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ISOLATION AND CHARACTERIZATION OF PI-3 VIRUS FROM SHEEP AND GOATS

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225 nasal swab samples were collected from diseased sheep and goats for the isolation of PI-3 virus. The samples were taken from diseased animal at seven different Governorates Kaffer el sheikh, Alexandria, EL Behaira, Port Said, Demiatta El-Qalubia and Giza through the winter seasons of years 1999 to 2003. The virus was successfully isolated from three ovine samples after three successive passages on MDBK cells. The isolated viruses were then titerated and identified using different biological, serological and molecular assay.

Key words: Avian influenza H7, backyard, Egypt, RRT-PCR; ELISA, HI

.INTRODUCTION

Parainfluenza-3 (PI-3) is the most common virus infection of the respiratory tract of sheep and goats throughout the world. The virus belongs to family Paramyxo-viridae of order Mononegaviralis, the non segmented negative single stranded RNA viruses **(Pringle, 1991)**. The virus is generally spherical and 150 to 350 nm in diameter but can be pleomorphic in shape. The genomic RNA contained within a helical nucleocapsid that is enclosed within the lipid bilayer. Inserted into the envelope are glycoprotein spikes that extended approximately 8 to 12 nm from the surface of the membrane **(Kingsbury, 1991; Field, 1996)**.

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PI-3 virus was first isolated from sheep in 1966 **(Hore,1966)** since then serological surveys have shown that this infection is widely spread in sheep population in many countries **(Fenner et al.,1996)**. In Egypt, the first isolation of PI-3 virus from lambs was carried out in 1971(**Baz 1971)**. The virus revealed symptoms like mild pyrexia, coughing and nasal discharge for several days and necropsy has shown area of pneumonia, particularly in ventral parts of the apical lobe **(Hore and Stevenson, 1967)**

The present study is directed to isolate the PI-3 virus from sheep and goats which naturally affected with respiratory manifestation, and to identify the isolated virus using several assays.

MATERIAL AND METHODS

Active surveillance

The surveillance was conducted in October, 2007 for three days, where 45 houses from 11 villages were included as shown in figure (1).

Samples

Serum samples: 207 serum samples were collected from chickens (73), ducks (67), geese (52), pigeons (6) and turkey (9).

Tracheal and cloacal swabs: 200 tracheal swabs were collected from (66) chickens, (64) ducks, (47) geese, (12) pigeons, and (11) turkey and 200 cloacal swabs were collected from chickens (66), ducks (64), geese (47), pigeons (12), and turkey (11). As shown in table (1)

The birds were of different ages. The vaccination status against H5N1 virus was doubtful as told by the villagers, where two confirmed villages only were vaccinated at this time.

Avian Influenza Virus detection

Cloacal and tracheal swabs were collected from chickens, ducks, geese, pigeons and turkey. The swabs were immersed in 1- 2ml viral transport media containing 2000000 I.U. penicillin, 2 mg streptomycin and 1000 I.U. mycostatin/ml. Briefly, five tracheal or Cloacal swabs were pooled together. RNA was extracted by using virus RNA Extraction Kit (QIAGEN, Valencia, Calif., USA).

The RNA were amplified using One-Step Real-time reverse transcription-PCR (QRT-PCR) kit (PG-Biotech; QIAGEN, Valencia,

Calif., USA) for detection of the matrix gene of influenza type A viruses.

Samples: 225 nasal swabs were collected from sheep and goats suffering from respiratory manifestation at seven Govenorates through the winter seasons of years 1999 to 2003; the dilated data are shown in table (1).

Table (1)Number of nasal swab samples collected from sheep& goats in different localities in Egypt

Governorates	Localities	No. of sheep	No. of goats	Total number
Kaffer el sheekh	El karada	27	0	27
Alexandria		32	0	32
El Behaira	Wady el Ntron	2	0	2
Port Said		20	0	20
Demiatta		7	13	20
El- Qalubia	Meshthor	10	0	10
	Tahanob	10	4	14
Giza	Abbatoir	55	0	55
	Oseem	16	4	20
	Nahia	11	14	25
Total number		190	35	225

The samples were collected in sterile tubes containing 2 ml of sterile PBS and 200ul of stock antibiotic solution per each sample. The samples were taken on ice bucket. Clarification was done by centrifugation at 5000 rpm for 5 minutes at 4° c in cooling centrifuge. The supernatant was taken and kept at -70°c till used.

Viruses: first local bovine Egyptian PI-3 virus strain 45 was (kindly supplied from Rinder Pest like disease Department at Veterinary Serum and Vaccine production Institute Abbassia Cairo. Second bovine PI-3 virus strain SF_4 was kindly supplied from Rabies Department in VACSERA.

Cells: MDBK cells were kindly supplied from department of Virology, Fac. of Vet. Medicine Cairo University.

Heamagglutination test:

Samples were subjected to heamagglutination test to exclude the non-heamaglutinatinating

samples from the isolation work. The test was conducted according to **Singh& Baz (1966)** using 1% G. pig RBC_s.

Isolation of PI-3 virus from HA positive samples:

The isolation was carried out according to **Rulka (1987)** using MDBK cells. Three successive passages were done for this isolation.

Titeration of isolates:

The harvest of the inoculated MDBK cells after three passages of the isolates was titrated. The test was performed according to **Fray and Liess (1971)** Briefly, in a microtiter plate 90ul of MEM free serum were dispensed into each well then 10ul of the suspected isolate suspension were added to the wells of the 1st column and serial ten fold dilutions were made.50.000 cells/ well were added while being in suspension. The plate was incubated at 37C in CO_2 incubator with daily microscopic examination. The $TCID_{50}$ was calculated according to **Reed and Muench (1938).**

Identification by heamadsorption technique:

The test was conducted according to **Elizabeth et al., (1997).** After 16 hours of inoculation of cells with isolates, the attached cells were fixed for 30 minutes with formaldehyde in PBS (pH 7.2) then 1% suspension of G. pig RBC_s was added. The cells were examined under inverted microscope.

Identification of PI-3 virus by detecting inclusion bodies:

The test was made using Zinker Solution as a fixative according to **Clayden (1971).**

Identification of the isolates using Immunofluorescence:

The test was performed according to **Vander Heide (1971)** using cell culture staining chamber (CCSC). Each cup was inoculated with 100ul of the isolate and uninfected cups were included as control. The cups were overlaid with 100ul of anti PI-3 serum conjugated with FITC (supplied from Department of Virology, Faculty of Veterinary Medicin Cairo University) then examined under fluorescent microscope.

Identification of PI-3 suspected isolates by virus neutralization test:

The test was performed according to **Murkami et al., (1983)** Beta neutralization procedure using antibovine PI-3 hyperimmune serum (supplied from Serum& Vaccine Research Institute Abbassia, Cairo). The serum was

originally supplied from Imes, Iowa, USA).

Detection of PI-3 virus by polymerase chain reaction (PCR):

The genomic RNA was extracted from inoculated tissue culture by means of TRIZOL Reagent according to their manufacture instructions (commercially supplied by Life Technologies CO.) The TRIZOL Reagent is improvement to single step RNA isolation method was declared by **Chromcznski and Sacchi (1987).** RT-PCR was employed using oligonucleotide primer pair designed from conserved site of fusion protein gene (F gene) as previously described by **Lyon et al., (1997).** The sequences of utilized primers as follow:

Sense (F)=5' CATTGAATTCATACTCAGCAC 3' Antisense (R) = 5'AGATTG-TCGCATTT(AG)CCTC3'

Table (2) Cycling protocol for amplification of F gene of PI-3

steps	Temp	Time	No. of cycle
RT	47c	30 min.	One cycle
Initial denaturation	94c	2 min.	One cycle
Denaturation	94c	45 sec.	35 cycle
Anealing	51c	45 sec.	
Extension	72c	1 min.	
Final extension	72c	5 min.	One cycle
Preservation	4c	∞	

Analysis of the PCR product using agar gel electrophoresis.

The analysis was carried out according to **Sambrook et al., (1989)** using ethidium bromide stained agarose gel 1.5%

RESULTS

Results of heamagglutination test are presented in table (3).Five HA-positive samples were cultivated and propagated on MDBK cells. Three passages in cell culture were applied and resulted three isolates which were designated as Oseem56/1999, Oseem184/2003 and Nahia70/2000 according to their localities and the year at which the samples were collected table (4) present the designation and passages results of the cell

culture work. The three isolates as well as local bovine strain 45 were titerated according to infectivity the results presents in table (5) It seem to be titers ranged from 10^5 to $10^{6.6}$ $TCID_{50}$/ml with the highest test titer was given by the isolate Oseem56/1999 followed by isolate Oseem 184/2003 and finally the isolate Nahia 70/2000. The isolated viruses were identified using hemadsorption, inclusion body detection, immunofluorescence, SNT as well as RT-PCR. Results of

Table (3) Haemagglutination titer of the five HA positive nasal swab samples and their localities.

Species	Serial number	designated number	Locality	HA titer (HAU)
Sheep	1	56	Oseem	2^6
	2	184		2^2
	3	105	Port said	2^2
	4	101		2^3
	5	70	Nahia	2^4

Table (4) Results of cultivation of the HA positive (ovine) nasal swab samples

o. of passage	No. of positive samples (CPE)	No. of negative samples (no CPE)	Designated name of isolate
1st. passage	1	4	Oseem56/1999
2nd passage	3	2	Oseem56/1999, Oseem184/2003 and Nahia70/2000
3rd passage	3	2	Oseem56/1999, Oseem184/2003 and Nahia70/2000

CPE = cytopathic effect

Table (5) Results of titration of PI-3 isolates on MDBK cells using infectivity test.

Designated name of isolate	Titer of virus ($TCID_{50}$/ml)
Oseem56/1999	$10^{6.6}$
Oseem184/2003	$10^{6.5}$
Nahia70/2000	10^{5}
PI-3 reference strain A^{45}	$10^{6.2}$

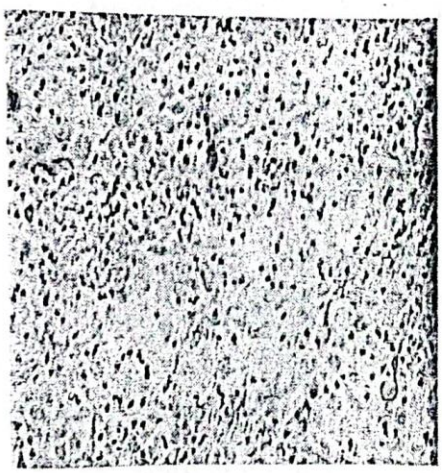

Photo (1)

Photo (2)

Photo (2) infected MDBK cells with the isolates after fixation and the positive heamadsorption.

Photo (3) normal non infected MDBK cells

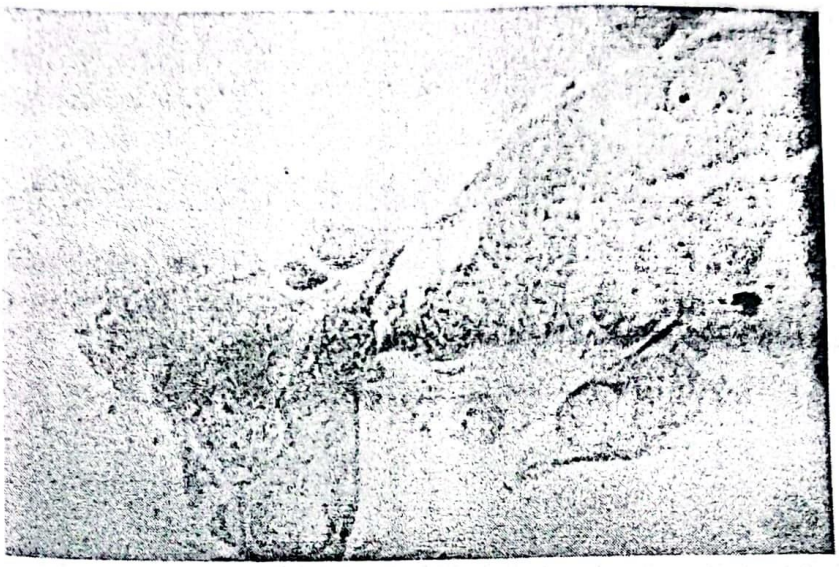

Photo (3) large esinophilic intracytoplasmic inclusion body

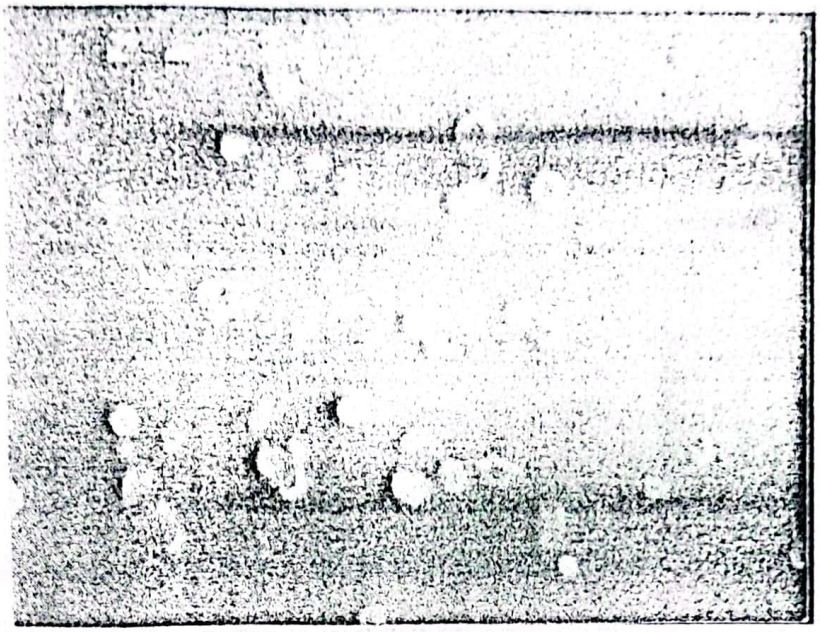

Photo (4) shows the observed intracytoplasmic perinuclear fluorescence in the in the positive FA-isolates on MDBK cells.Table (6) Results of virus neutralization test for PI-3 isolated viruses against antiparainfluenza-3 reference serum.

Photo (3) large esinophilic intracytoplasmic inclusion body.

Table (6) Results of virus neutralization test for PI-3 isolated viruses against antiparainfluenza-3 reference serum.

Isolate designated name	Neutralization titer on MDBK cell line
Oseem56/1999	512
Oseem184/2003	8
Nahia70/2000	256
(Reference A^{45}strain)	1024

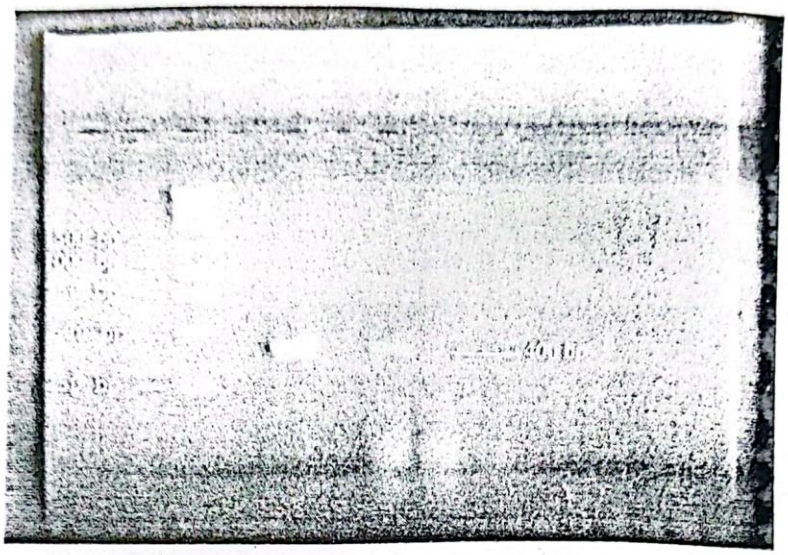

Photo (5) Shows the ethidium bromide stained 1.5% agarose gel electrophoresis of the PCR products. Lane M : represents the molecular DNA marker. Lane A: represents the amplified PCR product of approximately 400 bP, the conserved portion of F gene of PI-3 virus strain A^{45} (positive control). Lane 1 to 3 represents the PCR products of the isolated viruses. Only Oseem56/1999 revealed the expected band (lane 1).

DISCUSSION

PI-3 virus causes, different clinical syndromes in sheep and goat specially respiratory infections **(Ackermann and Brodgen, 2000)**. Serological surveys have shown that infection is widely spread among sheep population in many countries **(Fishman, 1965; Pop et al., 1979;Brako et al., 1984; Saddour, 1987 and Riedmann et al., 1991).** Many of serological data indicated that the ovine PI-3 virus is antigenically related to bovine and human strains of PI-3 **(Wood et al., 1975)**. In the presnt, work we tried to isolate PI-3 virus from naturally infected sheep and goats and RT-PCR was used as a recent tool for detection and identification of the isolated virus. The detection of PI-3 virus by heamagglutination, revealed five positive samples (all of them were from sheep samples representing 2.2% of the 225 collected samples. This low percentage of HA positive may be due to the high amount of virions (at least $10^{5.5}$-$10^{6.5}$ virions) required to give one

work we tried to isolate PI-3 virus from naturally infected sheep and goats and RT-PCR was used as a recent tool for detection and identification of the isolated virus. The detection of PI-3 virus by heamagglutination, revealed five positive samples (all of them were from sheep samples representing 2.2% of the 225 collected samples. This low percentage of HA positive may be due to the high amount of virions (at least $10^{5.5}$-$10^{6.5}$ virions) required to give one HA unit **(Redda 1964).** The isolation was achieved through three passage applied on MDBK cells giving rise to three isolates developed a clear CPE. The identification by detecting inclusion bodies revealed large esinophilic intracytoplasmic with no evidence to nuclear inclusions in contrast to bovine PI-3 virus that produced both cytoplasmic and nuclear inclusion similar results are previously reported **(Howard et al., 1982).** It was noticed that the three isolates were from lambs which may be indicate the prevalence of PI-3 virus among sheep than in goats. However, the failure to isolate the virus from goat may be due to the lower number of collected samples from goats compared with those from sheep and the fact that the animals were kept under relatively isolated conditions **(Erasmus et al., 1967).** The low titer of Oseem184/ 2003 isolate may be taken as criterion for heterogeneity **(Gaul et al., 1982).** This finding agreed with many of the previous studies, which have shown that at least three ovine PI-3 strains of different serotypes namely (G_2 strain, CsL6 strain and DH-1 strain), do exist **(Hore, 1966); St.George, 1969** and **Howard et al., 1982).** The heamadsorption used as an indicator system for the presence of PI-3 virus proved to be more rapid and sensitive than waiting until the cytopathic changes appear. This finding is similar to those described previously by **St.George (1969)** and **Toth and Jankura (1990).** RT-PCR technique waw performed in this work using two primers chosen from conserved site of fusion protein gene (F gene of whate PI-3 viruses). The primers were chosen in this site of genome as it is conserved among PI-3 viruses not specific for ovine PI-3 virus. Also, both sheep and goats can catch bovine PI-3 virus. Moreover, these primers could help us in identification of the standard bovine PI-3 virus strain and the

standardization of the technique. Also, the same primers were developed by **Lyon et al., (1997)** and **Maria et al., (1998).** The detection and differentiation of PI-3 virus could be studied by amplification and sequencing of another part of the genome for example HN gene **Ruth et al., (1994), Echevarria et al., (2000)** and **Vecherov et al., (2003).** Indeed, This study reports the isolation and characterization of parainfluenza-3 virus from sheep and goats in Egypt.

REFERENCES

Ackermann, M.R. and Brogden K.A. (2000): Response of ruminant respiratory tract to Mannheimia (pasterella) heamolytica. Microbes infect. 2 (9): 1079-1088.

Baz, Thanaa (1971): Isolation of PI-3 from sheep in Egypt. M.V.Sc. thesis Fac. Vet. Med., Cairo Univ.

Brako, E. E., Falton, R. W and Nicholson, S.S. (1984): Prevalence of bovine herps virus virus 1, B V D, PI-3 goat respiratory syncytial , bovine leukemia and blue tongue viral antibody in sheep.

Chomczynski, P. and Sacchi, N. (1987): Single-step method of RNA isolation by acidguanidium thiocyanate-phenol-chloroform extraction. Annal. Biochem. 162,1556.

Clayden, E.C. (1971): Practical section cutting and staining. Fifth Edition.

Echevarria, J.E. ; Erdman, D.D. Meissner, H. C. and Anderson, L. (2000): Rapid molecular epidemiologic studies of human PI-3 virus based on direct sequencing of amplified DNA from a multiplex RT-PCR assay. J. Virol. Method. 88 (1): 105-109.

Elizabeth, J Haanes, Paul G. and Richard W. (1997): The bovine PI-3 haemagglutinin,neuraminadase,glycprotein expressed in baculovirus protect against experimental BPIV-3 challenge. Vaccine, 15 (6/7): 730-738.

Erasmus, B.J.; Boshoff, S.T. and Pieterse, L.M. (1967):

Antibodies to PI-3 virus in sera of domestic and animals in South Africa. Bulletin De L` office International de Epizooties 68, 657-664.

Fenner, E. Paul J.G.; Frederick A. Murphy, Rudplf Rott, et al. (1996): Vet. Virology. Second Edition Academic Press. INC.

Field, B.N.; Knipe, D. M.; Howley, P.M.., et al. (1996): Parainfluenza viruses. Fields Virology Third edition 1205-1241.

Fishman, H.R. (1965): Presence of neutralizing antibody for myxovirus parainfluenza-3 in sheep sera. Proceeding of Society for experimental biology and medicine 118, 725-727.

Fray and Liess (1971): Growth curve studies and applicability of highly cytopathogenic BVD-MD virus strain for diagnostic purposes using microtiter method. Zbl. Vet. Med., 618. 61-71.

Gaul, S.K.; Simpson, T.F.; Wood, G.N. and Falton, R.W. (1982): Antigenic relationship among some animal rotaviruses. J. Clin. Microbiol. 16: 495-503.

Hore, D.E. (1966): Isolation of ovine strains of PI-3 virus serologically related to type3. Vet. Rec. 80:26-27.

Hore, D.E. and Stevenson, R.G. (1967): Experimental virus pneumonia in lambs. Vet. Rec. 80: 26-27.

Howard. Lehmkuhl, PhD.: Randall, C. and Cutlip, D.V.M, PhD (1982): Characterization of PI-3 virus isolated from the lung of lamb with pneumonia. Am. J. Vet. Res. Vol. 43 (4): 626-628.

Kingsbury, D.W. Ed. (1991): The paramyxoviruses. New York: Plenum Press.

Lyon, M.; Leroux, C.; Greenland, T. et al. (1997): Presence of unique PI-3 strain identified by RT-PCR in visna-maedi virus infected sheep. Vet. Microbiol. 51: 95-104.

Maria, Z.; Tim, B.; Carol, J. S.; John M.G. and Katherine, N.W. (1998): Moleucular epidemiology of consecutive outbreaks of parainfluenza-3 in bone Marrow Transplant Unit.

Murakami, Y.; Nishioka, N. et al (1983): Serotypes of bovine rota distinguish by serum neutralization. Infection & immunity, 40 (3): 851-855.

Pop, M.; Vasiu, C.; Rauntean, G; Slavoici, N.; Cirstet, I.; Baba, I.; Rotaru, O. and Maier, R. (1979): Serological survey of indigenous Romanian and Imported sheep for parainfluenza-3 virus. Facnatea de Zootehnie SiMedicina Veterinaria. 145-149.

Pringle, C. R. (1991): The order mononegavirales. Arch. Virol. 117: 137-140.

Redda (1964): Dissentation advanced to the faculty of veterinary medicine, Ciession Verhaltender virus spozizifischen komponenton in Verschiodenen. Arten Von Gewobokultu-Zlien.

Reed, L.M. and Muench, H. (1938): A Simple method of estimating 50 percent end points Am. J., 27: 493-495.

Riedmann, S.; Montecions, M.I.; Tadich, N. and Reinhardt, G. (1991): Serological survey for antibodies to parainfluenza-3 virus in sheep in chile. Veterinary Record 128 (24): 572.

Ruth, A k.; Jean, L.F.; Linda, B.B.; Robert, H.Y. (1994): Rapid detection of parainfluenza virus type 3 RNA in respiratory specimen. Use of reverse transcription- PCR-EIA. Journal of clinical microbiology, Feb. P.484-488.

Rulka, J (1987): Use of poultry erthrocytes in hemagglutination for PI-3 virus. Medycyna Weterynaryjna 43 (6): 342-345.

Saddour , M. (1987): Level of PI-3 antibodies in sheep serum. Medycyna Weternaryina 43 (2): 97-99.

Sambrook, J.E.; Fritsch, E.F. and Maniates, T. (1989): Molecular coloning Lab. Manual. 2nd edition.

Singh, K.V. and Baz, T.I (1966): Isolation of PI-3 from water buffaloes in Egypt. Nature Land., 210, 616-656.

St . G eorge , T . D .(1969) : [I] a bovine strain of

myxovirus parainfluenza type 3 .[II] The isolation of myxovirus parainfluenza type3 from sheep in Australia.

Toth , T .E . and Jankura , D . (1990) : Analysis of bovine parainfluenz -3 reblication in bovine embryonic lung cell by in direct fluorescent antibody and haemadsorption assays . J. virological methods, 27 (1) :113 – 119.

Vander Heide, L. (1971): The fluorescent antibody technique in the diagnosis of bovine respiratory diseases. Proc. 14th animal meeting of the virus united states, animal health association. 584-588.

Vecherov , A.E.; Aianot, P.K.; Timina, A. M. and Lisitsin, V.V. (2003): Detection and differentiation of bovine parainfluenza-3 virus strains studied by amplification and sequencing of the HN gene. Vopr. Virusol. 48 (5): 46-49.

Wood, G.T.; Mansfield, M.E. and Heitzman, P.J. (1975): Serological response of lambs to live virus vaccines containing infectious bovine rhinotracheitis, bovine virus diarrhea and bovine myxovirus parainfluenza-3. Veterinary medicine& small animal clinician 70 (4): 450- 454.