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MOLECULAR DIAGNOSIS OF RINDERPEST AND PESTE DES PETITS RUMINANTS VIRUS USING TRIZOL REAGENT

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ABSTRACT

Reverse transcription polymerase chain reaction was standardized to diagnose and differentiate between rinderpest (RP) and Peste Des Petits Ruminants (PPR) viruses. The RNA of these viruses was isolated using trizol reagent. The P and N-gene primers amplified DNA of RP and PPR viruses, giving 429bp and 238bp, respectively in regular PCR. The F-gene specific primers amplified RP and PPR viruses giving DNA product of 372bp in regular PCR, while F-gene nested primers differentially amplified RPV and PPRV giving product of 309bp and 235bp, respectively in nested PCR.

Key words: Rinderpest, PPR, molecular diagnosis.

INTRODUCTION

Rinderpest (RP) is a devastating disease generally affecting large ruminants, in particular cattle and buffaloes, and is currently the target of a global eradication programme (Rweyemamu and Cheneau, 1995). Pakistan has been declared as provisionally free from rinderpest in March, 2007 following Office International des Epizooties (OIE) pathway. Peste des Petits Ruminants (PPR) predominately affects small ruminants such as sheep and goats and is characterized by high fever, ocular and nasal discharges, pneumonia, necrosis, ulceration and inflammation of mucous membrane of gestro-intestinal tract, leading to severe diarrhoea (Gibbs *et al*., 1979). Morbidity and mortality rates vary but can be as high as 100 and 90%, respectively. Mortality can approach 100% when PPR is associated with other diseases, such as capri-pox (Kitching, 1988).

Rinderpest virus (RPV) and Peste des Petits Ruminants virus (PPRV) belong to genus morbilliviruses of family paramyxoviridae (Barrett *et al*., 1991; Barrett, 1994). Diseases caused by these viruses are among the most important transboundry animal diseases (Anderson *et al.,* 2006) and some times difficult to distinguish clinically from other diseases like bovine viral diarrhoea, malignant catarrhal fever, foot and mouth disease, pasteurellosis and broncho-pneumonia in ruminants (Barrett *et al*., 1993a). Various techniques are being used to differentiate RP and PPR, like nucleic acid hybridization using either radio labeled or biotinylated cDNA probes (Diallo *et al*., 1989; Pandey *et al*., 1992), competitive (Anderson *et al*., 1991) or immuno-capture ELISAs (Libeau *et al*., 1994) or differential immuno-histochemical staining of tissue sections (Saliki *et al*., 1994) by using monoclonal and polyclonal antibodies. However, polymerase chain reaction (PCR) is extremely sensitive, specific and can detect RPV in cattle as early as two days post infection (Anderson *et al.,* 2006).

In the present study, a reverse transcription polymerase chain reaction (RT-PCR) was standardized by targeting various morbilliviruses phosphoprotein (P), nucleocapsid protein (N) and fusion protein (F), using universal and specific primer sets both in regular and nested PCRs to identify and differentiate RPV and PPRV.

MATERIALS AND METHODS

Samples

Swabs (ocular, nasal and mouth erosions), organs (lungs, liver, spleen and eye ball), tissues (whole blood and lymph nodes) and tears were collected from either dead or live sheep and goats. These animals were clinically diagnosed to be infected with PPRV.

The RP samples (spleen, lungs, liver, lymphoid tissue and occulo-nasal swabs) from cattle and buffalos used in this study were collected from 1985-1994, kept at -70ºC at National Institute for Biotechnology and Genetic Engineering (NIBGE), Faisalabad, Pakistan. Canine distemper virus (Recombitek® Distemper, Merial) and measles virus (Sanofi Pasteur, Bulgaria) were taken as positive controls, while distilled water was used as negative control.

RNA extraction

Each tissue sample (50-100 mg) was homogenized in 1 ml of Trizol reagent using glass Teflon and incubated in 1.5 ml eppendorf tube for 5 minute on a hot plate at 30°C. Chloroform (200 µl) was added to this tube, vortexed for 75 seconds and re-incubated at

room temperature (15-30°C) for 2 minutes, followed by centrifugation at 12000g for 15 minutes at 4°C. After centrifugation, the upper aqueous phase was transferred into fresh eppendorf tube and RNA was precipitated by mixing with 1 ml iso-propyl alcohol. This eppendorf tube was incubated at 30°C for 10 minutes on a hot plate, followed by second centrifugation at previous speed, time and temperature. Supernatant was discarded and RNA pellet was dissolved on vortex-shaker in 2 ml of 75% ethanol and centrifuged at 7500g for 5 minutes at 4°C. This procedure was repeated twice and RNA pellet thus obtained was air dried, suspended in 1 ml RNase free DEPC (Diethyl pyro-carbonate) treated water (concentration was adjusted to 1 mg/ml, using an optical density reading 260 nm of 25 for pure RNA) and incubated for 10 minutes at 56°C in water bath.

Histopaque®-1077 (Sigma-Alderich, Inc., St. Louis, USA), 3 ml, was added to a 1 ml conical centrifuge tube and brought to room temperature. Heparinized whole blood (3 ml) was carefully layered onto Histopaque and it was centrifuged at 400g for 30 minutes at room temperature. The upper layer was aspirated with the help of a pasteur pipette and discarded. With a second pasture pipette, the opaque interface was carefully transferred into a clean conical centrifuge tube and 10 ml isotonic phosphate buffer saline solution (PBSS) was added and mixed, followed by centrifugation at 250g for 10 minutes. The supernatant was discarded and the cell pellet obtained was resuspended in 5 ml isotonic PBSS and mixed, followed by centrifugation at 250g for 10 minutes. The supernatant was again discarded and the cell pellet was resuspended in 1 ml trizol reagent and shifted to 1.5 ml eppendorf tube. Then the same procedure was followed for all tissue samples described earlier.

Reverse transcription/cDNA synthesis

An amount of 5 µl (approximately 5 µg RNA), 2 µl of random hexa-nucleotide primers (50 ng/µl) and 3 µl of DEPC treated water was taken in eppendorf tube and incubated at 70°C for 5 minutes. This solution was cooled at room temperature for 10 minutes to allow primer annealing and centrifuged for 16-20 seconds before opening. Then 4 µl of 5X RT buffer (50 mM tris HCl (pH 8.3), 3 mM $MgCl_2$ and 75 mM KCl), 2 µl of acetylated bovine serum albumin (BSA, 0.1 mg/ml; Sigma-Alderich, Inc., St. Louis, USA), 2 µl DTT (0.1M), 1 µl dNTPs (10 mM each) and 1 µl reverse transcriptase (200 units) were added to each tube. These tubes were centrifuged and incubated at room temperature for 5 minutes and then at 37°C for 45 minutes to obtain cDNA. This product was stored at -20°C until used for PCR.

PCR amplification

cDNA was amplified using different sets of primers by PCR. For this purpose, 5 µl of cDNA was added to the following 50 µl amplification mixture: 5 µl of 10X PCR buffer, 34 µl sterile distilled water, 1 µl of 10 mM deoxyribonucleotide triphosphate, 3 µl $MgCl_2$ (25 mM), 1 µl of forward and reverse primers (10 pM each). Finally, one hot start wax bead containing Taq-polymerase was added to each 0.75 ml reaction tube. The amplification profile used was 5 min at 94°C, followed by 40 cycles at 94°C for 30 seconds (denaturation), 30 seconds at 50°C (annealing) and 45 seconds at 72°C (extension) and a final extension at 72°C for 10 minutes in a thermal cycler (Master Cycler, Eppendorf, Germany). An amount of 10 µl first-round PCR product was then added to the second-round PCR mixture with the same composition but with a different set of primers. Each PCR product (10 µl) was mixed with 2 µl of 10 X loading dye (40% sucrose, 0.25% xylene cyanol and 0.25% bromophenol dye in water) was electrophoresed at 100V for 40 minutes in 1.5% agarose gel (containing 0.5 µg/ml ethidium bromide) in 1X TBE buffer [5X stock TBE: 54.0g Trizma base, 2705g boric acid, 20 ml EDTA (0.5M, pH 8.0)]. The gel for the presence of expected bands size was visualized under ultraviolet illuminator at a wavelength of 254 nm with Eagle Eye Gel documentation system (Stratagene, USA). A known negative and a positive control were also included in each run to ensure specificity along with 100bp DNA ladder.

RESULTS AND DISCUSSION

The RT-PCR test conditions were standardized before experimental work, including optimization of annealing temperature (50-60°C) and magnesium concentration (15-30 mM). The best amplification was obtained by using 25 mM $MgCl_2$ at 50°C.

The P and N-gene primers are "universal primers" for all morbilliviruses and these amplified a product size of 429bp and 238bp, respectively in regular PCR. Simultaneously, to check RNA quality, bovine β-actin gene primers (BA1/BA2) were used in the same PCR reaction which amplified a product size of 275bp (Table 1).

The F-gene primers of both PPRV (F1/F2) and RPV (F3/F4) amplified product size of 372bp in regular PCR, while F-gene PPRV (F1A/F2A) and RPV (F3A/F4A) primers amplified a product of 309bp and 235bp, respectively in nested PCR (Table 1).

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 have been reports from Africa and India of RPV

Table 1: Primers used for amplification of target sequences of PPRV and RPV

Amplicon	Target gene	Primer sequence (5'-----3')	Amplification			Sense/ antisense[a]
			PPR	RP	Size	
Regular universal (UP)	P	**P1:** 5′-ATG TTT ATG ATC ACA GCG GT-3′	Yes	Yes	429	S
		P2: 5′-ATT GGG TTG CAC CAC TTG TC-3′				AS
Regular universal (N)	N	**N1:** 5′-ACA AAC CCA GGA TTG CTG AAA TGA T-3′	Yes	Yes	238	S
		N2: 5′-CTG AAT TTG TTC TGA ATT GAG TTC T-3′				AS
Regular bovine beta actin	BA	**BA1:** 5′-GAG AAG CTG TCG TAC GTC GC-3′	No	No	275	S
		BA2: 5′-CCA GAC AGC ACT GTG TTG GC-3′				AS
Regular PPR specific	F	**F1:** 5′-ATC ACA GTG TTA AAG CCT GTA GAG G-3′	Yes	No	372	S
		F2: 5′-GAG ACT GAG TTT GTG ACC TAC AAG C-3′				AS
Nested PPR specific	Part of F-gene	**F1A:** 5′-ATG CTC TGT CAG TGA TAA CC-3′	Yes	No	309	S
		F2A: 5′-CTA TGA ACA GAG GGG ACA AG-3′				AS
Regular RP specific	F	**F3:** 5′-GGG ACA GTG CTT CAG CCT ATT AAG G-3′	No	Yes	372	S
		F4: 5′-CAG CCC TAG CTT CTG ACC CAC GAT A-3′				AS
Nested RP specific	Part of F-gene	**F3A**: 5′-GCT CTG AAC GCT ATT ACT AAG-3′	No	Yes	235	S
		F4A: 5′-CTG CTT GTC GTA TTT CCT CAA -3′				AS

[a] S = sense; AS = antisense.

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).

Universal primers recognize all the morbilliviruses and are based on sequence within P and N gene, both of which are highly conserved regions across the genus (Barrett *et al*., 1993b). The P-gene primer sets (F1/F2 and F3/F4) are based on the regions of F-protein gene, moderately conserved and are suitable to identify specific virus in genus morbillivirus (Evans *et al.*,1994; Forsyth and Barrett, 1995; Shiala *et al*., 1996; Kennedy *et al*., 2000) in regular PCRs, while other F-gene primers (F1A/F2A and F3A/F4A) used in nested PCRs, were specific within F-gene and these primers are used to differentiate PPRV and RPV. The control primers (BA1/BA2) were used to check the quality of extracted cellular RNA and were based on the conserved regions within bovine beta actin gene (Collins *et al*., 1995). These primers amplified cellular RNA of large and small ruminants and were used to confirm the extracted cellular RNA, while for amplification of viral RNA specific primers or random hexa-nucleotide primers were used.

A positive control was included in every test as a qualitative measure of reverse transcription and PCR amplification steps. Positive controls were used to reduce any risk of cross contamination, while a negative control was included to detect any possible contamination of reagents.

The RT-PCR distinguished individual morbillivirus but strain or lineage differentiation requires sequence analysis which is time consuming, expensive and labor intensive. Presently, nested PCR was used to confirm the identity of DNA product but by knowing the virus lineage it could help to pinpoint the origin of outbreak at unexpected place (Chamberlain *et al*., 1993; Barrett *et al*., 1993a, 1998). The usefulness of this technology first proved itself in 1993 when an unusual outbreak of RPV occurred on the Russian-Mongolian border and

the lineage was identified as being of Asian origin (Barrett *et al*., 1993a).

Trans-boundary animal diseases (TADs) may be defined as those epidemic diseases which are highly contagious or transmissible and have the potential for very rapid spread, irrespective of national borders, causing serious socio-economic and possibly public health consequences. FMD, RP, PPR, Rift valley fever, Classical swine fever, African swine fever, Newcastle disease, Highly pathogenic Avian Influenza and Contagious Bovine Pleuro-pneumonia are important TADs.

Accreditation of RP freedom is a five year process which is over seen by the Office International des Epizooties (OIE). The whole system is strongly surveillance based and proving RP freedom depends on applying a portfolio of surveillance techniques which include: routine animal disease reporting and follow up, emergency reporting with follow up, village disease searching, risk focused (on prior knowledge) and random sero-surveillance studies, wild life surveillance where sufficient number of susceptible wild species make this approach. To date, RP is the only animal disease for which there exists a global program (Global Rinderpest Eradication Programme, GREP) with the aim of world wide eradication. During the next six years, FAO and OIE will need to ensure that all five stages defined for the eradication of an infectious disease have been satisfied i.e. control, elimination of disease, elimination of infection, eradication and extinction. The aim of GREP is to declare the world free of RP by the year 2010. Before this declaration can be made, a global serological survey will take place to demonstrate the lack of any circulating RP virus. The last case of RP in Pakistan was detected in October, 2000 near Karachi and the vaccination against RP was withdrawn in same year (Hussian *et al.,* 2001).

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