounced from the 2nd passage.

precipitation line started to appear within 24-48 hours between the prepared hyper immune serum and 9 examined of 10 pooled field samples (90%) while only 5 of the 3rd egg passaged samples showed precipitation line (50%) as well as appeared in the control positive well, where control negative well showed no line of precipitin **(Fig. 4).**

Regarding to the result of IF AT, yellowish green color appeared in all examined samples (100%) **(Fig 5)** and in all inoculated CAMs (after 3rd passage) (100%) **(Fig. 6),** while in negative control sample (normal tissue and uninfected CAM) showed no yellowish green reaction **(Fig.7).** These results indicate that the IF AT is more sensitive than AGPT.

Titration of the field sample and the 3rd passages of the selected sample (No. 6) on CAMs of ECEs showed that the vims titer for both samples was (10^{515} and $10^{5,67}$ EID50/I ml).

Histapathological examination of the 3rd egg passage on CAM of samples (No.6) revealed esinoplilic intracytoplasmic inclusion bodies (Bollinger bodies) **(Fig.8).**

The result of amplified PCR products revealed that all 10-pooled field sample and their 3rd CAM passages show positive clear bands amplicon size 587 bp for P4b gene **(Fig.9).**

Table (1): Comparative results of the virus isolation in ECEs and identification in the pooled field samples and the 3rd passage using AGPT, IF AT.

Farm No.	Vaccination history	Virus isolation	AGPT		IFAT	
		3rd passage	Field samples	3rd egg passage	Field tissue	3rd egg passage
1	+ve	+	+	+	+	+
2	+ve	+	+	. +	+	+
3	+ve	+	-	-	+	+
4	+ve	+	+	-		+
5	+ve	+	+	-	+	+
6	+ve	+	+	+	+	+
7	+ve	+	+	-	+	+
Total +ve samples	100%		85.7%	42.8%	100%	100%
8	-ve	+	+	+	+	+
9	-ve	+	+	+	+	+
10	-ve	+	' +	-	+	+
Total +ve samples	100%		100%	66.6%	100%	100%
Control +ve (FPV vaccine)		+	+		+	
Control -ve		-	-	-	-	-

Virus isolation + = Pock lesion on CAM.

AGPT+ = White line of precipitin.

IF AT + = Yellowish green color.

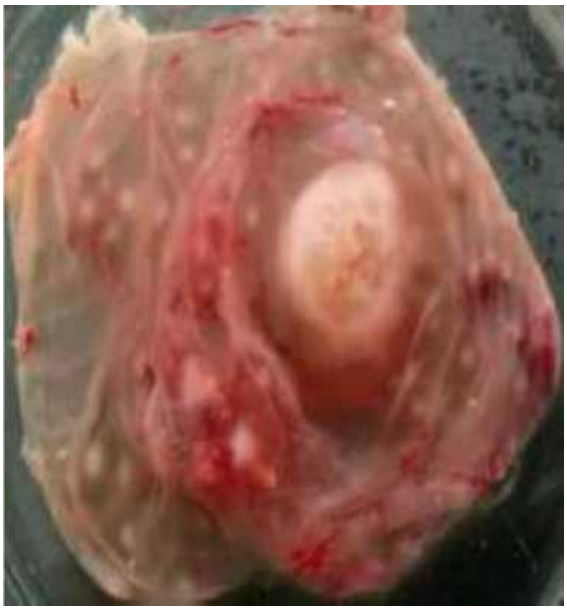

Fig.1- 17- day old harvested CAM showing diffuse pock lesions (after 3rd passage of field samples).

Fig.2- Control non-infected CAM of ECE.

Fig.3- Precipitin line between field tissue sample (1,2, 4), sample No., (5) is control positive sample (FPV vaccine). Sample No., (3) gave no precipitin line, sample No., (6) is control negative sample. (HIS) is prepared hyper immune FPV serum

Fig.4- 17- day old harvested egg embryo showing subcutaneous hemorrhage, stunted growth and dwarfing of embryo (vaccinal strain)

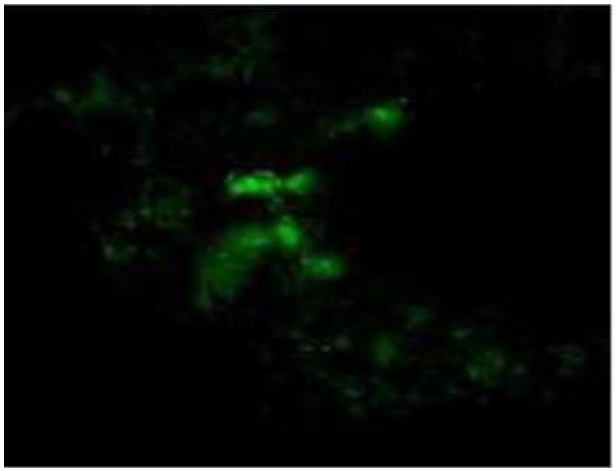

Fig.5- Immunoflourescent reaction, yellowish green color appeared in cryostat section of passage on field tissue (X40)

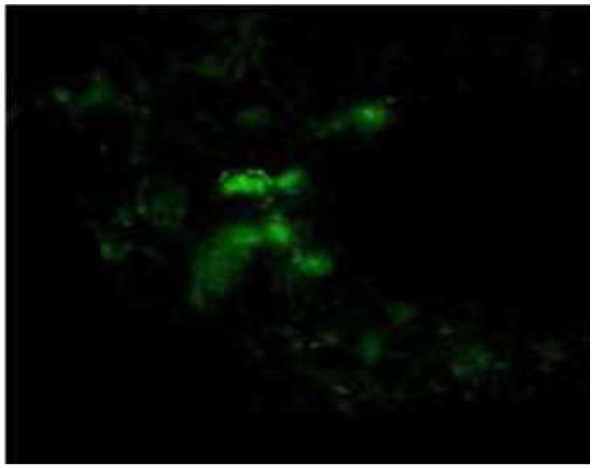

Fig.6- Immunoflourescent reaction, yellowish color appeared in cryostat section of 3rd egg CAM (X40)

Fig.7- Control negative (no yellowish green color appeared in cryostat section of normal CAM (X40)

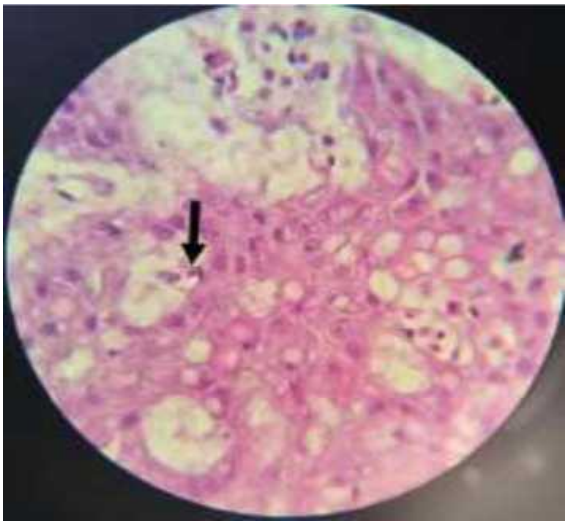

Fig.8- Histopathological picture of 3rd egg passage on CAM showing eosinophilic intracytoplasmic inclusion bodies (Bolliner bodies) and ballooning degeneration in CAM.

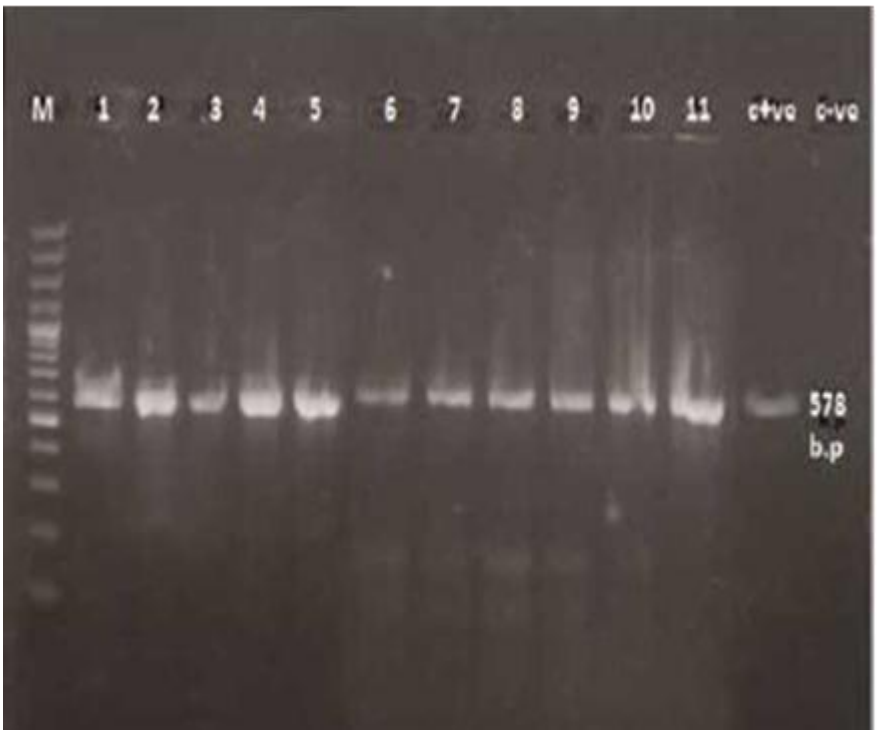

Fig.9-: Gel electrophoresis of PCR product of amplicon 587bp for P4b gene of field sample and 3r^{d} egg passage on CAM in agarose gel.
M: DNA marker.
Lane 1-5: The amplified product prepared from field samples.
Lane 6-11: The amplified product prepared from inoculated CAMs with isolated virus (after 3rd passage)
C+ve : The amplified product prepared from standard FPV vaccine.
C-ve: The amplified product prepared from un-infected CAM.

DISCUSSION

In this study, a trial for isolation and identification of FPV from the different poultry farms at Dakahlia and Damietta Governorates was investigated. A total of 10 pooled samples including nodules and diphtheritic membranes were aseptically collected from vaccinated and non-vaccinated chicken flocks aged from 30 to 60 days old. Isolation of suspected FPV on CAMs of ECEs was done for three passages. The lesions appeared on the inoculated CAMs were showed clear distributed pock lesions and thickening of the CAMs. Similar results were rep**orted by (Tripathy and Reed 2003; Manarolla et al. 2010 and Devi et al. 2016)** whose reported variable levels of thickening, ranging from mild to severe, in CAM infected with Avianpoxviruses isolates.

FPV was detected in field samples and after 3rd passages by AGPT. The obtained results revealed that clear white precipitin lines in 9 out of 10 pooled field samples (90%) and only 5 of the 3rd egg passaged samples (50%) and in a control positive, where control negative showed no line of precipitin against prepared FPV hyper immune serum, these results were in concurrence with the findings of (**Smits et al. 2005 and Ohore et al.**

2007) whom observed that sensitivity of AGPT appears to be low but highly specific in diagnosis pox virus infections compared with other detection methods. AGPT is a useful diagnostic test because its simplicity in reagents, equipment's and analysis with low budget but it requires high high concentration of both antigen and antibody.

Concerning the results of IFAT, the positive results appeared as the yellowish green color in which all 10 pooled field samples (100%) and in all 3rd passage of CAM. These results agreed with **(Tripathy 1993; Mishra and Mallick 1997 and Tripathy and Reed, 2003)** whom pointed that infected sections of CAM stained by IFAT showed green fluorescence by fluorescent microscope examination. Comparing between the result of AGPT and IFAT in FPV diagnosis showing that IFAT is more sensitive than AGPT as the later need more amount of antigen. Histopathological examination of 3rd CAM passage revealed balloning degeneration in CAM and esinoplilic intracytoplasmic inclusion bodies (Bollinger bodies). These data confirmed by the results obtained by (**Metz et al. 1985).** Inclusion bodies resemble Bollinger bodies which are described in most avian poxvirus infections by **(Eaves and Flewett 1955; Purcell et al. 1972).**

With regarding to the molecular detection for FPV using polymerase chain reaction (PCR) depending on P4b gene of FPV, it revealed the presence of amplified products of positive bands at the correct size (578 bp) detected by gel electrophoresis in which 10 pooled field samples and the 3rd CAM passage gave positive bands for FPV. These results agree with **(Lee and Lee 1997; Jarmin et al. 2006 and Manarolla et a l. 2010)**. The results indicate that PCR is more specific and accurate for identification of FPV. Further molecular studies are needed as sequencing to differentiate between field and vaccinal stain of FPV.

REFRENCES

Boulanger, D., Green, P., Smith, T., Czerny, C. P. & Skinner, M. A. (1998): The 131-amino-acid repeat region of the essential 39-kilodalton core protein of fowlpox virus FP9, equivalent to vaccinia virus A4L protein, is nonessential and highly immunogenic. Journal of Virology 72, 170-179.

Devi, T. G., Archaya, S., Gopinath, L. R., & Bhuvaneswari, R. (2016): Virulence and charecteristics of attenuated field fowl pox virus in Embryonated Chick Embryo (ECE). *Indian* Journal of Applied Research, 5:8.

Drury, R.A.B. and Wallington, E.A. (1980): Carleton's histopatho logical technique. 5th Ed. Oxford.

Eaves, G. and Flewett, T.H. (1955): The structure of fowl pox inclusions (Bollinger bodies). Journal of Hygiene (London), 53: 102-105.

El Hossiny. M. (2002): Study on pox virus infection in pigeons. MVSc thesis, Faculty of Veterinary Medicine Kafr El-Sheikh, Tanta University.

Huw, lee, L. and Hwa, Lee, K. (1997): Application of the polymerase chain reaction for the diagnosis of fowl

poxvirus infection. Journal of Virological Methods 63: 113-119.

Jarmin, S., Manvell, R.; Gough, R.E.; Laidlaw, S.M. and Skinner, M.A. (2006): Avipoxvirus phylogenetics: identification of a PCR lenghpolymoi*phism that discriminates between the two major clades. Journal of General Virology, 87: 2191-2201.

Lee, L.H. and K.H. Lee (1997): Application of polymerase chain reaction for the diagnosis of fowl pox virus infection. Journal of Virological Methods 63: 113-119.

Lu" schow, D., Hoffmann, T., Hafez, H.M., (2004): Differentiation of avian Poxvirus strains on the basis of nucleotide sequences of 4b gene fragment. Avian Diseses. 48: 453^-62.

ManaroUa, G.; Pisoni, G.; Sironi, G. and Rampin T. (2010): Molecular biological characterization of avian poxvirus strains isolated from different avian species. Veterinary Microbiology, 140:1-8.

Metz, A.L.; Hatcher, L.; Newman, A.J.& Halvorson, D.A. (1985): Venereal pox in breeder turkeys in Minnesota. Avian Diseases, 29(3): 3-850.

Mishra, S.S. and Mallick, B.B. (1997a): Detection of fowl pox virus using immunoperoxidase test and fluorescent antibody technique. Indian Veterinary Journal. 74:199-202.

Moss B. (1996): *Poxviriclae:* the viruses and then replication. *In* Field's virology (B.N. Field, D.M. Knipe, P.M. Flowley, R.M Chanock, J.L. Melnick, T.P Monath, B. Roizman & S.E. Straus, eds.). Lippincott-Raven, Philadelphia, Pennsylvania, 2637-2671,

Muller, H.K., Wittek, R., Schaffner, W., Schumperli, D and Wyler, R. (1977): Comparison of five poxvirus genomes by analysis with restriction endonucleases Hind III, BamH I, and EcoRI. .1. Gen. Viral. 38, 1377147.

Ohore, O.G.; Emikpe, B.O.; Oluwayelu, D.O.; Adeyemi, R.O. and M.A. Ockiya, (2007): Seroprofiling of antibodies to fowl pox in commercial and indigenous chicken in Southwestern Nigeria, Journal of Animal and Veterinary Advances, 6(5): 697-701.

OIE (2016): Fowl pox virus. Manual of Diagnostic Tests and vaccines for Terrestrial Animals. Office Internationa; des Epizooties (OIE)., Chapter 2.3.10:1-8

Purecell, D.A.; Clarke, J.K.; McFerran, J.B. and Hughes, D.A. (1972): The morphogenesis of pigeon pox virus. Journal of General Virology, 15:79-83.

Smits, J.F.; ATella, J.L.; Carrete, M; Serrano, D. and Lopez, G. (2005): An epizootic of avian pox in endemic short-toed lacrks (Calandrella rufescens) and Bertholt's pipits (Anthus berthelotti) in the Canary Islands, Spain. Veterinary Pathology, 42: 59-65.

Tripathy, D.N. (1989): Pox. In Purchase, H.G.; Arp, L.E. Domermuth, C.H. and Pearson, J.E. (Eds). A laboratory Manual for isolation and identification of avian pathogens. 3rd Edition.

American Association of Avian

Pathologists Kennett square, PA. Page number: 103-105.

Tripathy, D.N. (1993): Avipoxviruses. In Mcferran J.B. and Mcnulty M.S. eds. Virus infections of birds, page number:

• 5-15. Elsevier science publisher B.V.

Tripathy, D.N. and Reed, W.M. (2003): Pox. In: Y. M Saif. Diseases of Poultry, (edition) 10th edition IOWA State University Press page number: 253 — 269.

Young D.S. (2001): Effect of disease on clinical lab..test; 4th edition A.A.