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A Single-Tube RT-PCR Amplification for Detection of Rift Valley Fever Virus

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Abstract: A reverse Transcriptase (RT) Polymerase Chain Reaction (RT-PCR) was developed and evaluated for detection of Rift valley fever virus in cell culture and serum samples from acute hemorrhagic fever cases. A pair of primers selected from the Medium (M) RNA gene segment of RVFV was used for RT-PCR amplification. The RT-PCR produced a 342-base pair (bp) from RVF RNAs extracted from smith burn vaccine strain propagated *in vero* cell culture and sera from infected humans and animals. However, the RT-PCR assay did not amplify the specific 342 bp product from RNAs extracted from closely related hemorrhagic fever viruses including Crimean Congo Hemorrhagic Fever Virus (CCHFV), Dengue virus, total RNA extracted from non infected vero cells. The described RT-PCR provides rapid, sensitive and specific method for detection of RVFV in cell culture and directly in serum samples. The RT-PCR assay should be recommended for inclusion during epidemiological surveys and outbreaks of the disease among humans and susceptible animal populations.

Key words: Epidemiology, Viral hemorrhagic fevers, RVFV, RT-PCR, Sudan, animal population

INTRODUCTION

The Rift Valley Fever (RVF) Virus (RVFV), a member of the genus *Phlebovirus* belongs to the Bunyaviridae family and possesses a negative sense single stranded RNA genome composed of a Large (L), a Medium (M), and a Small (S) segment (Giorgi, 1996; Garcia *et al*., 2001). The L segment codes for the viral polymerase. The M segment codes for the precursor to the envelope glycoproteins G_1 and G_2 and the non structural proteins 14 and 78 kDa (Collett, 1986; Muller *et al*., 1994). The S segment codes for the nucleocapsid protein N and the non structural proteins (Vialat *et al*., 1997). The virus was first isolated in 1930 in the Rift Valley Province, Kenya, associated with large epidemics in domestic ruminants and humans (Daubney *et al*., 1931). The virus is transmitted to livestock and humans by the bites of infected mosquitoes of the genus *Aedes* or exposure to tissues or blood of infected animals (Meegan and Bailey, 1989). Rift Valley fever is an emerging epidemic disease of humans and livestock and constitutes a major problem in sub-Saharan Africa, North Africa and recently in Saudi Arabia and Yemen (Ahmad, 2000).

RVF outbreaks have been reported in many African countries including Kenya, Somalia, Tanzania, Egypt, Senegal, Mauritania, Nigeria and South Africa (Arthur *et al*., 1993; Olaleye *et al*., 1996; Sall *et al*., 1998, 1999, 2001). In 2000, R VF cases were confirmed in Saudi Arabia and Yemen, marking the first reported occurrence of the disease outside the Africa (Shoemaker *et al*., 2002; Ahmad, 2000; Elfadil and Ali, 2006). In Sudan, the virus was first isolated from infected calves during an out break of the disease in Kosti, White Nile State (Eisa and Obeid, 1977). Subsequently, the virus was isolated from sporadic cases of infected calves in Hilat Koko (Eisa *et al*., 1980). Recently, out breaks of the disease were reported in different states of Sudan including Gazeera, Kassala, Khartoum, River Nile, Sinnar and White Nile (Elageb, 2010). RVFV is currently diagnosed by serology, virus isolation and molecular based techniques (Shope *et al*., 1980; Meegan *et al*., 1987; Garcia *et al*., 2001; Sall *et al*., 2001; Jansen van Vuren *et al*., 2007).

Serological assays are complicated by cross reactions and usually require some times for antibodies to be produced by the susceptible host. RVFV isolation is hazardous, laborious, expensive and time consuming and a final result required 2-4 weeks.

In addition, due to biological hazard, high containment laboratory facilities (BSL-4) are required for handling virus isolation attempts. These limitations encourage the development of reliable, sensitive, inexpensive and rapid methods for detection of the virus genome by molecular based techniques.

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Sensitive assays are critical to the success of any monitoring program such as prevention and control programs. In previous studies, rapid, sensitive, inexpensive diagnostic assays for detection of RVFV using molecular techniques were developed and evaluated based on different gene segments of the virus (Espach *et al*., 2002; Aradaib *et al*., 2010; Sall *et al*., 2001; Youssef, 2001; Le Roux *et al*., 2009). In previous study, a Reverse Transcriptase (RT) polymerase Chain Reaction (RT-RCR) amplification assays targeting the S-segment of the virus were recently described (Ibrahim *et al*., 1997; Sall *et al*., 1999, 2001; Aradaib *et al*., 2010).

A simple and reliable method for detection of RVFV is urgently needed for rapid diagnosis in developing countries. It is well known that the M segment of the virus is less conserved among cognate genes of RVFV strains and is frequently associated with reassortment.

Therefore, it is suggested that detection of the virus targeting this M segment should enhance rapid diagnosis RVFV and would facilitate differential diagnosis of hemorrhagic fever viruses. In the present investigation, a conventional gel-based RT-PCR assay was developed and evaluated, for direct detection of viral RNAs using primers derived from the M RNA segment of RVFV.

MATERIALS AND METHODS

Cell culture and virus propagation: The South African vaccine strain of RVFV (Smithburn) was provided by Dr. M. Abdelrazig, Ministry of Animal resource, Sudan. Crimean Congo Hemorrhagic Fever Virus (CCHFV) RNA was extracted from sera of infected patients during a recent nosocomial outbreak of the disease in a rural hospital, West Kordufan, Sudan (Aradaib *et al*., 2010). Dengue virus RNA was kindly provided by Dr. Abubaker Shazaly, Department of Microbiology, Faculty of Medicine, University Malay, Malaysia. The RVFV vaccine strains were propagated on confluent monolayers of Vero cells as described previously (Aradaib *et al*., 2010). The infectious material was harvested and centrifuged at 1,500× g for 30 min and the supernatant was used for viral RNA extraction.

Collection of blood and serum samples: All samples were collected during the epizootic of RVFV in late fall and early winter of 2007. The most seriously affected areas of the Gezera State and the White Nile State of the Central Sudan were considered in this study. Serum samples, used in this study were collected from acute hemorrhagic fever cases and were kindly provided by Dr. Karsani, the National Medical health laboratory, Sudan. Serum samples and infected cell culture were used for detection of viral genome by RT-PCR.

Extraction of viral nucleic acid from infected cell culture: Viral RNAs were extracted from the infected cell cultures and serum samples using QIAamp viral RNA kit (QIAamp, GmHb, Germany) as per manufacturer's instructions. Briefly, 140 μL of virus suspension or sera were added to 560 μL AVL buffer containing carrier RNA into a 1.5 mL micro-centrifuge tube and mixed by pulse-vortexing for 15 sec followed by incubation at room temperature for 10 min. About 560 μL of absolute ethanol were added and mixed by pulse-vortexing for 15 sec.

About 630 μL of the mixture were transferred to QIAamp spin column mounted on 2 mL collection tube and centrifuged at 6000×(8000 rpm) for 1 min. The column was then transferred to another collection tube and the other 630 μL of the mixture were passed through it. The column was then washed twice with 500 μL of washing buffers WB1 and WB2, respectively. Finally, RNAs were carefully eluted by 60 μL of buffer AVE equilibrated to room temperature. Viral RNAs and total nucleic acids extracted from Vero cell cultures were quantified using a spectrophotometer at 260 nm wavelength.

Extraction of viral nucleic acid from serum samples: Extraction of viral nucleic acid from serum samples was performed as described for extraction of RNAs from cell culture supernatnts.

Primer selection: A pair of primers (RV1 and RV2) was selected from the published sequence of the Medium (M) RNA segment which codes for envelop glycoprotien (Bird *et al*., 2008) and used for the synthesis of the PCR amplification product. Thus, selection of the primers was based on nucleotide sequence of the M segment of Smithburn vaccine strain, complete nucleotide sequences with Gene Bank accession number DQ 380193. The primers specific for amplification of RVFV M segment were designed based on multiple sequence alignment of 20 published conserved sequences of the genes using Bio Eidit software (Carlsbad, CA, USA). Primer RV_2 included bases 2163-2182 of the positive sense strand of the M RNA gene 5-CTG TCT GGC ACA GCA TTG AT-3. RV4 included bases 2485-2504 of the complementary strand: 5-CAC ATT GAA ACA CCC ACA CC-3. Using primers RV_1 and RV2, the RT-PCR would produce a 342-bp nested PCR product. All primers were synthesized on a DNA

synthesizer (Milliigen/Biosearch, a division of Millipore Burlington, MA) and purified using oligo-pak oligonucleotide purification columns (Glen Research Corporation, Sterling, VA.) as per manufacturer's instructions.

Reverse Transcriptase (RT) polymerase Chain Reaction (RT-PCR) Protocol: A single tube RT-PCR amplification was carried out using One Step Access RT-PCR system (QIA-Gen, California, USA). Briefly, a standard 50 μL reaction mixture contained in final concentration of 1×enzyme mix reaction buffer, 5.0 μL of 10 mM dNTP mix, 5.0 μL of 25 mM $MgCl_2$, 1.0 μL of 5.0 Units enzyme mix, 2.0 μL of 20 picomole of each outer primers (RVF1 and RVF2), 5.0 μL of target RNA were used. The total volume was brought to 50.0 μL using RNase free water. Target genes were amplified in low profile 0.2 mL tube (MJ Research, California, USA). Dengue virus and CCHFV templates were used as negative controls. The cycling program consists of a reverse transcription step at 50°C for 30 min. A pre-denaturation step at 95°C for 15 min was performed to inactivate excess RT enzyme and to activate the Taq DNA polymerase in the enzyme mix. This was followed by 40 cycles of denaturation at 94°C for 1 min, annealing temperatures at 56°C for 30 sec each, extension at 72°C for 45 sec. The reaction mixture in each PCR tube was then subjected to a final extention step at 72°C for 10 min. All PCR amplifications were carried out at a 50 μL volume per tube. Thermal profiles were performed on a Techne PHC-2 thermal cycler (Techne, Princeton, New Jersey, USA).

Following amplification, 12 μL from each PCR tube containing amplified product were loaded onto gels of 1.0% Seakem agarose (FMC Bioproduct, Rockland, Maryland, USA) and electrophoresed. The gels were stained with ethidium bromide and the PCR products were visualized under UV light.

RESULTS AND DISCUSSION

The RT-PCR provided sensitive and specific detection of RVFV in cell culture and directly in serum samples from infected humans and animal cases. The pair of primers, designed from the M RNA segment produced a 342 bp primary PCR product from ≥100 fg RNA of RVFV. The DNA band at 100 fg dilution is faint but is still visible.

The PCR amplification products were visualized onto an ethidium bromide-stained agarose gels (Fig. 1). Application of this RT-PCR to 1.0 pg of RVFV RNA target resulted in direct detection of RVFV infection in acute phase sera collected from infected humans and animals and in RVFV-infected cell culture (Fig. 2).

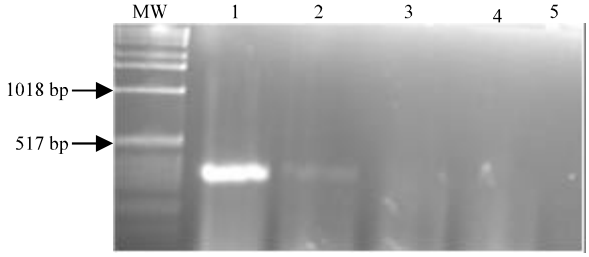

Fig. 1: Sensitivity of the RT-PCR for detection of the 342 bp PCR product using the outer pair of primers (RV1 and RV2). MW: Molecular weight marker (100 bp DNA ladder); Lane (1-4): RVFV RNA extracted from RVFV infected Vero cells at concentrations of 10 pg, 1 pg, 100 fg, 1.0 fg; respectively; Lane 5: total nucleic acid extracted from non-infected Vero cells (negative control sample)

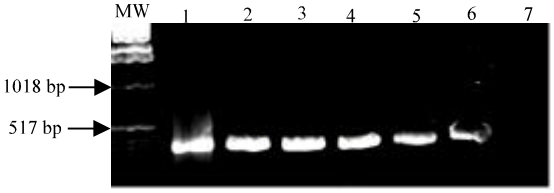

Fig. 2: Sensitivity of the PCR for detection of the 342 bp PCR product of RVFV from different strain of the virus. The RT-PCR detected different strains of RVFV. Molecular weight marker (100 bp DNA ladder); Lane (1-6): RVFV RNA extracted from RVFV infected Vero cells and serum samples. Lane 1: RVFV RNA extracted Smith burn vaccine strain grown on Vero cells (positive control); Lane 2-3: RNA extracted RVFV strain grown on Vero cells; Lane 4-5: RVFV RNA extracted from human serum samples. Lane 6: RVFV RNA extracted from spleen sample of infected goat; Lane 7: total nucleic acid extracted from non-infected Vero cells (negative control)

The specificity studies indicated that the specific 342 bp PCR products were not amplified from 1.0 pg RNA extracted from, closely related hemorrhagic fever viruses, dengue virus RNA; Crimean Congo Hemorrhagic Fever Virus (CCHFV) RNA and total nucleic acid extracts from non infected Vero cells (Fig. 3). Rift Valley Fever (RVF) is a devastating disease characterized by sweeping and abortion storms in livestock. Humans are also susceptible to infection and hence infection with the virus is of public health importance. Clinical manifestations of RVF are indistinguishable from those caused by other viral

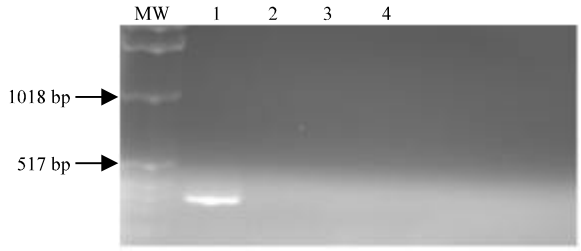

Fig. 3: Specificity of the PCR for detection of the primary 342 bp PCR product from RVFV. MW: Molecular weight marker; Lane 1: RNA extracted from RVFV vaccine strain (positive control); Lane 2: RNA extracted from dengue fever virus; Lane 3: RNA Extracted from Crimean Congo hemorrhagic fever; Lane 4: non-infected Vero cell s; RNA-free sample (negative controls)

hemorrhagic fever viruses and hence cannot reliably identify infected patients (Meegan *et al*., 1987; Aradaib *et al*., 2010). Serology is useful to identify past (IgG) and recent (IgM) during an epidemiological survey of the disease. However, antibodies are likely to be produced at a later course of the disease. In Africa, the presence of RVFV and the emergence of new viral hemorrhagic fever causing pathogens such as reassortant bunyaviruses of the family Bunyaviridae, necessitate the need for more efficient differential diagnostic assays. The reassortant bunyaviruses, designated as KV-66 and KV-141 were isolated from the blood of suspected malaria patients in Kassala, Eastern Sudan and were reported to be associated with an outbreak of viral hemorrhagic fever (Nashed *et al*., 1993).

These viruses were retrospectively identified as batai viruses of the family Bunyaviridae. The Batai viruses should be considered for differential diagnosis during an out break of acute hemorrhagic illness in humans and susceptible animals (Briese *et al*., 2006). Viremia in animals following infection with RVFV is well documented which provides virus for vector transmission to more susceptible populations.

In Sudan, RVF is of concern to public health officers because of outbreaks of the disease among human populations. The disease is also of concern to dairy producers and wildlife managers because of a possible epizootic among susceptible domestic livestock and wildlife populations (Eisa and Obeid, 1977). In the present study, RT-PCR developed and evaluated RT-PCR for direct detection of RVFV in cell culture and serum samples from humans and animals. The specificity studies indicated that the specific 342 bp PCR product was not amplified from a concentration of 1.0 pg RNA from dengue virus; CCHF virus or total nucleic acid extracts from non infected 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 which required high containment laboratory facilities.

The rapidity, sensitivity and specificity of the RT-PCR would greatly facilitate detection of RVFV during an outbreak of the disease among humans and susceptible animals. The described assay is a rapid procedure as the time required from submission of samples to final results of definitive diagnoses could be affordable within the same working day. The described RT-PCR based detection assay does not required nucleic acid hybridization confirmation which usually takes overnight. In addition, the RT-PCR could be easily performed in developing countries in East/Central Africa such like Sudan. Samples preparation and DNA extraction using QIAamp extraction kit was a simple procedure which takes half an hour. The thermal cycling profiles for amplification of the PCR products were consistently 4 h. Running of the agarose gel and electrophoresis usually takes 1 h. In contrast, the sensitivity studies of the conventional gel-based RT-PCR indicated that the described nested RT-PCR protocol was capable of detecting the amount of 1.0 Pg of total RVFV genomic ssRNA. This level of sensitivity is comparable to that of virus isolation.

Never the less, the described conventional RT-PCR is a simple procedure that can easily be adopted in developing African countries. It does not require labeling of the primers and no need for sophisticated laboratory equipments such as DNA sequencing machines or hybridization facilities.

Further studies are currently under way to improve the sensitivity of the described RT-PCR assay to detect RVFV in a variety of clinical samples from experimentally and naturally infected animals and to evaluate its potential as a sensitive and specific diagnostic assay through comparison with current diagnostic test used for detection of RVFV.

CONCLUSION

In conclusion, the described RT-PCR, using primers derived from the M RNA gene segment of RVFV provides a simple, rapid, sensitive and specific diagnostic method for detection of RVFV in cell culture and serum samples. This RT-PCR assay should be recommended for inclusion during epizootic of the disease among humans and susceptible livestock in the African continent.

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