Detection of Palyam Virus dsRNA in Blood Samples from Experimentally Infected Goats Using RT-PCR

Abdalla*, M. A; Elamin,S.M.M.; Karrar,***A.E.; Mohammed E.H. M.;*** Idris**, S. H.; Hago***,B.E. Salih**,M. M. and Aradaib***, I. E.**

*** College of Veterinary Medicine and Animal Production, Sudan University. P.O. Box,204 32 Khartoum North, Sudan.**

****Central Veterinary Research Laboratory, P.O. Box, 8067 Amarat, Khartoum, Sudan.**

***** Faculty of Veterinary Medicine, P.O . Box 32 Khartoum North, Sudan.**

Summary

A reverse transcriptase (RT) polymerase chain reaction (RT-PCR)-based assay was evaluated for detection of palyam virus ribonucleic acid (RNA) in blood samples from experimentally infected goats. A pair of olignucleotide primers (pml and pm2). selected from genome segment 3 of Chuzan virus was used as a target for PCR amplification. Using the designed primers, the RT-PCR resulted in amplification of a 350-bp product. Palyam dsRNAs

from experimentally infected goats 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 or whole blood from non-infected goats. The described RT-PCR-based assay could provide a rapid diagnostic method for detection of palyam virus.

Corresponding author:

Imadeldin Aradaib

Molecular Biology Laboratory (MBL), Department of Medicine, Pharmacology and

Toxicology, Faculty of Veterinary Medicine, P.O . Box 32 Khartoum North, Sudan.
E-mail: aradaib@yahoo.com

Introduction

The prototype palyam virus, a double stranded RNA (dsRNA) virus, is a member of the orbivirus genus in the family Reoviridae (Borden et al., 1971; German ;1979). The virus has a genome composed of 10 dsRNA segments. The genome segments code for the viral proteins (Bodkin and Knudson, 1985). 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 enzootic (Mohamed et al., 1999; 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 previously it was demonstrated that at least three isolates are serotypically distinct as determined by serum neutralization test. They have different profiles of their dsRNA genome segments on polyacrylamide gel electrophoresis (Mohammed et al., 1999). Clinical manifestations of palyam virus serogroup infection in cattle and goats include inappetence, abortion and fetal malformation in pregnant cattle (Swanepoel and Blackburn, 1976). The disease potential of the Sudanese isolates of palyam serogroup of orbiviruses remains unknown. However, reduced productivity and reproductive performance have greater economic effect than overt clinical disease (Aradaib et al., 2003; Abdalla et al; 2000). Segment 3 (VP3) of the virus was reported to be the most conserved genome segment among cognates of palyam virus serogroup (Yamakawa et al., 1999). Therefore, it was suggested that this genome segment could be targeted for detection of palyam virus serogroup, in cell culture, using RT-PCR amplification. In the

present study, we described a simple, rapid, sensitive and specific assay for detection of palyam virus in experimentally infected goats.

Materials and Methods

Experimental animals

Four 6-8-months-old Nubian goats were purchased and after repeated clinical examinations for evidence of clinical disease, each goat was subjected to virological and serological examination to eliminate the possibility of palyam virus infection. The goats were found to be healthy and free of infection. Four goats were inoculated with 2 ml of palyam virus at a dose of $1x10^6$ TCID50/ml. Six goats were injected with 2 ml of growth media (placebo) and kept as non infected controls. The inoculation of the virus was by subcutaneous injections in the shaved flank region. During the course of the experiment the animals were housed in insect-prove enclosures and were fed a ration of concentrate and hay with free access to water.

Blood samples

Heparinized blood samples were collected from the jugular vein for virus isolation (VI) and detection of virus nucleic acid sequence by PCR. Processing of the blood samples for VI was as described previously (Aradaib et al., 1994). Briefly, the blood cells were washed twice with phosphate buffered saline (PBS) containing 100 units penicillin and 200 units meg streptomycin/ml. The washed blood cells were restored to the original volume with 2mM Tris
buffer pH 8.0 to lyse the blood cells.

Cell culture

Vero cells were prepared in minimal essential medium (MEM) containing 100 units penicillin/ml and 100 mg streptomycin/ml, 10% tryptose phosphate broth and 10% foetal bovine serum (FBS) that was heat inactivated at 56 C for 30 minutes. Cell

cultures were incubated at 37 C and in humidified with 5% carbon dioxide until confluent monolayers were obtained (usually 2-3 days).

Virus Isolation and Identification

The vero cell monolayers were inoculated with lysed blood diluted 1:10 in MEM. After incubation at 37C for 1 hoor, the inoculated cell cultures were supplemented with MEM containing 2%fetal bovine serum (FBS). The cell cultures were again incubated at 37C and observed daily until cytopathic effect was 80%complete . cultures with no cytopathic effect were blind passaged . all cytopathic agents wereidentified by serum neutralization test (Aradaib et al .,1994) . the remaining lysed blood samples were stored at 4C for further analysis by PCR assay (Aradaib et al .,1995).

Extraction of Viral Nucleic Acid

The palyam virus, the EHDV and the BTV ds RNA s were extracted from the infected cells as previously described (Aradaib et al .,1994). Totalnucleic acid was resuspended in 100 ul double distilled water, and quantified using a spectrophotometer at 260 nm wavelength.

Primer Selection

Primers (pm1 and pm2) were selected from the published sequence of genome segment 3 of Japanese palyam virus (Yamakawa et al., 1999) pm 1 included bases 161-180 of the positive sense strand of genome segment : (5) - CCAGGAATTAGCAACACACGC-(3).

Pm2 included bases 491-510 of the complementary strand: (5)-AATGCATCTAACGCCGCAAC-(3). The RT–PCR using primers pm 1 and pm 2 would result in a 350 –bp PCR product.

1 primers were synthesized on a DNA synthesizer (Milliigen/ Biosearch, a division of Millipore Burlington,MA). Purified using oligo- pak oligonucleotide purification columns (Glen

Research corporation, Sterling, VA.) as per manufacture's instruction.

Reverse Transcriptase Polymerase chain Reaction (RT-PCR)

The protocol for RT-PCRbased assay was performed basically as described previously (Aradaib et al., 1994). The thermal cycling profiles were follow: 2-minute incubation at 95C, followed by 40 cycles of 95C, for 1 mint, 55C, for 30 sec and 72C, for 45 sec and a final incubation at 72C, for 10 min, thermal profiles were performed on a technePHC-2 thermal cycler (techne, Princeton, NJ.). Following amplification, 15 microliters from each PCR reaction containing amplified product were loaded onto gels of 1.5% Seakem agrose (FMC Bioproduct, rockland ME) and electrophoresed. The gels were stained with ethidium bromide, and the PCR products were visualized UV light.

Results

The specific 350-bp PCR product was visualized on ethidium bromide-stained agrose gel (Fig. 1). The amount of 1.0 ng RNA from epizootic hemorrhagic disease virus (EHDV) serotypes 1; bluetongue virus (BTV) serotype 1; and total nucleic acid extracts from uninfected vero cells failed to demonstrate the specific 350-bp product (Fig. 2).

Discussion

Infection with palyam virus in cattle and goats is usually subclinical particularly in focal areas of endemicity. However, viremia in infected ruminants is well documented, providing virus for insect transmission to more susceptible ruminants (Mohammed and Mellor., 1990; Abdalla et al., 2000). In Sudan, the presence of palyam serogruop of orbiviruses is of concern to dairy producers and wildlife managers because of a possible epizootic among susceptible livestock and wild ruminants. Diagnostic methods currently applied for detection of palyam

virus infection include serology and virus isolation. Serology is useful in epidemiological studies to identify previous infection. 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 (Bodkin and Knudson, 1985).

Members of the Palyam serogroup of 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 (Mohammed and Mellor., 1990). In the present study, we described a simple, rapid reproducible, sensitive and specific assay for detection of palyam virus serogroup in blood samples from experimentally infected goats using RT-PCR amplification technology.the RT-PCR assay reproducibly and specifically detected palyam viruses in all experimentally infected goats: 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 350-bp PCR products were not amplified even from a relatively high concentration of 1.0 ng of RNA from BTV serotypes 1; EHDV prototype serotype 1; 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 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.In conclusion, this palyam RT-PCR assay, using primers derived from VPS genome segment of provides a simple, rapid, sensitive and specific

diagnostic method for detection of palyam virus in susceptible ruminants.

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Figure 1.

Detection of the 350 bp-PCR product from the experimentally infected goats using palyam virus (isolate GP18).

Lane MW: molecular weight marker; Lanes 1-4: blood samples from experimentally infected goats. Lane 5: Total nucleic acid extracted from non infected Vero cells.

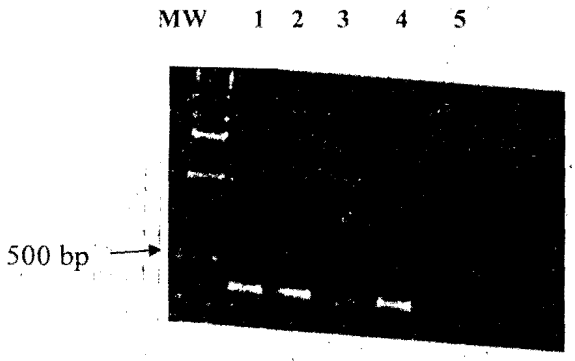

Figure 2.

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; and BTV serotype 1; 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: EHDV serotypes 1; Lane 3: BTV serotype 1; Lane 4: non infected Vero cell total nucleic acid extract; Lane 5: totao dsRNA extracted from non-infected goats.