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DETECTION OF RINDERPEST VIRUS BY POLYMERASE CHAIN REACTION (PCR) AND SOUTHERN BLOT HYBRIDIZATION

M.S. SABER * and A.M. ABBAS **

* Faculty of Veterinary Medicine, Cairo University, Giza, Egypt.
** Genetic Engineering Unit, Veterinary Serum and Vaccine Research Institute, Abasssia, Cairo, Egypt.

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SUMMARY

Rinderpest is an economically highly important disease affecting cattle and buffalo. It's endemic throughout large parts of Africa, Asia, and the Middle East (Egypt). The reverse transcription polymerase chain reaction (RT-PCR), using phosphoprotein (P) gene specific primers set, was used to detect rinderpest virus extracted from infected VERO cells. The amplified 429 bp PCR product was subjected to digestion with restriction endonucleases enzymes (Alu I & Hae III) which cut at specific sequence sites derived from rinderpest phosphoprotein (P) gene. The PCR product served as a probe for detection of cDNA derived from infected VERO cells with rinderpest, so PCR assay when combined with southern blot hybridization hopefully will pave the way for rapid detection, monitoring, and surveillance of rinderpest infection.

INTRODUCTION

Rinderpest is an acute, febrile, highly contagious viral disease of ruminants, particularly of cattle and buffalo. The disease is characterized by inflammation, hemorrhage, necrosis, and erosion of gastro-intestinal tract accompanied by bloody diarrhea, and death (Plowright 1968).

The causative virus belongs to Paramyxoviridae family, genus morbillivirus, and shares this group with human measles virus (MV), distemper virus of dogs (CDV), peste des petits ruminants (PPRV), and phocine distemper virus (PDV) (Gibbs et al., 1979 and Mahy et al., 1988).

More recently another highly pathogenic morbillivirus of uncertain origin caused an epidemic among striped dolphins in Mediterranean (Van Bressem et al., 1993).

The rinderpest virus in enveloped and of spherical

morphology with a helical nucleocapsid. It consists of negative sense ssRNA genome of approximately 16 Kb, organized into six structural genes which encode the six structural proteins with the gene order from the 3' -end to the 5' of the genome being nucleocapsid (N), phosphoprotein (P), matrix (M), fusion (F), attachment (H), and large polymerase (L), protein genes: the non-structural proteins (V and C) are both expressed from the (P) gene (Diallo et al., 1987, Grubman et al., 1988, and Catteneo et al., 1989).

The phosphoprotein (P) gene was the fewest conserved nucleotides among genus morbillivirus. Although the overall nucleotides and protein homology of the phosphoprotein (P) genes were less than that of other genes, there were long stretches of strictly conserved nucleotides with account for cross hybridization seen within P genes among all morbilliviruses (Sheshbeeradaran et al., 1986).

The rinderpest isolation using conventional cell culture techniques is a difficult and time-consuming process that impairs the rapid and reliable demonstration of virus. Therefore we need direct and fast viral nucleic acid detection methods such as the polymerase chain reaction (PCR) (Tenovr, 1988) and hybridization technique (Haas et al., 1991).

The high sequence variability found in RNA viruses makes it difficult to design primers for reverse transcription-polymerase chain reaction amplification which will be certain to work with all new field isolates. To overcome this probl for the detection of rinderpest virus, they desig several sets of primers based on well-conser sequences in the P and F genes using rand hexanucleotide primers and aliquots of the cD were then amplified using a panel of primer to identify the virus nucleic acid in the samp (Forsyth and Barrett 1995).

In our study, we used the previously mentio techniques: PCR analysis for the detection virus specific nucleic acid, and a rinderpest gene specific cDNA probe was used identification of RP cDNA derived from infe VERO cells.

MATERIAL AND METHODS

Preparation of viral mRNA:

The rinderpest virus (RPV) Kabete "O" strain propagated in primary bovine kidney monolayers in Joklik's modified med supplemented with 10% calf serum. The were infected with RPV at a multiplicit infection of 0.001-0.05 $TCID_{50}$/cell. at 48 hr infection, cells were harvested for extraction. Viral RNA was isolated from inf cells by a modification of the guanidi thiocymate procedure (Chirgwin et al., poly (A)+ RNA was purified using oligo cellulose column according to (Aviv and 1972).

Construction of RP cDNA:

RP cDNA was constructed and supplied gratefully by Yamanaka et al., (1988) using ploy (A)+ RNA template isolated from RP virus-infected cells. Double stranded cDNA was synthesized by a modification of the RNAse H procedure using random primered method for synthesis of RP cDNA (Gubler and Hoffman 1983).

PCR amplification:

Morbillivirus specific sequences were amplified using broadly reactive morbillivirus as (P) gene specific primers to amplify a 429 bp DNA fragment which spanned the RNA editing site (Barrett et al., 1993). The PCR primer set was designed according to Bellini et al. (1985) and was mentioned in Table (1). The primers were synthesized by (Protein Department, Davis, California, USA).

The amplification reaction of PCR was carried out as previously described by (Hass et al., 1991). The reaction mixture was done in 50µl total volume 1Pmole primers conc.., 1.5 mM $MgCl_2$, 200-400. µm nucleotides mix (dNTPs), 100-200 ngm of random primered RP cDNA, 2.5 units of Taq polymerase enzyme and the final volume was completed by double distilled water. The program of thermal cycler was done as follows: 1 cycle: denature at 94°C for 1 min. followed by 35 cycles: denature at 94°C for 1 min; annealing at 55°C for 1.5 min; extension period at 72°C for 1.5 min., and a final extension at 72°C for 10 minutes for 1 cycle.

Table (1): The primers sequence for amplification of 429 bp DNA PCR product of phosphoprotein (P) gene of rinderpest virus .

primer Name	Nucleotide position in RNA sense	primers sequence
Forward primer (2246)	400 - 419	5`-ATGTTTATGATGATC ACA GC GGT-3`
Reverse primer (2247)	808 - 828	5` ATT GGG TTG CAC CAC TTG TC-3`

Analysis of the amplified DNA:

The amplified product was separated by electrophoresis through 1.5% TAE-agarose gels and stained with ethidium bromide using standard DNA molecular marker (Ikb NDA Iadder, Cat. No. 15615-016, Gibco-BRL, USA).

PCR product purificaiton:

The PCR product was purified on 1.5% low melting agarose using (Magic^{TM} PCR prep DNA purification Kit, Cat No. A7170, Promege, USA).

Digestion of the amplified 429 bp DNA with restriction enzymes:

To confirm the specificity of the amplified DNA fragment, we used specific restriction endonucleases enzymes (Alu 1 and HaeIII) to cut at specific sites inside the phosphoprotein (P) gene of rinderpest virus.

A) Digestion of PCR product with ALu I restriction enzyme:-

The digestion reaction was done in a total volume of 20 ul: 2ul 10 X reaction buffer, 5 ul of purified PCR product, 12 ul distilled water, 1 ul of Alu I enzyme (Cat. No. 239275, Boherniger Mannheim, USA). The reaction was incubated at 37°C for 1hr. The digested products were separated on 3% low melting agarose (Gbco-BRL company, USA) and stained with ethidium bromide using standard DNA molecular marker (100 pb DNA ladder, Cat. No. 15628-019, Gibco BRL, USA).

B) Digestion of the PCR product with Hae I enzyme:

The reaction was carried out as the following: 2 of 10X reaction buffer, 5ul of purified DN product, 12ul of distilled water, and 1 ul o HaeIII enzyme (10u/ul) (Cat. No. 68393 Boherniger Mannheim, USA). The reaction w incubated at 37°C for 1 hr. It was separated on 3 Nusieve low melting agarose (Gibco-BR Company, USA) and stained with ethidiu bromide.

Southern blot hybridization technique:

The random primed cDNA of rinderpest w blotted to a presoaked (20 X SSC) Nylo membrane (Schleicher & Schull Company, USA The DNA was denatured with alkali (0.2M N OH, and 1.2 M NaCl) and neutralized with 0.4 Tris-HCl pH 7.6, Finally they were washed wi 2% SSC. The denatured DNA was permanentl fixed with ultraviolet crosslinkers (Stratalinke Stratagene Company, USA).

a- Probe preparation:

The amplified PCR product was prepared as specific rinderpest probe using (random primer DNA labeling Kit, Cat No. 1004 760, Bohernig Mannhinem, USA). Briefly, 3ul DNA P product was denatured by heating for 10 minu at 95°C and subsequent cooling on ice. A

following to a microfuge tube on ice and make up to a final volume 20ul: 3ul dATP, dGTP, dTTP mixtures, 5ul of 50 μ ci ^{32}P dCTP (Amersham Company, USA), 1ul Klenow enzyme, and 8ul double distilled water. The reaction was incubated at 37°C for 30 minutes. It was stopped by heating at 65°C for 10 minutes.

b- Prehybridization/hybridization steps:

Prehybridization was done in a solution containing 50% deionized formamide, 5X SSC, 50 mM sodium phosphate buffer pH 6.5, 5X Denhardt's solution, and 300 ngm denatured salmon sperm DNA per ml. The nylon membrane filters were sealed with poly propylene bags and incubated at 42°C for 4 hr in a shaking water bath. Hybridization was done in a solution containing 50% formamide, 5X SSC, 2X Denhardt's solution, 25mM sodium phosphate buffer pH 6.5, 10% dextran sulphate, and 50 ng denatured salmon sperm DNA per ml. The DNA probe (2ng/ml) was denatured by heating at 95°C for 10 min and added to the hybridization solution. The incubation was performed overnight at 42°C in a shaking water bath. After removal of the hybridization mixture, the filters were washed three times for 10 min each in 2 X SSC, 0.1% SDS at 50°C, again for 10 minutes each wash. The filters were dried and autoradiography was carried out at- 80°C with VAR-5X- ray film (Eastman Kodak, USA).

RESULTS AND DISCUSSION

Standard methods of virus isolation and differential neutralization are very lengthy procedures to carry out and give variable results depending on the quality of sample, hence the need for effective techniques which are rapid and independent on the isolation of infectious virus. Recent advances in molecular biology have led to the development of techniques such as RT-PCR method is simpler to use than hybridization and more sensitive than ELISA (Forsyth and Barrett 1995).

There is no abundant literature on the isolation of nucleic acids, particularly of the morbillivirus RNA for use in the PCR assay (Jackson et al., 1990).

For successful detection, at least a part of the viral RNA must be present in intact form. The ubiquitous presence of RNases in tissues and the necessity of carrying out a reverse transcription step make the enzymatic amplification of RNA more difficult than that of DNA (Haas et al., 1991).

In the present paper, we described fast rinderpest nucleic acid detection methods such as PCR and southern blot hybridization technique. The results of the reverse transcription polymerase chain reaction (RT-PCR), using primers set designed from the highly conserved sequence data of the morbillivirus phosphoprotein (P) gene, to detect

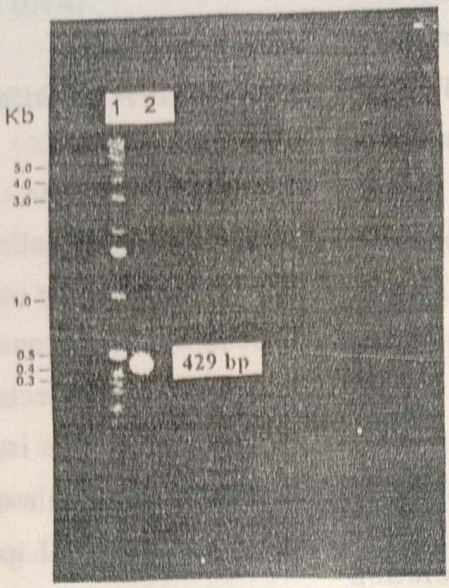

Fig. (1) : Agarose gel electrophoresis of ampilified PCR product derived from the phosphoprotein (P) gene of rinderpest virus. *lane (1)*:1Kb DNA Ladder as a marker. *lane (2):* A positive 429 bp PCR product *lane (3):* No amplified DNA product was detected as a negative control.

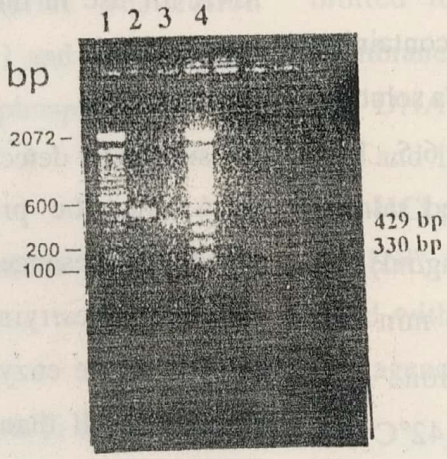

Fig. (2): Digestion of the ampilified 429 bp PCR product with Hae III restriction enzyme.. *Lane (1)* :100 bp DNA ladder as standard marker. *Lane (2)* : Ampilified 429 bp PCR product.(undigested DNA fragment as a control). *Lane(3)* :The digested PCR product with Hae III enzyme.

rinderpest virus extracted from infected VERO cells, were shown in Fig (1). They showed that a positive 429 bp PCR product was seen in lane (2), no amplified DNA product was detected in the Lane (3) as a negative control., while in lane (1) showed 1Kb DNA Ladder as a standard marker.

These results agreed with those obtained by (Forysth and Barrett 1995) who used random hexanucleotides primers and aliquots of the RP and cDNA were then amplified using a panel of primers set, based on well-conserved sequences of P and F genes, to identify the virus nucleic acid.

To confirm the specificity of the amplified PCR product, it was purified and digested with restriction enzymes. The digestion of 429 bp amplified DNA fragment with Alul enzyme gave two fragments (246 and 183 bp respectivley), while the digestion with Hae III enzyme gave three fragments (330, 67, and 32 bp) respectively as shown in fig. (2). The results from the digestion with restriction enzymes (Alu 1 & Hae III) indicated that the amplified PCR product was specific for the phosphoprotein (P) gene of RPV. These results agreed with those data obtained by the restriction mapping of phosphoprotein (P) gene of RPV from (Gene bank sequence, using the computer program of California Davis University, Davis, USA).

For more confirmation of the specificity of the amplified PCR product, southern blot analysis was performed. The cDNA derived from infected VERO cells with rinderpest virus were blotted to nylon membrane and hybridized with ^{32}P-labeled 429 bp PCR product which used as a specific probe for Rinderpest virus. The strong positive signals indicated the specificity of this probe to rinderpest virus, which no signals were detected when we used cDNA derived from uninfected VERO cells as a negative control. These resutls agreed with Taylor et al. (1990) who mentioned that the hybridization technique used to clearly identify the virus involved in outbreak. Also these results were similar to those obtained by (Haas et al., 1991) who used phosphoprotein (P) gene specific cDNA served as a probe detection of phocid distemper virus as a member of genus morbillivirus.

In conclusion, we recommend in the future work the use of PCR assay and virus specific cDNA probes in hybridization (southern blot analysis) for rapid detection of rinderpest virus, monitoring and surveillance of RP infection.

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