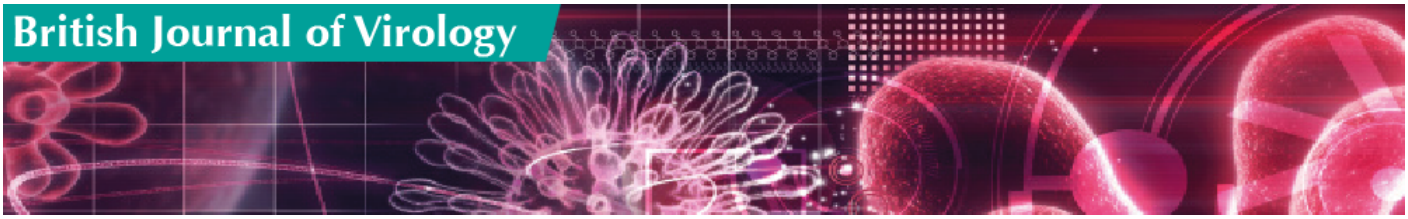

Virus interference between H7N2 low pathogenic avian influenza virus and lentogenic Newcastle disease virus in experimental co-infections in chickens and turkeys.

Journal	British Journal of Virology
Manuscript ID	SANDF20140108160101
Manuscript Type	Minireview
Date Submitted by the Author	Wed, 31 Dec 1969, 06:00 PM
Complete List of Authors	■ Dr Muhammad Munir ■ Dr Gill Khan

Detection and phylogenetic analysis of *Peste des Petits Ruminants Virus* isolated from outbreaks in Punjab, Pakistan

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Keywords: Peste des Petits ruminants, sheep, goats, Pakistan, DNA sequencing

Running title: Characterization of PPR from Pakistan

Summary

Peste des Petits Ruminants (PPR) is an important viral disease of small ruminants and is endemic in Pakistan. In the following study, two outbreaks of PPRV in goats have been subjected to laboratory investigations. The PPRV genome was detected using both conventional and real-time PCR. Genetic characterization of the local PPRV field isolates was conducted by sequencing 322 bp of the fusion (F) and 255 bp of the nucleoprotein (N) genes. The phylogenetic tree, based on the F gene, clustered isolates from both outbreaks into lineage 4 along with other Asian isolates and more specifically into subclusters 1 along with isolates from Middle East. Conversely, analysis of N gene revealed a different pattern in branching where Pakistani isolates clustered with Chinese, Tajikistani and Iranian isolates which probably represent the true geographical pattern of circulation of the virus. This is the first report presenting the phylogenetic tree based on N gene and performing a parallel comparison of the trees of F and N gene together from Pakistani isolates, despite the fact that PPRV is one of the most devastating diseases for small ruminants in the country. The results of this study shed light on the PPR virus population in Pakistan and emphasize the importance of using molecular methods to understand the epidemiology. Such understanding is essential in any efforts to control the number and impact of outbreaks that are occurring in endemic countries such as Pakistan especially in the current scenario when OIE and FAO are eager to control and subsequently eradicate PPR from the globe, as it has been practices for Rinderpest.

Introduction

Peste des petits ruminants (PPR) is a highly contagious and often fatal morbilli-viral infection of both wild and domestic small ruminants, and is characterized by high pyrexia (up to 106 °F), anorexia, nasal and ocular discharges, erosive stomatitis, conjunctivitis, gastroenteritis and pneumonia. *Peste des Petits Ruminats virus* (PPRV), a causative agent for PPR, is a single-stranded negative sense RNA morbillivirus. Clinically, PPRV affects

goats severely but sheep undergo a mild form of the disease while cattle may experience sub-clinical infection (Anderson and McKay, 1994). PPR causes serious economic losses in Asia, the Middle East and Sub-Saharan Africa.

Based on specific regions of the nucleocapsid (N) and fusion (F) gene, PPRV isolates are grouped into four distinct lineages while only one serotype is reported (Shaila et al., 1996). Lineages II and I are found exclusively in West Africa while lineage III has been isolated in eastern Africa (Ethiopia), Arabia (Oman, Yemen) and unexpectedly once from southern India. The fourth lineage (IV) is believed to be confined completely in the Middle East, Arabia, south Asia and China (Shaila et al., 1996; Wang et al., 2009).

Since the first confirmation of PPRV in Pakistan in 1994, a huge number of outbreaks have occurred in the country; however only a limited number of these outbreaks are documented due to a lack of awareness. After successful eradication of Rinderpest from Pakistan in 2007, PPRV gained attention and it's economic importance and transboundary nature was recognised. Recently, a number of studies, including this one, have been conducted to ascertain the magnitude of the PPR problem, characterize the virus population and to define the trends and causes of its occurrence in Pakistan (Hussain et al., 1998; Ahmad et al., 2005; Khan et al., 2008; Munir et al., 2008; Abubakar et al., 2008; Abubakar et al., 2009; Munir et al., 2009; Abubakar et al., 2010). Previous studies have almost always been based on either laboratory confirmation by detecting antibodies/antigen using ELISA or based on clinico-epidemiological observations. However, given the increase in circulation of PPR virus among susceptible populations and for future eradication concern of the FAO and OIE (Normile, 2010), it is of paramount importance to delve into the molecular and genetic characterization of the field PPR viruses. The objective of this communication was to detect PPRV in suspected outbreaks in the region by employing conventional and real-time PCR and then to use sequencing to provide phylogenetic analysis of nucleoprotein and fusion protein gene segments to get an insight genetic picture of PPRV.

Materials and Methods

Clinical history and sample collection

A total of 12 samples including nasal swabs and blood were collected from two outbreaks in Pakistan. A representative sample from each outbreak was subsequently taken for sequencing. The detailed information of samples is presented in Table 1. Lyophilized freeze-dried inactivated PPR vaccine (Nigeria 75/1) obtained from CIRAD, France was used as the reference virus during the course of study. Biological material was stored on QIAcard FTA Indicator Four Spots (Qiagen), which preserve nucleic acids and inactivate the samples. The samples were shipped at ambient temperature from Pakistan to the Swedish University of Agricultural Sciences, Uppsala, Sweden for processing.

Screening of the samples using real-time PCR

The RNA was extracted from Qiacard FTA Indicator (Qiagen) impregnated with clinical samples as recommended by manufacturer (preparation of isolated RNA from FTA® Cards, Rev 1 10/17/07, Whatman) with the following modifications. Using a 2.0mm diameter Harris micro punch (Whatman), one punch for each sample was removed according to manufacturers protocol (BD09, Whatman) and placed in separate 1.5 ml microfuge tubes. 200µl Tris-EDTA buffer (10mM Tris-HCl, pH 8.0, 0.1mM EDTA) was used instead of RNA processing buffer and incubated for 15 minute (flicking tubes 3 times over the course of incubation) on ice, not at room temperature.

PCR amplification and detection was performed in Rotor-Gene 6000 real-time analyzer (Qiagen) using AgPath ID one-step RT-PCR kit (Applied Biosystem) as recommended. The primers in the N region named NPPRf TAQ: GAG TCT AGT CAA AAC CCT CGT GAG and NPPRr TAQ: TCT CCC TCC TCC TGG TCC TC and probe sequence NPPRp TAQ: FAM-5'-CGG CTG AGG CAC TCT TCA GGC TGC-3'-BHQ1 were used. The reporter dye (FAM) signal was measured at the annealing step of each

cycle and the threshold cycle (C_t) for each sample was calculated. The samples having C_t value <35 of samples were considered positive.

PCR amplification, TOPO cloning and sequencing of F and N genes

One sample from each outbreak (Multan and Faisalabad) was selected for sequence analysis. A fragment of 380 bp was amplified from the most variable region at the 3′ end of the N gene using primers; PPR-N-F (5′- TCTCGGAAATCGCCTCACAGACTG-3′) and PPR-N-R (5′-CCTCCTCCTGGTCCTCCAGAATCT-3′). The F gene was amplified using PPRV-F-F 5'-ATCACAGTGTTAAAGCCTGTAGAGG-3', PPRV-F-R 5-'GAGACTGAGTTTGTGACCTACAAGC-3' primers. In both PCRs, 3µl of RNA was used in a 25µl reaction using One-Step RT-PCR kit (Qiagen) as recommended. The amplified PCR products were gel extracted and cloned in pCR3.0 using TOPO TA cloning kit (Invitrogen) following the manufacturer's instructions. At least 3 recombinant plasmids were processed with Miniprep (Promega) for DNA extraction and processed for sequencing using ABI PRISM BigDye Terminator version 3.1 (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's instructions. Sequences were analyzed with an automated nucleic acid analyzer (ABI PRISM 3100, Applied Biosystems, Foster City, CA). Each DNA fragment was sequenced at least twice in both directions.

Sequence and phylogenetic analysis

Sequence assembly and editing were performed using the SEQMAN programme of DNASTAR Lasergene 8 (version 8.0.2 13). Construction of phylogenetic trees was performed with the Neighbour-Joining method using Kimura two-parameter model in Mega5 version 5. BLAST program was used to search GenBank for homologous gene sequences in NCBI database. To look for variation among the sequences under comparison, a phylogenetic network was drawn for the nucleotide (F gene) or amino acid (N gene). The data was processed by star contraction algorithm (Forster et al., 2001) and then by Median-Joining (MJ) network algorithm (Bandelt et al., 1999) (www.fluxus-engineering.com). The sequences of the genes analyzed here have been

submitted to the GenBank. The accession numbers for F gene are JN009671 and JN009672 and for N gene JN009673 and JN009674.

Results

The Qiacard FTA Indicator (Qiagen) appeared to have been an appropriate sampling system for this study. The results from the clinical material revealed that all the samples tested from each outbreak were positive when tested with real-time PCR. However, conventional PCR showed two and three positive from Multan and Faisalabad districts, respectively (Table 1).

The analysis of 322 bp F gene sequences revealed that the homology between the two isolates in the present study ranged between 99-100% whereas the similarity of F gene with previously characterized Pakistani isolates range from 95% to 98%. Similarly, the sequence homology of N gene between isolate of each outbreak range between 99-100%. Since there is no N gene sequence available from Pakistani origin, no similarity can be calculated for previously characterized Pakistani PPRV isolates.

A phylogenetic tree for each gene (F and N) was constructed based on the sequences retrieved from GenBank, representing all the lineages. The F gene tree depicted that Pakistani isolates under study clustered together with isolates of Middle East (Fig. 1). Further, lineage IV in F gene tree can be divided into subclusters (SC): SC1, SC2 and SC3. Pakistani isolates were grouped in either SC2 (Pakistan/SAH/Pak/07 and Pakistan/FSD/PK/07) or in SC1 (Pakistan/94 and the isolates under study). The N gene based tree shows that all the four lineages are clearly separated from each other, however in this tree, the Pakistani isolates clustered together with isolates from Tajikistan, Iran and China (Fig. 2).

The network analysis based on F gene sequences showed that all the four lineages are well separated from each other (Fig. 3), each represented by different colour. A central

node was formed by 26 isolates of lineage IV, which is mainly constituted by Indian isolates. Another isolate (IN00 AF464879) made a second node, outside the central node. The previously characterized Pakistani isolate differs from the central node by 3 mutations, with the new isolates differing by further 5 and 7 mutations. Isolates from Saudi Arabia and Kuwait were also clustered in this minor taxon. The network analysis for the N protein showed that the isolates from the present study were separated from a central node made by Indian isolates in lineage IV with a difference of 3 and 6 mutations (Fig. 4). Lineage 1 showed a close relationship with lineage IV, however all the lineages are clustered in separate branches.

Discussion

There have been several outbreaks of PPR in Pakistan throughout the year. Several studies have confirmed the diagnosis of PPR based on immunoglobulin detection from clinical samples (Hussain et al., 1998; Ahmad et al., 2005; Khan et al., 2008; Munir et al., 2008; Abubakar et al., 2008; Abubakar et al., 2009; Munir et al., 2009; Abubakar et al., 2010). In order to understand the epidemiology of these PPRV outbreaks, it is essential to characterize these viruses to get better insight into the virus population. For the conservation of biological samples, especially where cold chain is unavailable, filter papers with inactivation properties containing dried blood or clinical samples appears to be a practical sampling system. It has been practiced for many RNA and DNA viruses (Michaud et al., 2007). The fact that all the samples in this study were detected by real-time PCR indicates that it is a convenient, inexpensive and appropriate source of sample collection and transportation for PPRV from goats, which have clinical signs.

PPRV has enormous economic importance, which is due to its highly infectious nature (Abubakar et al., 2011) and serious clinical implications. The high morbidity (100%) and mortality (90%) in PPRV susceptible populations is mainly contributed by secondary infections, which cause symptoms such as pneumonia or gastroenteritis. The majority of animals in the outbreaks were either malnurished or had heavy parasitism. It is plausible that malnutrition/parasitism are factors for increasing the clinical relevance of

PPRV infections. A compromised immune status caused by parasites and/or malnutrition may make animals more susceptible to catching and have serious symptoms result from PPRV.

To assess the genetic similarity and divergence between the isolates of these two outbreaks and their relationship with previously characterized isolates submitted to GenBank, the partial F and N gene sequences were phylogenetically analyzed. An overall topology of the trees indicated that PPRVs from different geographical regions vary to a larger extent in their N gene than F gene sequences. Previously characterized Pakistani PPRV isolates were grouped in either subcluster 1 or 2 in the lineage IV (based on F gene) where as the isolates in current study were grouped into subcluster 1 which constitute the majority of PPRV isolates sequenced so far (Shaila et al., 1996; Kerur et al., 2008). Because the N sequences of Pakistani PPRV isolates are not available (or not sequenced before) it is hard to predict the homology and rate of variations over the time. It appears from the tree constructed on N gene that Pakistani isolates are closely related to Chinese, Tajikistani and Iranian isolates. It is logical that PPRV could have come from these countries, as they are geographically proximate to Pakistan. On the other hand, the F gene based tree showed that Pakistani isolates are close to isolates from Middle East with whom Pakistan has immense trade. Recent evidence indicates that N gene appears to provide the better epidemiological picture of PPRV and is now in favour over F gene classification (Shaila et al., 1996). However, given the fact that PPRV has tendency to mutate, phylogenetic analysis based on both F and N genes should provide a more informative characterization. This is demonstrated clearly in results of this study, which has the new isolates clustering differently for the two genes.

Due to the high number of silent mutations in the 3rd codon, the amount of amino acidic variations in the F-gene MJ network was too low to get a good geographical resolution (data not shown). The network based on the nucleotide variation gives a better resolution (Fig. 3). The F gene based network analysis indicated that branching of PK10_MULTAN and PK10_FAISALABAD matched the branching pattern shown by

Neighbour-joining method for the corresponding sequences. Further, the new isolates clustered with Indian and Iranian isolates in a different taxon than previously characterized Pakistani PPRV isolates. The isolates in the present study along with another Pakistani isolate characterized in 1994 branched in a separate taxon with in lineage IV. This indicates either a number of introductions into Pakistan or, a high mutation rate, or both. In any case, further studies at larger scale are warranted. Network analysis of for the N protein was in concordance with the branching pattern shown by the consensus tree.

To conclude, based on clinical signs and the detection of PPRV using real-time and conventional PCR, the disease outbreaks in the current study were caused by PPRV. The phylogenetic analysis of the F and N genes showed that Pakistani PPRV isolates clustered into lineage IV, as expected. In order to address the problem, real-time or RT-PCR and sequencing should be added to regular serological surveillance to allow characterization of PPRV in the region. The OIE and FAO should recommend this test to enable investigations into the cause and control of outbreaks. Given the economic importance of PPRV due to high case fatality, morbidity and mortality and due to high production losses, it is extremely important that such monitoring take place. To further reduce the economic impact of PPRV on small ruminant industry in Pakistan, an awareness campaign to the livestock owners should be considered.

Acknowledgements

The authors are thankful to EPIZONE and Geneviève Libeau from CIRAD, France for providing PPR vaccine. We are also grateful to Martì Cortey for helping in network analysis. Muhammad Munir is recipient of fellowship from Higher Education Commission of Pakistan (HEC).

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Table. 1: Description of the samples used in the study.

Outbreak districts	Clinical picture	Samples collected	Parasitism	Malnutrition	Real-time PCR	Conventional PCR
Multan	Beetal Breed, Diarrhoea, Weakness, Oral necrosis, Fever (104 °F)	4	Yes	Yes	Positive (all)	Positive=2, Negative=2
Faisalabad	Desi Breed, Sever diarrhoea, Weakness, Shivering, Lesions around oral cavity, Fever (103 °F)	8	No	Yes	Positive (all)	Positive=3, Negative=5

Fig. 1. Majority rule consensus tree of PPR viruses based on the variable region of the F gene (322 bp) constructed using the neighbour-joining method in the Kimura-two-parameter model in Mega5 version 5. Numbers indicate the bootstrap values (1000 replicates) and only the values above 50% are shown in the figure. Horizontal distances are proportional to sequence distances. Figure indicates the clear division of the four lineages of PPRV. The samples used in this study are marked with round black spot (●).

Fig. 2. Majority rule consensus tree of PPR viruses based on the variable region of the N gene (255 bp) constructed using the neighbour-joining method in the Kimura-two-parameter model in Mega5 version 5. Numbers indicate the bootstrap values (1000 replicates) and only the values above 50% are shown in the figure. Horizontal distances are proportional to sequence distances. Figure indicates the clear division of the four lineages of PPRV. The samples used in this study are marked with round black spot

(●).

Fig. 3. Phylogenetic network analysis based on the F gene of the field isolates with corresponding sequences published in the GenBank. Numbers along the branches represent the nucleotide changes separating the nodes. Based this network analysis, the isolates from the present study clustered in the single taxon with 7 mutations (PK10_MULTAN) or 5 mutations (PK10_FAISALABAD) compared to previously characterized Pakistani isolate (PK94_FR667646). Samples used in this study are marked in circle.

Fig. 4. Phylogenetic network analysis based on the N protein of the field isolates with corresponding sequences published in the GenBank. Numbers along the branches represent the amino acid changes separating the nodes. Samples used in this study are marked in circle.