Journal of Animal and Veterinary Advances 2 (12): 671-674, 2003
© Grace Publications Network, 2003

Application of RT-PCR for Detection of African Isolates of Palyam Orbiviruses Serogroup in Cell Culture

[1]Abdel Rahim E. Karrar, [1]Mohamed A. Abdalla, [2]Ali M. Majid, [3]Mohammed M. M. Salih, [3]Salah M. M. Elamin, [4]Bennie I. Osburn and [1]Imadeldin E. Aradaib
[1]Molecular Biology Laboratory (MBL), Department of Medicine, Pharmacology and Toxicology, Faculty of Veterinary Medicine, P.O . Box 32 Khartoum North, Sudan; [2]National council for Research and [3]Central Veterinary Research Laboratory, Ministry of Science and Technology, Republic of Sudan; [4]School of Veterinary Medicine, University of California, Davis, 95616

Abstract: A reverse transcriptase (RT) polymerase chain reaction (RT-PCR)-based assay, for detection of African isolates of palyam virus ribonucleic acid (RNA) in cell culture, was developed. A pair of oligoribonucleotide primers (pal1 and pal2), selected from genome segment 3 of Chuzan virus, an isolate of the palyam serogroup, was used as a target for PCR amplification. Using RT-PCR, the pair of primers (pal1 and pal2) resulted in amplification of a 660-bp product. RNA samples from African isolates of palyam virus serogroup, propagated in cell cultures, were detected by this RT-PCR-based assay. Amplification product was not detected when the palyam RT-PCR-based assay was applied to RNA from, closely related orbiviruses, bluetongue virus (BTV) and epizootic hemorrhagic disease virus (EHDV); total nucleic acid extracts from uninfected Vero cells. The described RT-PCR-based assay provides a rapid, sensitive and specific method for detection and differentiation of palyam serogroup of orbiviruses in cell culture.

Key words: Orbiviruses, serogroup, palyam, VP3, PCR

Introduction

The prototype palyam virus, a double stranded RNA virus, is a member of the orbivirus genus in the family Reoviridae (Borden *et al.*, 1971). The virus has a genome composed of 10 double-stranded (ds) RNA (dsRNA) segments. The genome segments code for the viral proteins (Bodkin and Knudson, 1985). Palyam virus is related to bluetongue virus (BTV) and epizootic hemorrhagic disease virus (EHDV). The virus has been isolated from sentinel cattle herds in Australia, Asia and Africa (Gorman, 1979; Mohammed and Mellor, 1990 and Gorman, 1992). There are ten distinct serotypes of the palyam serogroup, distributed world wide, and differentiated by serum neutralization test (Gorman., 1992). In Sudan, five untyped isolates of palyam virus serogroup are enzootic (Mohamed *et al.*, 1999 and Abdalla *et al.*, 2000). The isolates were recovered from a healthy sentinel cattle herd at the University of Khartoum farm, Shambat, Central Sudan (Mohammed and Mellor, 1990). In previous report, we were able to demonstrate that at least three isolates are serotypically distinct as determined by serum neutralization test (Mohammed *et al.*, 1987). They have different profiles of their ds RNA genome segments on polyacrylamide gel electrophoresis (Mohammed *et al.*, 1999). Whereas cattle and goats are susceptible to infection, association of palyam virus with clinical disease in sheep is yet to be reported. Clinical manifestations of palyam virus serogroup infection in cattle and goats include inappetence, abortion and fetal malformation in pregnant cattle (Swaneopoel and Blackburn, 1976). Recently, Chuzan virus, a member of the palyam orbiviruses serogroup, was isolated from apparently healthy sentinel cattle in Japan. Subsequently, Chuzan virus was described as a cause of Chuzan disease characterized by congenital abnormalites of cattle (Yamakawa *et al.*, 1999). The economic impact of most orbiviruses infections is mainly attributed to clinical disease in susceptible animal populations and restrictions on the international trade of livestock and associated germplasm (Gorman, 1992; Osburn *et al.*, 1994; Aradaib *et al.*, 1994 and Aradaib *et al.*, 1995). The disease potential of the Sudanese isolates of palyam serogroup of orbiviruses reains unknown. However, reduced productivity and reproductive performance have greater ecomomic effect than overt clinical disease (Aradaib *et al.*, 2003 and Abdalla *et al.*, 2000). Segment 3 (VP3) of the virus was reported to be the most conserved gene among cognates of different members of palyam virus serogroup (Yamakawa *et al.*, 1999). Therefore, it was suggested that a fragment of this genome could be targeted for detection of palyam virus serogroup, in cell culture, using a nested RT-PCR amplification technology.

In the present study, we described a simple, rapid, and sensitive and specific assay for detection of African isolates of palyam orbiviruses serogroup, based on VP 3 genome sequence analysis of Chuzan virus.

Materials and Methods

Cell culture and Virus Propagation: The Sudanese isolates of palyam virus serogroup Su 48, 3838, 3843, 3863 and GP18; South African isolate of palyam virus; bluetongue virus (BTV) serotype 1, 2, 4, and 16; epizootic hemorrhagic disease virus (EHDV) serotypes

1, 2 and 4; were obtained from the Faculty of Veterinary Science, University of Khartoum, Sudan. The viruses were isolated and processed as described previously (Aradaib *et al.*, 1995). All viruses were propagated on confluent monolayers of Vero cells. The infectious material was harvested and centrifuged at 1,500 x g for 30 min and the cell pellet was used for the dsRNA extraction.

Extraction of Viral Nucleic Acid from Infected Cell Culture: The palyam virus isolates, the EHDV and the BTV dsRNAs were extracted from the infected cells as previously described (Aradaib *et al.*, 1994). Total nucleic acid was ethanol-precipitated. Viral dsRNA was purified by differential lithium chloride ($LiCl_2$) iprecipitation, and resuspended in 100 ul double distilled water, and quantified using a spectrophotometer at 260 nm wavelength.

Primer Selection and Synthesis of the Probe: Primers (pal1 and Pal2) were selected from the published sequence of genome segment 3 of Chuzan virus (Yamakawa *et al.*, 1999) and used for the synthesis of the RT-PCR amplification product. Pal1 included bases 61-80 of the positive sense strand of genome segment 3: (5)- AGCTCCCTATTTGGATGGTG-(3). Pal2 included bases 701-720 of the complementary strand: (5)-CTCTGTATTACGGCTTGCTG-(3). RT-PCR using primers Pal1 and Pal2 would result in a 660-bp product. All primers were synthesized on a DNA synthesizer (Milligen/Biosearch, a division of MilliporeBurlington, MA) and purified using oligo-pak oligonucleotide purification columns (Glen Research Corporation, Sterling, VA.) as per manufacturer's instructions.

Reverse Transcriptase Polymerase Chain Reaction: The protocol for RT-PCR based detection assay was performed basically as described previously (Aradaib *et al.*, 1994). The thermal cycling profiles were as follows: a 2-min incubation at 95 °C, followed by 40 cycles of 95 °C for 1 min, 55 °C for 30 sec and 72 °C for 45 sec, and a final incubation at 60 °C for 10 min. Thermal profiles were performed on a Techne PHC-2 thermal cycler (Techne, Princeton, NJ.). Following amplification, 20 microliters from each PCR reaction containing amplified product were loaded onto gels of 1.5% SeaKem agarose (FMC Bioproduct, Rockland ME) and electrophoresed. The gels were stained with ethidium bromide, and the PCR products were visualized under UV light.

Results

The Palyam RT-PCR-based assay afforded sensitive and specific detection of all isolates of palyam serogroup of orbiviruses. The specific 660-bp PCR product was visualized on ethidium bromide-stained gel from as little as 0.1 pg of RNA from Sudanese GP 18 isolates (Fig.1).

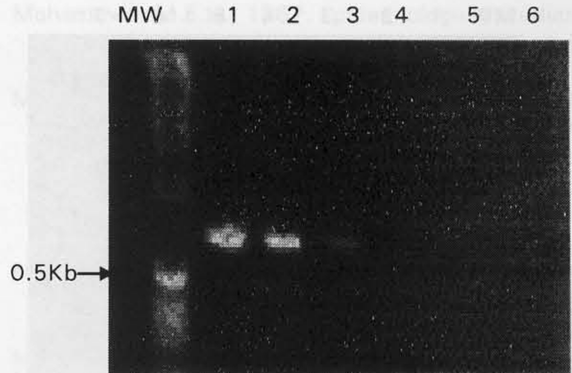

Fig. 1: Sensitivity of the reverse transcriptase polymerase chain reaction (RT-PCR) for detection of the 660 bp-PCR product from Sudanese isolate of palyam virus (isolate GP18). Lane MW: molecular weight marker; Lanes 1-5: (GP18) 100 pg, 10 pg, 1.0 pg, 100 fg, 10 fg of GP18 RNA, respectively. Lane 6: Non infected Vero cells total nucleic acid extract

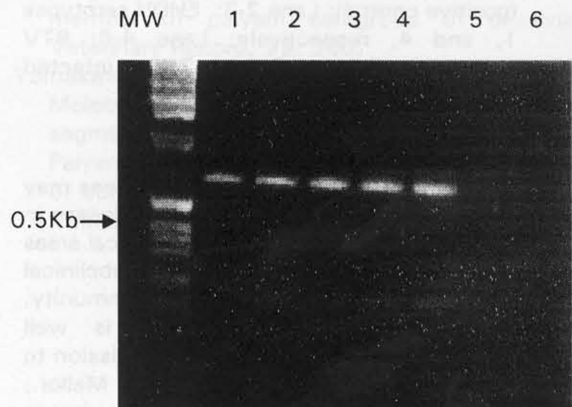

Fig. 2: The specific 660 bp-PCR product from 1.0 pg RNA of African isolates of palyam virus serogroup. Lane MW: molecular weight marker; Lane 1-4: GP18 ; 3838; 3843; 3856; Lane 5: South African isolate of palyam virus; Lane 6: Non infected Vero cell total nucleic acid extract

The 660-bp specific PCR product was detected from 1.0 pg of RNA target in all isolates by ethidum bromide-stained agarose gel electrophoresis including the Sudanese and South African virus isolates (Fig 2). The amount of 1.0 ng RNA from epizootic hemorrhagic disease virus (EHDV) serotypes 1 and 2; bluetongue virus (BTV) serotypes 1, 2, 4, and 17; and total nucleic acid extracts from uninfected Vero cells failed to demonstrate the specific 660 bp-PCR product (Fig.3). All palyam viruses which were PCR positive were also positive by conventional virus isolation on vero cell culture.

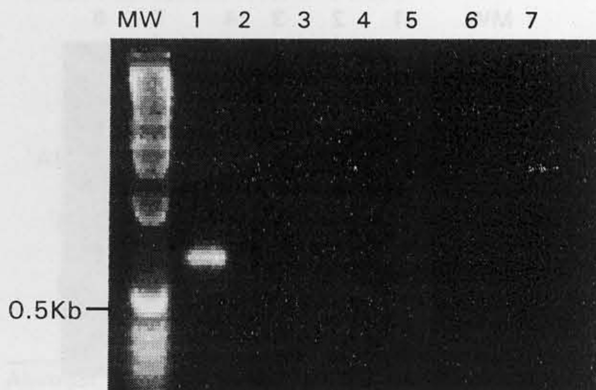

Fig. 3: Specificity of the RT_PCR for detection of palyam serogroup of orbiviruses RNA. Amplification product was not detected from a relatively high concentration of 1.0 ng of RNA from EHDV serotype 1, 2 and 4; and BTV serotype 1, 2, and 17; or total nucleic acid extracts from Vero cells. Lane MW: molecular weight marker; Lane 1: 1.0 pg of RNA from PG18 isolate of palyam virus (positive control); Lane 2-3: EHDV serotypes 1, and 4, respectively; Lane 4-6: BTV serotype 1, 2, and 17; Lane 7: non infected Vero cell total nucleic acid extract

Discussion

Members of the Palyam serogroup og orbiviruses may cause an infectious non contagious clinical disease in cattle (Swanepoel and Blackburn, 1976). In focal areas of endimicity, however, the virus produces subclinical infection, which could be attributed to herd immunity. In general, viremia in infected cattle is well documented, providing virus for insect transmission to more susceptible ruminants (Mohammed and Mellor., 1990). In the Sudan, the presence of palyam virus is of concern to dairy producers and wildlife managers because of a possible epizootic among susceptible animal populations. In addition, international movement of livestock and/or their associated germplasm may be restricted unless the animals are certified free of infection by virus isolation or serology (Osburn *et al.*, 1994).

Diagnostic methods currently applied for detection of palyam virus infection include serology and virus isolation. Serology is useful in epidemiologic studies to identify previous infection. The agar gel immunodiffusion (AGID) test, currently used as standard serologic test, is complicated by cross reactions between all members of the orbivirus serogroup (Aradaib *et al.*, 1995). The problems associated with the use of AGID test have been solved in regard to BTV serogroup-specific detection (Osburn *et al.*, 1992) and for EHDV serotype-specific detection (Aradaib *et al.*, 1994) by using monoclonal antibodies (MAb) in competitive ELISA (cELISA) technique. Conventional virus isolation is tedious, time consuming, labor intensive and expensive. To address these problems, specific complementary RNA probes derived from different genome segments have been developed (Bodkins Knudson, 1985). Recently, we have reported on application of reverse transcriptase (RT) polymerase chain reaction (RT-PCR) amplification technology for detection of BTV and EHDV, closely related orbiviruses, in cell cultures and clinical samples. These RT-PCR assays proved highly sensitive and specific method for simultaneous detection and differentiation of BTV and EHDV viral RNAs (Aradaib *et al.*, 1994; Aradaib *et al.*, 1995 and Aradaib *et al.*, 2003). In the present study, we described the first report on the application of a rapid reproducible, sensitive and specific assay for detection of palyam virus serogroup using RT-PCR amplification technology.

The RT-PCR assay using primers derived from genome segment 3 of Chuzan virus, which codes for VP3, reproducibly and specifically detected all the Sudanese and the South African isolates of palyam viruses in cell cultures. Selection of the primers was based on the observation that the VP3 genome has the most conserved nucleotide sequences from among cognates of palyam virus serogroup (Yamakawa *et al.*, 1999). The specificity studies indicated that the specific 660-bp PCR products were not amplified even from a relatively high concentration of 1.0 ng of RNA from BTV serotypes 1, 2, 4, 16 and 17; EHDV prototype serotype 1 and 2 ; or total nucleic acid extracts from uninfected Vero cell controls, under the same stringency condition described in this study. This RT-PCR-based assay could serve as a supportive diagnostic assay to the time consuming and cumbersome conventional virus isolation laboratory procedure. The rapidity, sensitivity and specificity of the RT-PCR assay would greatly facilitate detection of palyam virus infection during an outbreak of the disease among susceptible ruminants.

Conclusion

This palyam RT-PCR assay, using primers derived from VP3 genome segment of Chuzan virus provides a simple, rapid, sensitive and specific diagnostic method for detection of African isolates of palyam orbiviruses serogroup in cell culture.

Acknowledgments

This work also was supported by special fund from the Vice Chancellor office, University of Khartoum (U.of K), Sudan and the Ministry of Science and Technology, Republic of the Sudan, contract number (MOST-1).

References

Abdalla, M. A., R. A. Omer, A. E. Karrar, M. E. H. Mohammed, I. E. Aradaib and B. I. Osburn, 2000. Experimental palyam virus infection of Nubian goats with Sudanese isolates of palyam serogroup of orbiviruses. Sudan J. Vet. Sci. Anim. Husb., 39: 116-121.

Aradaib, I.E., G.Y. Akita and B.I. Osburn, 1994. Detection of epizootic hemorrhagic disease virus serotypes 1 and 2 in cell culture and clinical samples using polymerase chain reaction. J. Vet. Diag. Inves., 6: 143-147.

Aradaib, I.E., G.Y. Akita, J.E. Pearson and B.I. Osburn, 1995. Comparison of polymerase chain reaction and virus isolation for detection of epizootic hemorrhagic disease virus in clinical samples from naturally infected deer. J. Vet. Diag. Inves., 7: 196-200.

Aradaib, I.E., W.L. Smith, B.I. Osburn and J.S. Cullor, 2003. A multiplex PCR for simultaneous detection and differentiation of bluetongue and epizootic hemorrhagic disease viruses. Comparative Immunology, Microbiology and Infectious diseases 26: 77-87

Bodkin, D.K. and D.L. Knudson, 1985. Sequences relatedness of palyam virus genes to cognate of the palyam serogroup viruses by RNA-RNA blot hybridization. Virology, 143: 55-62.

Borden, E.C., R.E. Shope and F.A. Murphy, 1971. Physicochemical and morphological relationships of some arthropod-borne viruses to bluetongue virus-a new taxonomic group. Physicochemical and serological studies. J. of General Virology, 3: 261-271.

Gorman, B.M., 1992. An overview of the orbiviruses, in bluetongue, African horse sickness and related Orbiviruses, ed. Walton TE, Osburn BI, CRC press, Boca Raton, Fl, 335-347.

Gorman, B.M., 1979. Variation in Orbiviruses. J of General virology , 44: 1-15.

Hammami, S. and B.I. Osburn, 1992. Analysis of genetic variation of epizootic Hemorrhagic disease virus field isolates by coelecterophoresis of their double stranded RNA segments. Amer. J. Vet. Res., 63: 636-642.

Mohammed, M.E.H., 1987. Epidemiological studies on some Sudanese Arboviruses. Ph.D. thesis, University of Reading. United Kingdom.

Mohammed, M.E.H., A.E. Karrar, K.E.E. Ibrahim, M.M. Mukhtar and B.I. Osburn and I.E. Aradaib, 1999. RNA genome profiles of Sudanese Isolates of palyam virus serogroup using agrose gel and polyacrylamide gel electrophoresis (SDS-PAGE). The Sudan J. Vet. Sci. Anim. Husb., 38: 37-48.

Mohammed ,E.H.M. and P.S. Mellor, 1990. Further studies on bluetongue-related Orbiviruses in the Sudan. Epidemiology and infection, 105: 619-632.

Mohammed, M.E.H. and W.P. Taylor, 1987. Infection with bluetongue and related orbiviruses in the Sudan detected by the study of sentinel calf herds. Epidemiology and Infection, 99: 533-545.

Osburn, B.I., I.E. Aradaib and C. Schore, 1994, Comparison of Bluetongue, and epizootic hemorrhagic disease complex. Proceedings of the 13th international symposium of the world association of Veterinary Microbiologist, Immunologist and Infectious Disease Specialist, perugia, Montova, Italy.

Swanepoel, R. and N.K. Blackburn, 1976. A new member of palyam serogroup of orbiviruses. Veterinary Record, 99: 360.

Yamakawa, M., S. Furuuchi and Y. Minobe, 1999. Molecular characterization of double-stranded RNA segments encoding the major capsid proteins of a Palyam serogroup orbivirus that caused an epizootic of congenital abnormalities in cattle. J. Geneneral Virology, 80: 205-208.