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Isolation and Identification of Field Isolate of *Peste des Petits Ruminants* Virus in Egypt

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Abstract: Peste des petits ruminants (PPR) is an acute and highly contagious viral disease of small ruminants causing high morbidity and sometimes high mortality rates. Samples were taken from 270 animals (170 Sheep and 100 goats) in three different governorates of Egypt which are Al Daqhaliya, Al Qalyoubiya and Al Sharqiya. The samples were inoculated on Vero cells for cytopathic effects (CPE). Hemagglutination assay (HA), was used for primary identification of the virus. The causative agent was also identified by sandwich-ELISA and In-Situe RT-PCR. 109 (39.63) out of 270 animals showed signs of the disease. 33.53% of sheep and 52% goats clinically examined, Showed signs of the disease. The observed symptoms were very severe in goats than sheep. Vero cells showed rounding and syncytial formation and they were primarily identified as PPRV with HA titer 1:64. ELISA results revealed 35.29% of the sheep and 56% of the goats as positive against PPRV. Age analysis of data revealed significantly ($p < 0.05$) higher seroprevalence of PPR in >12 months age group (15.88 and 31), followed by 8-12 months (9.41 and 15), while 4-8 months, were (8.24 and 6) percentage in sheep and goats, respectively. In-Situe RT-PCR showed infected tissue culture cells with cytoplasmatic staining (Mainly perinuclear) whose intensity correlated directly with the degree of viral cytopathic effect during examination of cells under light microscope. The current study recommends ELISA technique as a screening test for the disease and In-Situe RT-PCR as a confirmatory test for fixed tissue and to avoid handling of the virus.

Key words: ELISA · Peste Des Petits Ruminants · In-Situe RT-PCR

INTRODUCTION

Peste des petits ruminants (PPR) is an acute and highly contagious viral disease of goats and sheep causing high morbidity and sometimes high mortality rates [1-4]. The causative agent of the disease, Peste Des Petits Ruminants Virus (PPRV), is classified as a member of the genus Morbillivirus in the family *Paramyxoviridae* [5, 6].

The PPRV genome encodes six structural proteins which are N, P, M, F, H and L. The genome also encodes two nonstructural (C and V) proteins [7, 8]. The fusion (F) and hemagglutinin (H) proteins, which constitute the majority of the viral envelope, are considered to be largely responsible for the induction of protective host immune responses [9]. Based on sequence analysis of the F gene, phylogenetically, there are four different lineages (I-IV) of PPRV whereas lineage IV is believed to be prevalent in India; other lineages are prevalent in African countries [10].

In 1989 the disease was reported in Egypt, but incidence was very low [11, 12]. El-Hakim [13] and Abd El-Rahim *et al.* [14] reported an outbreak of PPRV in Egypt. Since then, PPR has become one of threatening diseases in Egypt.

The present study was designed to gain an insight into the present situation of PPR disease, isolation and identification of PPRV among sheep and goats and maintain suitable, accurate and sensitive diagnostic method for disease detection.

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MATERIALS AND METHODS

The current work was carried out during a period of six month (January-June, 2013). Collection of samples was carried out from 270 animals (170 Sheep and 100goats) in three different governorates of Egypt which are Al Daqhaliya, Al Qalyoubiya and Al Sharqiya. These animals were in small holder farms where there is no regular system of vaccination.

Animals and Sample Collection: The affected animals (Sheep and goats) exhibited vesicular lesions on the gums and/or the tongue, lacrimation, nasal discharge, dyspnea, severe diarrhea and death. Various specimens, swabs from tongue and gum lesions, nasal and lacrimal discharge and tissues from intestine, lungs, lymph nodes, heart and stomach were collected from the affected goats and sheep and were transported to the laboratory on ice. Swabs were immersed into Minimum Essential Medium (MEM) according to the procedures [15] and filtered through a 0.45 µm syringe filter prior to use. Tissue were homogenized by means of homogenizer and centrifuged at 5000 rpm for 5 min and the supernatant was filtered through a 0.45 µm syringe filter [15]. The filtrate was stored in-70°C till use in various virological assays.

Virus and Sera: Reference virus and sera was kindly supplied Animal Vaccines and Sera Institute, Abasia, Cairo, Egypt.

Virus Isolation and Propagation: The samples were inoculated on Vero cells (Supplied by Vaccines and Sera Institute, Agoza, Egypt). Vero cells were grown in MEM (Sigma, USA) supplemented with 10% Fetal Bovine Serum (FBS) and antibiotics (penicillin and streptomycin following instructions of Kumara *et al.* [16]. Confluent monolayers of cells were infected with the virus isolated from the specimens (Except for samples taken from gastro-intestinal tract due to cytotoxic factors) for 2 h at 37°C after which the cells were washed with phosphate buffer saline (PBS) and fresh MEM was added (First passage). The cells were incubated at 37°C and monitored daily for cytopathic effects (CPE) caused by the virus [16]. Several passage of the virus into Vero cells till CPE became noticed. Infected cells were subjected to hemagglutination test.

Hemagglutination Assay: For primary identification of the virus, hemagglutination assay (HA) were performed using 0.5% suspension of chicken red blood cells according to standard procedures of Brian *et al.* and World Organization for Animal Health [15,17].

Detection of PPR antigen by ELISA: Sandwich-ELISA technique was applied according to Singh *et al.* [18] and Madbouly *et al.* [19]. Each sample was tested individually in triplicate. The optical density (OD) at 450nm was read using an automated plate reader (Bio-Tek EL312E reader, Bio-Tek Instruments).

In-Situe RT-PCR: Oligonucleotide primers, corresponding to 'N' gene sequence (Gen Bank, Accession No. X74443) [20], were used. Upstream primer sequence: 5`TCT CGG AAA TCG CCT CAC AGA CTG 3`. Downstream primer sequence: 5`CCT CCT CCT GGT CCT CCA GAA TCT 3`. This primer pair amplifies 1232-1255 and 1538-1560bp region of N gene [21]. The viral RNA was isolated by means of Qiagen purification kit (QIAGEN, GERMANY, Cat no 52304) and PCR was performed by one step RT-PCR Qiagen kit. In-Situ RT-PCR technique was performed step by step as described by Murphy *et al.* [21]. The infected cells was fixed by formalin and embedded in paraffin. The paraffin was then sectioned and a paraffin sections were loaded on slides, the sections were digested by protease then D Nase then RT-PCR was performed (GeneAmp In Situ PCR System 1000, Perkin Elmer) and cells were examined under light microscope.

RESULTS AND DISCUSION

Field Observations: The disease was clinically characterized by sudden death of apparently healthy animals and yellowish then bloody diarrhea, vesicular lesions on the gums and tongue, lacrimation, nasal discharge and dyspnea (Table 1). The clinical signs matched with the report of Hammouchi *et al.* [22]. Out of 270 animals: 109, animals 39.63 showed the characteristic signs of the disease. The results showed that 57 (33.53%) out of 170 sheep and 52 (52%) out of 100 goats, Showed signs of the disease. The observed symptoms were very severe in goats than sheep.

Virus Isolation and Propagation: Cell rounding and syncytia formation was observed only in cells infected with virus Five days post inoculation (PI) (Fig. 1). In cells showed no CPE, cells were freeze-thawed twice and the resulting suspension was used for a second passage. Passages were continued until the obvious appearance of CPE. The fifth passage showed very clear CPE. These results match the finding of Truong *et al.* [23].

Hemagglutination Assay: HA gave the best result in the fifth passage of the samples, where the HA titer was of 1:64. Some samples did not give high titers. This may be

Table 1: Signs of PPR in sheep and goats

	Sheep		Goats	
Symptoms	Number	Percent	Number	Percent
Diarrhea	39	22.94%	41	41%
Lesions on the gums and/or tongue	42	24.71%	46	46%**
Ocular and nasal discharges	31	18.24%	45	45%**
Dyspnea and signs of broncho-pnomonia	11	6.47%	21	21%
x^2 value	32.17**			

**P<0.05

Table 2: Shows correlation age risk of PPR

	Sheep		Goats	
Age	Number	Percent	Number	Percent
4-8 months	14	8.24%	6	6%
8-12 months	16	9.41%	15	15%
>12 M	27	15.88%**	31	31%**
x^2 value	30.9**			

**P<0.05

attributed to its virus titer which in turn decrease HA sensitivity. These results come in agreement with Hammouchi *et al.* [22].

Detection of PPR antigen by ELISA: ELISA results revealed 60 (35.29%) out of 170 sheep and 56 (56%) out of 100 goats as positive (Fig. 2). This may be attributed to higher recovery rate in infected sheep than goats [24]. Mahajana *et al.* [25] reported high survival and lower case fatality rate in sheep (34.4%) than goats (46.9%). Similarly, Sharma *et al.* [26] observed significantly higher ($p < 0.05$) morbidity and mortality in case of goats as compared to sheep. Age analysis of data revealed significantly ($p < 0.05$) higher seroprevalence of PPR in >12 months age group 15.88 and 31, followed by 8-12 months 9.41 and 15, while 4-8 months, were 8.24 and 6 percentage in sheep and goats, respectively (Table 2). The lower prevalence of PPR in young stock may be due to the passive immunity passed to them by colostral antibodies up to 4 months of age. Susceptibility to PPRV infection in kids increases thereafter corresponding with the natural decline in maternal antibodies. Ata and Al-Sumry [27] reported a lower prevalence of PPR in lambs of dam with previous history of PPR up to 3-5 months of age. The findings are also in agreement with Agrawal *et al.* [28], Shukla *et al.* [29] and Abubakar *et al.* [30] those observed an increase in seropostivity of PPR with the advancing of age.

In-Situe RT-PCR: RT-PCR amplified parts of N gene and to confirm this amplification, electrophoresis of RT-PCR products were performed and the expected band was recorded at 350 bp (Fig. 3). The cells were examined under light microscope without staining. Infected tissue culture cells showed cytoplasmatic staining (Mainly perinuclear) whose intensity correlated directly with the degree of viral

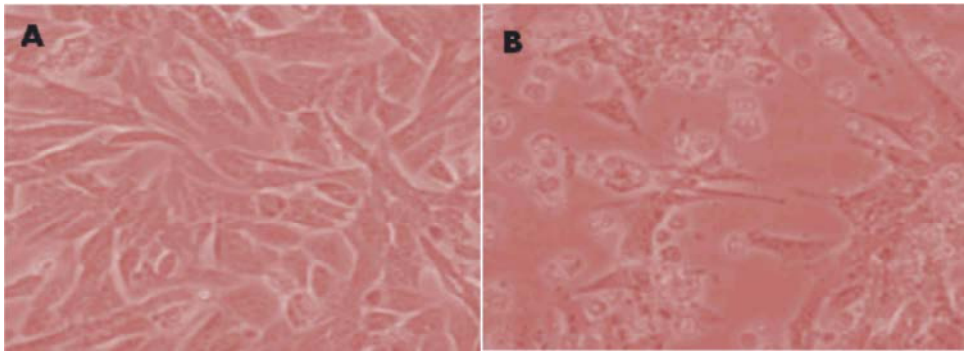

Fig. 1: Shows normal Vero cells (A). Vero cells shows rounding syncytia formation and cell degeneration 5 days PI (B).

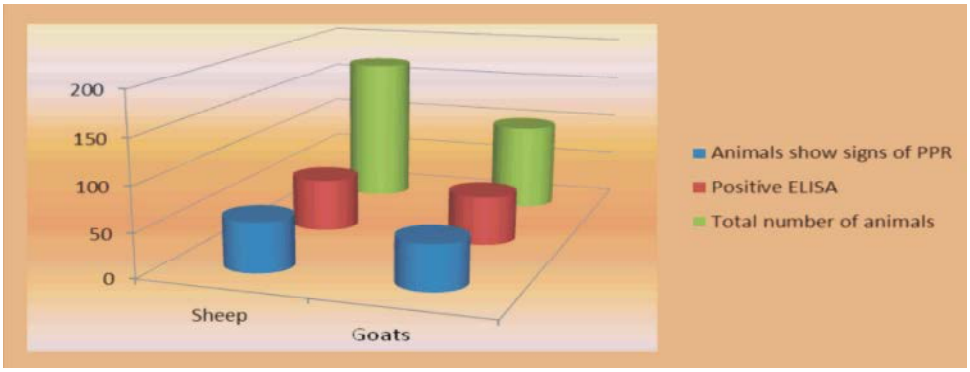

Fig. 2: Shows numbers of animal with signs of PPR and confirmed by ELISA test

Fig. 3: Amplified PPR after agarose gel electrophoresis, ethidium bromide staining and UV trans-illumination. Lane 1, contain 100pb marker while lane 2, 3, 4, 6, 7 contain PPR where an illuminating band at 350bp. Lane 8 contain control positive

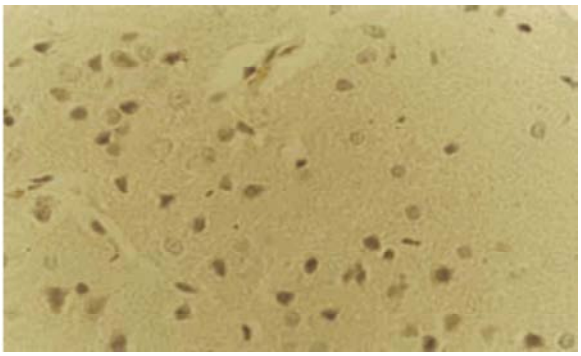

Fig. 4: Shows In-Situe RT-PCR, where infected cells show dark staining granules which represent the virus inside the cells,

cytopathic effect. In other words, rounded up cells depicted stronger staining in the form of dark granules around the nuclei (Fig. 4).

The current study recommends ELISA technique as an accurate screening test for diagnosis of PPR also recommends the direct In-Situ RT-PCR method for the confirmatory test of PPRV as it could be useful in several situations. In a diagnostic measure, it would allow confirmation of viral isolation in tissue culture, but most importantly, it would allow PPRV detection in formalin-fixed samples, thus avoiding handling and amplification of live virus. On the other hand, the ability to localize viral RNA at the cellular level might help to elucidate the pathogenesis of PPR persistent infection. This study also recommends vaccination against PPR in young age to avoid age risk.

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