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Full Length Article

Molecular Based Diagnosis of Rinderpest and Peste Des Petits Ruminants Virus in Pakistan

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ABSTRACT

Differential diagnosis of Rinderpest (RP) and Peste des petits ruminants (PPR) by host species and clinical signs is not always exact; hence, laboratory confirmation is necessary to establish the cause of disease and to distinguish these two viruses, where small animals are source of infection for large ruminants. In this study, reverse transcription polymerase chain reaction (RT-PCR) was standardized against RP and PPR. RNA of these viruses was isolated using denaturing solution (Solution D) containing guanidium thiocynate, sarcosyl, sodium citrate and ß$_2$ mercapto-ethanol. Regular PCR using morbilli specific oligonuclotides based on the sequences of conserved region of F and P-gene. These F and P gene primers amplified 429bp and 372bp, respectively for both RP and PPR. Nested PCR was performed to differentiate RP and PPR using F-gene amplicons by F3A/F4A and F1A/F2A primers, respectively. These set of primers amplified a product of 235bp and 309bp for RP and PPR, respectively. The results of the study indicated that RT-PCR can be successfully used for detection and differential diagnosis of RP and PPR.

Key Words: Reverse transcription polymerase chain reaction (RT-PCR); Rinderpest (RP); Peste des petits ruminants (PPR)

INTRODUCTION

Rinderpest (RP) and Peste des petits ruminants (PPR) are important viral trans-boundary diseases of ruminants in the developing world caused by two closely related morbilliviruses genus of paramyxoviridae (Barrett *et al*., 1991; Barrett, 1994). The RP has been and is still one of the most devastating diseases of the large ruminants in certain parts of the world and was first appeared as a recognized disease in late 1980's. It has now been found across the Middle East and Indian sub-continent reaching as far as Nepal and Bangladesh (Banayard *et al*., 2006). RPV is controlled by vaccination but in many areas war or civil unrest means that, this can not always be carried out effectively, resulting in reservoirs from which new virus outbreaks spread to neighboring areas and countries (Forsyth & Barrett, 1995). PPRV is found in west Africa, the Middle East, Bangladesh, Pakistan and India (Taylor, 1984; Shaila *et al*., 1989) but as yet no large scale vaccination programs have been established to eradicate the disease, which is still wide spread. Morbidity and mortality rates vary, but can be as high as 100 and 90%, respectively. These levels are usually lower in endemic areas and mortality can be as low as 20% (Roeder & Obi, 1999).

As differential diagnosis of RP and PPR by host species and clinical signs is not always definitive, laboratory confirmation is necessary to establish the cause of the disease and differentiate between both viruses in case where small animals are source of infection for large ruminants. Indian buffaloes have also been reported to have died from PPRV infection (Banayard *et al*., 2006). Cattle that are sero-positive for PPR have been found in West Africa, with up to 80% prevalence in some herds, but there is no evidence that it can cause disease in bovids. Nucleic acid hybridization using either radio labeled or biotinylated cDNA probes are being used to identify these viruses and this has greatly speeded up differential diagnosis (Diallo *et al*., 1989; Pandey *et al*., 1992). Competitive (Anderson *et al*., 1991) and immuno-capture ELISAs (Libeau *et al*., 1994) and differential immuno-histochemical staining of tissue sections (Saliki *et al*., 1994) by using monoclonal and polyclonal antibodies are also being used. However, these techniques are less sensitive than polymerase chain reaction (PCR) for the detection of viruses. In this study, RT-PCR was standardized, using phosphoprotein (P) and fusion (F) protein gene specific primer sets to detect and differentiate RPV and PPRV both by regular and nested PCR.

MATERIALS AND METHODS

Tissue samples. Eye swabs, nasal swabs, whole blood, lung, liver, spleen, lymphoid tissue, tears, eye, mouth erosion swabs from goat, sheep, cattle and buffalo (Table I).

RNA isolation. Tissue (antigen) was finely minced and homogenized in 4 mL of denaturing solution (Solution D)

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Table I. List of diagnostic samples tested showing type of samples from animal species

Tissue Samples	Buffalo	Cattle	Sheep	Goat	Total
Eye swabs	4	4	3	2	13
Nasal swabs	5	3	3	2	13
Whole blood	20	13	10	8	51
Lung	14	10	3	2	29
Liver	2	2	1	1	6
Spleen	3	2	1	1	7
Lymphoid tissue	18	10	6	6	40
Tears	6	5	1	1	13
Eye	2	1	-	-	3
Mouth erosions	3	2	2	2	9
Total	77	52	30	25	184

containing guanidium thiocynate, sarcosyl, sodium citrate and ß$_2$ mercapto-ethanol before the addition of sodium acetate and subsequent extraction with phenol-choloform-isoamyl alcohol. RNA was precipitated by the addition of 2.5 volumes of cold ethanol. For the eye, mouth, gum swabs and tears 1ml of sample including the swab, was vortexed vigorously in 2 mL solution D before a similar extraction and precipitation of RNA. Peripheral blood lymphocytes from 5-10 mL whole blood were purified on ficoll gradient and the cell pellets taken up in 0.4 mL solution D and extracted using the above method. The pellet obtained was air dried. The RNA was dissolved in RNase free water and incubated for 10 min at 55-60°C. In RT-PCR both known positive and negative RNA samples were taken as controls. The canine distemper and measeals virus were taken as positive control, while distilled water was taken as negative control. Initially, P or F gene specific primers were used for reverse transcription of viral RNA, but later random hexa-nucleotide primers were used for reverse transcription due to cost and efficiency.

Reverse transcription/cDNA synthesis. Extracted tissue RNA was dissolved in RNase free DEPC (Diethyl pyro-carbonate) treated water 1 mg mL^{-1}. RNA solution 5 µL (Approximately 5 µg RNA), 2 µL of random hexa-nucleotide primers (50 ng µL^{-1}) and 3 µL of DEPC treated water was taken in eppendorf tube. After 5 min of incubation at 70°C the solution was cooled at room temperature for 10 min to allow primer annealing and centrifuged briefly for 16-20 seconds before opening.

An amount of 4 µL of 5X RT buffer (50 mm tris HCl (pH 8.3), 3 mm $MgCl_2$ & 75 mm KCl), 2 µL of acetylated bovine serum albumin (BSA, 0.1 mg mL^{-1}), 2 µL DTT (0.1 M), 1 µL dNTPs (10 mm each) and 1 µL reverse transcriptase (200 Units) was used for each RNA sample. It was mixed gently, centrifuged briefly and incubated at room temperature for 5 min and then at 37°C for 30-60 min to obtain cDNA and this product was stored at -20°C until used for PCR.

PCR amplification. A 5 µL cDNA was taken and added with 45 µL of the mixture containing 5 µL 10X PCR buffer, 3 µL $MgCl_2$ (25 mm), 34 µL sterile distilled water, 1 µL dNTPs (10 mm each), 1 µL F-gene primer (10 pmol each) and 1 hot start wax bead containing Taq-polymerase was added to each 0.75 mL reaction tube.

The thermal cycler program was: step1, 1 cycle, 94°C for 5 min; step2, 40 cycles, 94°C for 30 seconds, 50°C for 30 seconds, 72°C for 45 seconds; step3, 72°C for 10 min.

Primers. The morbillivirus specific oligo-nucleotides were based on the sequences from conserved regions of the P gene, which were previously found to act as "universal primers" for all morbilliviruses (Barrett *et al*., 1993a). RPV or PPRV virus specific oilgo-nucleotides were based on well conserved regions of the F gene sequences (Evans *et al*., 1994; Mayer & Diallo, 1995), which selectively primed the homologous virus RNA and so could distinguish between the two viruses.

The universal P gene specific primers were:

UPP1, 5′-ATG TTT ATG ATC ACA GCG GT-3′

UPP2, 5′-ATT GGG TTG CAC CAC TTG TC-3′

The F gene specific primers were:

PPRVF1, 5′-ATC ACA GTG TTA AAG CCT GTA GAG G-3′

PPRVF2, 5′-GAG ACT GAG TTT GTG ACC TAC AAG C-3′

RPVF3, 5′-GGG ACA GTG CTT CAG CCT ATT AAG G-3′

RPVF4, 5′-CAG CCC TAG CTT CTG ACC CAC GAT A-3′

The "nested" F gene specific primers were:

PPRVF1A, 5′-ATG CTC TGT CAG TGA TAA CC-3′

PPRVF2A, 5′-CTA TGA ACA GAG GGG ACA AG-3′

RPVF3A, 5′-GCT CTG AAC GCT ATT ACT AAG-3′

RPVF4A, 5′-CTG CTT GTC GTA TTT CCT CAA -3′

Control primers to check RNA quality were based on the conserved bovine beta-actin gene (Collins *et al*., 1995).

BA1, 5′-GAG AAG CTG TCG TAC GTC GC-3′

BA2, 5′-CCA GAC AGC ACT GTG TTG GC-3′

Photo-documentation. An amount of 75 µL PCR product was mixed with 2 µL of 10X loading dye (40% sucrose, 0.25% xylene cyanol & 0.25% bromophenol dye in water) was run on 1.5% agarose gel (containing 0.5 µg mL^{-1} ethidium bromide) at 100V for 0.5-1 h and visualized under UV light for the presence of bands of expected size.

RESULTS

The RT-PCR test conditions were standardized before experimental work including optimization of annealing temperature and magnesium concentration. The P gene primers (UPP1/UPP2) are universal primers for all morbilliviruses and these amplify the product of 429bp size. Simultaneously, to check RNA quality bovine β-actin gene (BA1/BA2) were used, which gave a product of 275bp. The F gene PPRV primers (F1/F2) amplify the product size of 372bp. Similarly, F gene RPV primers (F3/F4) also amplified a product of 372bp size. For nested PCR F gene amplicons was used and PPRV primers (F1A/F2A) amplified a product of 309bp, while RPV primers (F3A/F4A) amplified a product of 235bp.

DISCUSSION

The RP and PPR are some times difficult to distinguish clinically from other diseases in ruminants. Virus disease such as bovine viral diarrhea, malignant catarrhal fever and foot and mouth disease and bacterial diseases such as pasteurellosis and broncho-pneumonia (Scott *et al*., 1986; Barrett *et al*., 1993b) can have clinical signs similar to those of RP and PPR.

Morbilliviruses are a group of enveloped viruses forming a separate genus with in the family, paramyxoviridae. Two viruses with in the genus, RP virus and PPR virus cause disease in ruminants. RP is a devastating disease generally affecting large ruminants, in particular cattle and buffalo and is currently the target of a global eradication programme (Rweyemamu & Cheneau, 1995). PPR predominantly affects small ruminants, such as sheep and goats, but in Asia infection of small ruminants with both viruses in known to occur and small ruminants can transmit RPV to cattle and other large ruminants (Anderson *et al*., 1990). Previously PPRV in sheep and goats may have been attributed mistakenly to RPV and exposure of cattle to PPRV can interfere with the take of the RPV vaccine (Anderson & McKay, 1994). PPR was originally described in West Africa in 1940's, however, as RPV vaccination programmes to eliminate that disease is Asia have taken effect, PPR has been found to be more widespread than originally thought (Nanda *et al*., 1996; Shaila *et al*., 1996). Molecular techniques, such as RT-PCR and nucleotide sequencing, have improved the speed and specificity of diagnosis of these viruses and have made it possible, not only to detect morbilliviruses as a group, but also to rapidly differentiate further the individual morbilliviruses. Universal primer sets, which recognize all morbilli viruses have been developed, which are based on sequence with in the phosphor-protein (P) and nucleocapsid protein (N) genes, both of which have regions that are highly conserved across the genus. Other primer sets, based on regions of the fusion (F) protein gene, which are moderately conserved, were found to be suitable for identifying the specific viruses (Forsyth & Barrett, 1995; Shiala *et al*., 1996; Kennedy *et al*., 2000). Sequencing of the DNA amplicons from RT-PCRs has made it possible to perform phylogenetic analysis on virus isolates and by this means, three separate lineages of RPV (RPV-L1-RPVL3) and four of PPRV (PPRL1-PPRL4) have been identified (Shaila *et al*., 1996; Barrett *et al*., 1998; Dhar *et al*., 2002). RT-PCR can distinguish the individual morbilliviruses but strain or lineage differentiation requires sequence analysis, which is time consuming, expensive and labour intensive.

Two morbilliviruses induced diseases are currently targated for the global eradication, measles by World Health Organization and RP by Office International des Epizooties (OIE) and Food and Agricultural Organization. As RP disease in cattle is economically important and global vaccination programme have been implemented in order to eradicate the disease (Rweyemamu & Cheneau, 1995). Efficient, sensitive and rapid diagnostic tests like RT-PCR are required to aid the eradication programme strategies in various parts of the world. Pakistan has been declared as RP free country in March, 2007 following OIE pathway. All the samples of RP in this study were collected from 1985-1994.

The disease caused by PPRV, commonly referred to as "PPR", was first reported from west Africa in early 1940's (Gargadennec & Lalanne, 1942) and was later found in Senegal (Gilbert & Monnier, 1962) and subsequently recognized as been endemic in west and central Africa (Scott, 1981). It has also been reported in Sudan (El Hag Ali & Taylor, 1984; Taylor, 1984) and in east Africa in Kenya and Uganda (Wamwayi *et al*., 1995) and in Ethiopia (Roeder *et al*., 1994). PPR was first reported in 1987 in Southern India, where it caused epidemics for several years with out apparent further spread (Shaila *et al*., 1989; Taylor *et al*., 2002). The Arabian Peninsula, the Middle East and the remaining parts of the Indian sub-continent were swept by an epidemic of PPR in 1993-1995. Since, then the disease has remained endemic in most of these regions and on much of the Indian sub-continent (Shaila *et al*., 1996; Nanda *et al*., 1996).

These diseases are important due to their trans-boundary nature. The current RT-PCR used for diagnosis is both robust and sensitive but, although capable of differentiating between different morbilliviruses, it cannot differentiate between the lineages, which circulate in different geographical regions. Knowing the virus lineage can help pin point the origin of an outbreak when it occurs in an un-expected place (Chamberlain *et al*., 1993; Barrett *et al*., 1993a, 1998).

At present the DNA product must be sequenced to determine the virus lineage, but this can take time and not all laboratories are equipped to carryout sequence analysis. In the absence of a sequencing facility, nested PCR was used to confirm the identity of the DNA product. In RT-PCR viral RNA can be detected as early as 2 days post infection in cattle and major advantage is the ability to sequence PCR product (Anderson *et al*., 2006).

Generally RPV only causes disease in large ruminants, such as cattle and buffaloes and PPRV only in small ruminants such as goats and sheep. However, there are reports from Africa and India of RPV causing clinical and sub-clinical infection in small ruminants (Taylor, 1986), which can then be transmitted to cattle causing a more serious disease (Anderson *et al*., 1990), while PPRV is known only to cause a sub-clinical infection in cattle (Diallo *et al*., 1989).

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