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Isolation and characterization of lineage-IV *Peste des Petits Ruminants* (PPR) virus strains from Pakistan

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ABSTRACT: A total of 62 Peste des Petits ruminants (PPR) outbreaks in sheep and goat flocks were investigated in Pakistan during 2005-2007. The presence of PPR virus (PPRV) was confirmed by clinical picture, necropsy examination, Ic-ELISA, virus isolation and RT-PCR. Of 397 tissue samples, 65% tested positive by Ic-ELISA. Six PPR virus isolates were obtained through cell culture on VERO or GKC cell from 61Ic-ELISA positive samples identified by characteristic CPEs and confirmed by testing the cell culture supernatant by Ic-ELISA and RT-PCR using PPRV specific F gene based primers. The sequence data of F gene from 6 isolates was analyzed for identities and a phylogenetic tree was generated based on 372bp F gene sequences of PPRV. The isolates were clustered into lineage 4 along with other Asian isolates. The recent isolates and a previous isolate from Pakistan (PAK-2004) were found to be monophyletic having close relationship with an Indian isolate (IND-PON).

KEYWORDS: PPR, isolation, Pakistan, phylogenetic analysis, Ic-ELISA.

1 INTRODUCTION

Peste des Petits Ruminants (PPR) is an "acute, highly contagious and frequently fatal viral disease of sheep, goats and wild small ruminants" [1]. The disease is characterized by fever, muco-purulent ocular and nasal discharges, necrotizing and erosive stomatitis, severe enteritis and pneumonia leading to death [2]. PPR is a trans-boundary animal disease (TAD) of significant economic importance [3], ranking among the top ten diseases affecting small ruminants [4]. It has been described as the most important single cause of morbidity and mortality in small ruminant in Nigeria [5]. The causal agent, PPR virus (PPRV), is an enveloped ribonucleic acid virus of the genus *Morbillivirus* of family *Paramyxoviridae*[6]. The disease was first reported in Cote d'Ivoire in 1942 [7]. At present, it is endemic across the Sub-Saharan Africa, Morocco, Arabian Peninsula, Middle East, Turkey, Iran, Iraq, Pakistan, India, Bangladesh, Nepal, Tajikistan and Kazakhstan in Central Asia and Tibet, China [8-12]. The disease was first recorded in Pakistan during 1991 [13]. However, there are limited reports, which have attempted to document the prevalence and demonstrated continuing activity of PPRV in the country [14-16]. Bearing in mind the impact of PPR in the background of subsistence farming, the present study was conducted with the objective to isolate and characterize local PPR virus strains circulating among small ruminants population of Pakistan with special emphasis on monitoring the changes in virulence.

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2 MATERIALS AND METHODS

2.1 OUTBREAKS REPORTING

A total of 62 suspected PPR outbreaks reported by transboundary animal diseases (TAD) officers in their respective regions were investigated during 2005-2007. Each reported outbreak flock was examined for collection of data and samples.

2.2 CLINICAL EXAMINATION

The affected or "morbid" animals were defined as those showing anorexia, depression and an elevated body temperature over 104ºF. Animals were examined for the presence of clinical signs of PPR i.e. fever, discharges (ocular and nasal), erosive/necrotic mouth lesions, bronchopneumonia and diarrhea. Clinical examination of the affected animals was carried out in each affected flock and clinical signs were recorded.

2.3 NECROPSY EXAMINATION

The post mortem examination was conducted in those flocks where one or more dead animals were available. During necropsy, particular attention was given for evidence of discharges from the nostrils and eyes, diarrhea and gross pathological lesions in the oral mucosa, respiratory tract and alimentary tract. The gross pathological lesions were recorded.

2.4 SAMPLE COLLECTION

A total of 397 tissue samples were collected from dead goats and sheep at the time of necropsy using aseptic procedures. These included lungs, spleen and lymph nodes (mesenteric, bronchial, retropharyngeal and mesenteric lymph nodes). Each sample was marked with a unique identifier with date, and place. The samples were transported to the laboratory in cold condition. In the laboratory, the samples were stored at -70^{o}C till further processing.

2.5 SAMPLE ANALYSIS

The presence of PPRV antigens in the tissue samples was considered as evidence of PPRV infection in affected animals. The tissue samples (n=397) collected at necropsy were examined using an immune-capture ELISA (Ic-ELISA) kit (BDSL, France). Each of the tissue sample was processed to make a slurry and was tested by Ic-ELISA using the standard protocol [17].

The optical densities of the samples were measured at 492nm with an automated ELISA reader Immunoscan plus (Flow laboratories) using ELISA Data Interchange (EDI) software [18]. The samples with percent positivity (PP) value greater than 18% were considered positive.

2.6 ISOLATION AND PROPAGATION OF PPRV

Two types of cell culture were used to obtain PPR virus isolates *viz*. primary goat kidney cells (GKC) and Vero cells (African Green Monkey Kidney Cells). A total of 18 tissue samples (lungs, spleen and lymph nodes), that tested positive for PPRV antigen by Ic-ELISA, were inoculated onto primary GKC [19, 20]. The remaining 43 tissue samples that tested positive for PPRV antigen by Ic-ELISE, were processed and inoculated onto Vero cells [9, 20]. The control flask was inoculated with sterile phosphate buffer saline (PBS) only. The inoculated cell cultures were observed twice daily (morning and evening) under an inverted microscope for the appearance of cytopathic effects (CPEs). A sample was considered negative for PPRV if no CPEs were observed within 12 days post inoculation. In flasks, where CPEs developed within 12 days, the supernatant was harvested when 80% of the cells showed specific CPEs. The isolates thus obtained on GKC were adapted to grow on Vero cells by propagating for three passages. However, in case of Vero cells where there were no CPEs, the inoculated cultures were subjected to freezing and thawing three times to disrupt the intact cells (blind passages) and 1 ml of this suspension was added to a new 25 cm^2 flask of confluent monolayer of Vero cells. A sample was considered negative if no CPEs were observed after five blind passages.

2.7 PPR VIRUS IDENTIFICATION

The virus isolates were identified by characteristic CPEs observed on GKC and Vero cells and the presence of PPR virus was confirmed by testing the cell culture supernatant using Ic-ELISA. The isolates were catalogued on the basis of outbreak location and year of isolation. All the six isolates were lyophilized and are available in Animal Health Laboratories repository. The six PPR virus isolates obtained on cell culture and 46 field samples collected during PPR outbreaks reported during 2005-07 that tested positive by Ic-ELISA were confirmed using RT-PCR. The field samples comprised of ocular and oral swabs (18) and tissue samples (28) (lungs 7, Spleen 4 and lymph nodes 17). The PPR virus specific RNA from isolates and tissue and swab samples was extracted using an RNeasy kit (Qiagen GmbH, Hilden, Germany) according to manufacturer'sinstructions. A negative control was included to detect any possible contamination of reagents. The extracted RNA was eluted in 40 µl of nuclease-free water and stored at -70°C until use. The purity and quantity of the RNA was determined using Nanodrop (Nano Drop 1000, Thermo scientific, Wilmington, DE, USA). Vaccinal virus (Nigeria 75/1) was used in one step RT-PCR as control to confirm the successful extraction of RNA [11]. One step RT-PCR was performed to amplify extracted RNA by using PPR virus specific primers based on highly conserved sequences within F gene of PPR virus Forsyth and Barrett (1995).

2.8 SEQUENCING

The PCR products from the agarose gel was purified using Wizard® SV Gel and PCR clean-Up System kit (Promega Corp. Madison, WI, USA) according to the manufacturer's instructions. Sequencing was performed using the Dye Terminator Cycle Sequencing GenomeLab™ DTCS Quick Start Kit (Beckman Coulter, USA) following manufacturer's instructions. Approximately 100ng of purified PCR product was used as template with 5 pM of a single sequencing primer. The reactions were capillary electrophoresed on the CEQ™ 8000 Genetic Analysis System (Beckman Coulter, USA). For all sequencing reactions specific primers were used to sequence both the strands.

2.9 SEQUENCE ANALYSIS

The sequence data was compiled manually with the help of Blast 2 sequences (Tatusova and Madden, 1999; at http://www.ncbi.nlm.nih.gov/blast/bl2seq/wblast2.cgi), for both the strand separately for each isolate. The nucleotide sequences from both the strands were complementary aligned to each other. In this way a consensus sequence from one isolate was determined. The sequencing data was submitted in the Gene Bank.

2.10 PHYLOGENETIC ANALYSIS

The sequences of F gene of PPRV isolates were obtained from the nucleotide data base in the GenBank updated till 18-01-2010 and used for analyses. The sequences were aligned in ClustalW Sequence Alignment program using ClustalW 1.6 matrix for DNA alignments [21] in the Molecular Evolutionary Genetics Analysis Program (MEGA) version 4.0.1 [22] and only those parts which were aligned with the sequences of isolates sequenced in this study, were used further for phylogenetic analysis. Phylogenetic Neighbor-Joining (NJ) analysis was carried out using Maximum Composite-likelihood model with uniform rates among the sites; the 1000 bootstraps replicates were used to evaluate the significance of generated tree.

3 RESULTS

3.1 CLINICAL OBSERVATIONS

The clinical examination of affected animals indicated depression, pyrexia (39°C - 41.6°C), swollen lips, conjunctivitis, catarrhal ocular and nasal discharges along with cough and diarrhea. Almost all the affected animals appeared dehydrated. The animals examined in early stages of disease showed an erosive and necrotic lesion on lower gums just beneath the insertion of incisors. Similar lesions were also noted on the inner side of the lips, cheek papillae, dorsal surface of the tongue and hard palate.

3.2 NECROPSY OBSERVATIONS

The postmortem examination of carcasses showed sunken eyes, rough and dehydrated/dry skin with soiling of hindquarter. The internal examination revealed pneumonic lungs, hemorrhages on mucosal surfaces of abomasum and large intestine (caecum and colon) and inflamed lymph nodes, especially mesenteric lymph nodes.

3.3 DETECTION OF PPRV ANTIGEN

Of 397 samples tested, 258 were found positive for PPR virus with a positive percentage 64.99%.

3.4 VIRUS ISOLATION AND IDENTIFICATION

A total of six cytopathic PPR viruses (PPRV) were isolated from six different outbreaks using primary GKC and Vero cells. The PPRV isolates were identified by the characteristic CPEs induced by PPRV on both cell types (Table 1). Of these, two isolates were obtained of the 18 samples inoculated on primary GKC. The CPEs were observed on day 5 post-inoculation and onward. The CPEs included cell rounding, retraction, vacuolation, and formation of small syncytia. Of 43 samples inoculated on Vero cells, four PPRV isolates were obtained. Of these, one isolate was obtained after single blind passage while three isolates were obtained after two blind passages. The CPEs on Vero cells included cells of increased refractivity, rounding and detachment from the surface, clumping into grape like clusters and the appearance of fine spindle cells with elongated processes. The PPRV was harvested when 80% of the cells showed CPEs. The control flasks did not show any CPEs and the monolayers remained intact during the observation period. The cell culture supernatant of each isolate was tested positive by Ic-ELISA confirming that CPEs were induced by PPRV. The percent positivity values ranged between 78 to 133% (Table 1).

3.5 PHYLOGENETIC ANALYSIS

The sequences were submitted to GeneBank under accession numbers GU980858, GU980859, GU980860, GU980861, GU980862, GU980863, GU980864, GU980865, GU980866, GU980867 and GU980868**.** The results of the sequence analysis indicated that all the present study isolates of PPRV from 4 different provinces/ regions of Pakistan fall into lineage 4 (Figure 1). The nucleic acid sequences analysis of 372 F gene sequences of PPRV revealed identity among the 11 field isolates of the present study and previous isolate from Pakistan (Pak 2004) ranging between 100-98.8%. The sequence identities among recent isolates and two other previous isolates (Pak-Shah-Pk07 and Pak-Fsd-Pk07) ranged between 99.7- 96%. The identity score between recent isolates and a closely related Indian isolate (IND-PON) was 99.1- 97.8%. The identity scores between isolates from Pakistan and isolates from India, Turkey, China, Iran, Nigeria and Cote d Ivoire ranged between 99.1- 92%, 97.8- 96.6%, 97.8- 96.3%, 98.1-97.2%, 94.4- 91.6% and 89.8- 87.9% respectively (Table 2). The phylogenetic tree of PPRV isolates from Pakistan is shown in figure 2.

4 DISCUSSION

The current study was undertaken to isolate PPR virus circulating in small ruminant's population and monitor the changes in virulence of PPR virus involved in the outbreaks occurring in different parts of the country. The presence of PPRV virus in these outbreaks was demonstrated by clinical picture, necropsy examination, Ic-ELISA and virus isolation and RT-PCR.

The Ic-ELISA is an effective test for diagnosis of PPR from field samples and identification of PPRV in cell culture supernatant. A higher positive percentages or PI found in this study have been reported previously using Ic-ELISA in nasal secretions of clinically affected goats [23]. The monoclonal antibody based Ic-ELISA is now accepted as an alternative of virus isolation for diagnosis of Rinderpest virus (RPV) and PPRV by Office Internationale des Epizooties [24] for being simple, rapid, highly specific and sensitive assay [25].

Initially, primary GKC were employed for the isolation of PPRV from the field samples to increase the sensitivity of this technique. Later, Vero cells were preferred because of their continuity and lesser chances of contamination. The Vero cells also proved suitable for the isolation of PPRV. CPEs observed in this study due to PPRV on Vero and GKC were initial cell rounding, detachment from the surface, retraction, vacuolation and multinucleate syncytia formation. Other studies also reported similar findings[19, 20, 26].The six PPRV isolates obtained on primary GKC and Vero cells were confirmed using Ic-ELISA that can detect as little as $10^{0.6}$ $TCID_{50}$/well of PPRV particles[24].

Despite detection of viral antigen in all the tissue samples processed for virus isolation, only six isolates of PPRV were obtained. This low success rate for the isolation of PPRV may be attributed to the fact that the samples were collected at the time of necropsy after disease had run its full course. Successful isolation of RP/PPRV depends on various factors including the phase of the disease during which samples were collected from donor animals [27]. The samples collected during the infectious period i.e. in febrile phase are ideal for the isolation of RPV/PPRV and the titers of infectious virus decline exponentially after fever has regressed [20]. The isolation of PPRV using Vero cells is reported to be difficult since it requires one or more blind passages to become tissue culture adapted even from very fresh clinical samples or isolated virus [20, 28].

Moreover, PPRV is difficult to isolate from infected animals despite detection of PPRV antigen by Ic-ELISA in tissue samples [17].

The RT- PCR using primers based on highly conserved sequences within F gene of PPRV proved suitable for diagnosis and/or confirmation of the PPRV isolates obtained on cell cultureand effectively tracked the changes in virulence of PPR virus. The assay described by Forsyth and Barrett, (1995) has extensively been used for the specific diagnosis and molecular epidemiological studies of PPR virus [8, 9, 29].

High sequence variability's found in RNA viruses [10, 30] necessitate the need to carry out molecular epidemiological studies focusing on sequence identities and distances among local field isolates and isolates of different geographic origin. The phylogenetic analysis revealed that lineage 4 PPRV is circulating in Pakistan. These results are in agreement with those reported earlier [8, 9, 11, 29]. The data also suggested that two strains of PPRV are circulating among small ruminants population in Pakistan. The existence of two viral subgroups among lineage 4 PPRVs circulating in Asian countries has already been reported [29]. The most related sequences to those of Pakistani isolates originated from India. This clearly is indicative of cross border transmission of PPRV infection into Pakistan. The border between Pakistan and India was used to be porous and there was frequent movement of livestock prior to the fencing of border by India. It was revealed during field investigations that sheep and goats were brought from India for trade purposes during shortage of mutton in Pakistan and also before Eid festival. The cross border transmission of PPRV was held responsible for the introduction of PPR in Tibet, China [11]. However, the Indo-Pakistan subcontinent was swept across by an epidemic of lineage 4 PPRV during 1993-95 [8, 10, 31]. With this common history the sequence identities and close genetic relationship among PPRV isolates can be expected.

It is evident from the phylogenetic analysis that source of PPR outbreaks in south of Punjab province and upper Sindh province is from central Punjab (Faisalabad). The isolates from these regions were found 100% similar to a previous isolate (PAK-2004). The most likely source of PPR outbreaks in Azad Jammu and Kashmir (AJK) was from northern Punjab. These further inter and intra provincial spread of PPR virus within the country may also be attributed to the animal movement. The prevailing production systems for small ruminants in Pakistan are traditional and extensive. Majority of the farmer's have very smallholdings with livestock production varying in three broad areas *viz.* canal irrigated areas, arid/ semi-arid areas and desert areas where crop production is not possible. There have been four main systems namely nomadic (44%), semi migratory transhumant (38%), sedentary and/or household (18%) [32]. All of these systems require a lot of stock movement in search of pastures and water. It can be speculated that the transhumant stock movement may had contributed to the transmission of PPRV from central Punjab into south of Punjab and upper Sindh. Likewise the nomadic and transhumant movement from northern Punjab to summer alpine pastures may possibly be held responsible for the transmission of PPR virus into AJK.

During the course of this study it was observed that PPR outbreaks exhibited two different epidemiological and pathological trends in the country. The first trend was seen in case of epidemic outbreaks where the affected animals presented classical clinical signs (acute form) of disease. The affected animals were dying within two weeks' time with a mortality rate of 50- 70%. Such trend was seen during 2005-06 in south Punjab and upper Sindh. The second trend was observed during the following years i.e. in 2006-07 especially in north of Punjab and AJK where the outbreaks of PPR were less explosive in terms of severity of clinical signs and mortality. The duration of disease was longer and many of the affected animals recovered. Such changes in virulence of PPR virus were depicted in sequence analysis of F gene of the isolates e.g. PAK- Att-07-NARC5, PAK- FJg-07-NARC4 and PAK- Mzd1-07-NARC where these isolates varied in identity ranging between 99.1-98.8% from the virulent virus PAK- Veh- 05-NARC1 and PAK- KP1- 06-NARC1. These changes are indicative of immune pressure and greater weight of infection in the field. The genetic variations in F protein gene coupled with the epidemiological picture strongly suggest that PPR has established as an endemic infection in the country. This also elucidated the evolving nature of PPR virus.

5 CONCLUSION

Based on the results of the present study, it is concluded that lineage 4 PPR virus infection has established as an endemic infection in the country. Now, it constitutes a major threat to the small ruminant's production and food security in the region. This calls for a comprehensive strategy to improve the disease reporting and surveillance activities, farmers' awareness regarding sanitary prophylaxis for PPR and other TADs and further studies to understand epidemiology and pathogenesis of PPR in small ruminants to devise an optimal control strategy.

Table 1. Isolation and identification of PPR virus isolates using goat kidney cells (GKC) and Vero cells (Vero-76) based on cytopathic effects (CPEs).

Isolate	Cell Type	CPEs						PP (Ic-ELISA)
		Cell rounding	Detachment from Surface	Vacuolation	Syncytia formation	Clumping of cells	Cells with Elongated processes	
PAK-VEH-05/NARC1	GKC	+	+	+	+	-	-	94
PAK-ICT-06/NARC2	GKC	+	+	+	+	-	-	133
PAK-KP1-06/NARC3	Vero-76	+	+	-	-	+	+	78
PAK-FJg-07/NARC4	Vero-76	+	+	-	-	+	+	106
PAK-Att-07/NARC5	Vero-76	+	+	-	-	+	+	89
PAK-Mzd1-07/NARC6	Vero-76	+	+	-	-	+	+	121

PP = Percent positivity
Ic-ELISA = Immuno-capture Enzyme Linked ImmunoSorbant Assay
PAK = Pakistan
VEH = Vehari
ICT = Islamabad Capital Territory
KP1 = Khairpur
FJg = Fateh Jang
Att = Attock
Mzd1 = Muzaffarabad
NARC = National Agricultural Research Centre

Table 2. Percentage sequence identities of F gene sequences between PPRV isolates of different geographical origins

	Pakistan	India	Turkey	China	Iran	Nigeria	Cote d' Ivoire
Pakistan Lineage 4	100- 96.0	99.1-92.0	97.8-96.6	97.8-96.3	98.1-97.2	94.4-91.6	89.8-87.9
India Lineage 4	99.1-92	100-92.3	99.1-92.9	99.4-92.0	99.4-93.5	95-88.6	90.1-88.2
Turkey Lineage 4	97.8-96.6	99.1-92.9	99.4-98.1	97.2-96.9	99.1-98.8	94.4-92.5	89.1-88.5
China Lineage 4	97.8-96.3	99.4-92	97.2-96.9	100-99.7	97.5-97.2	93.8-92.5	88.8-88.5
Iran Lineage 4	98.1-97.2	99.4-93.5	99.1-98.8	97.5-97.2	100	94.1-93.2	89.4
Nigeria Lineage1	94.4-91.6	95.0-88.6	94.4-92.5	93.8-92.5	94.1-93.2	97.8-90.4	97.8
Cote d' Ivoire Lineage 2	89.8-87.9	90.1-88.2	89.1-88.5	88.8-88.5	89.4	97.8	90.4-91.9

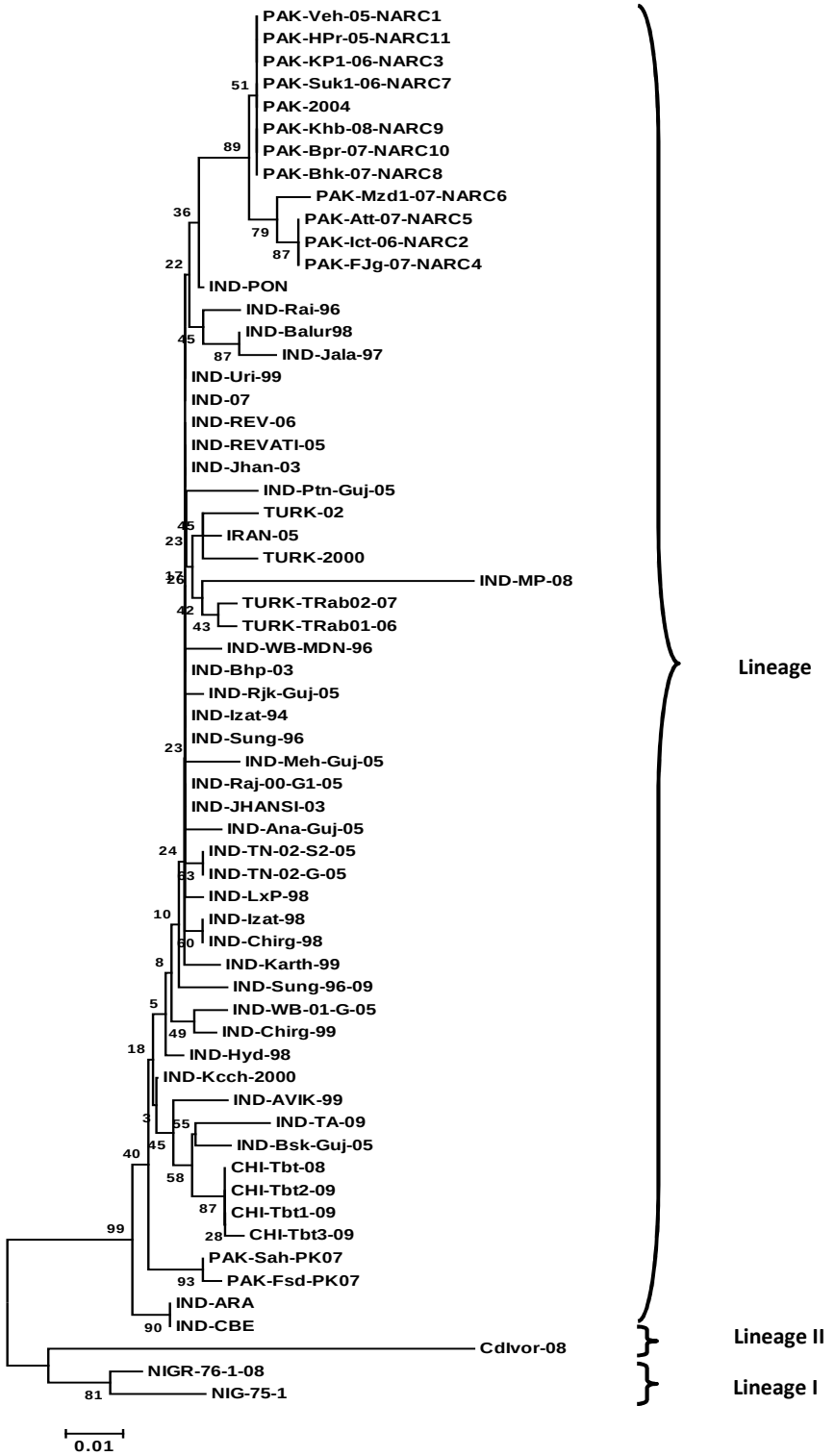

Fig. 1. Unrootedneighbor joining phylogenetic tree based on 372 bp partial sequence of F gene of PPRV detected in Pakistan and other isolates. Bootstrap values are shown on the branches. Bar indicates substitution per site.

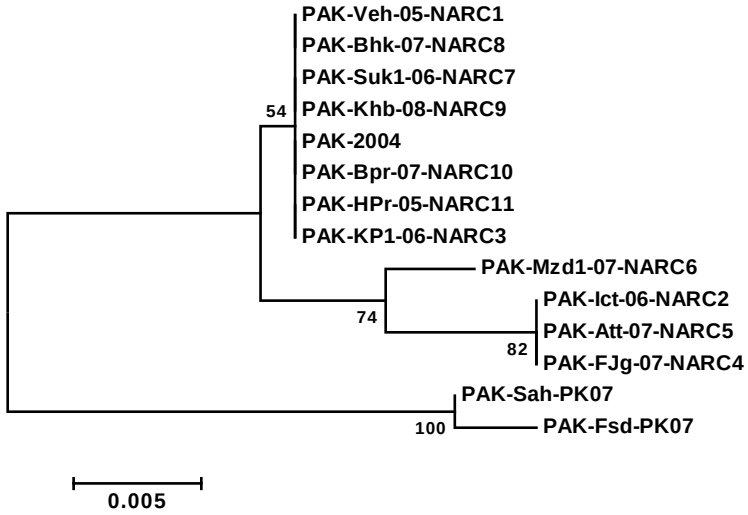

Fig.2. Phylogenetic relationship based on 372 bp partial sequence of fusion (F) gene of Pakistani isolates of PPRVs.

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