ISOLATION OF BOVINE VIRAL DIARRHEA VIRUS 1, A PESTIVIRUS FROM AUTOPSIED LAMB SPECIMEN FROM TAMIL NADU, INDIA

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Summary. – An epizootic of febrile illness among the Madras red breed of sheep had occurred in 1994 in Verrapuram, Chennai, India. The epizootic was suspected as Rift Valley fever (RVF)-like sickness based on clinical features. However, its etiological agent could neither be isolated nor implicated conclusively. During the post-epizootic period a male lamb died of similar clinical features and the spleen was immediately collected. Inoculation of spleen suspension in infant mouse brain yielded a virus that was serially passaged in infant mice and rhabdomyosarcoma (RD) cells. Electron microscopic observations revealed virus particles resembling flaviviruses. RT-PCR performed on extracted total RNA from infected cells and mouse brains with flavivirus-specific or RVF-specific primers gave negative results. However, an amplicon of 280 bp was obtained with pestivirus-specific primers from the 5'-UTR. Further, a nested PCR yielded a product of 157 bp. Nucleotide sequencing of the 157 bp product showed 100% homology to BVDV-1. Western blot analysis with a flavivirus envelope protein-specific MAb revealed three proteins of 33 K, 45 K and 55 K. Further studies suggested that the 33 K and 55 K proteins were glycosylated. This is the first report of isolation of BVDV-1 from a lamb in India.

Key words: Bovine viral diarrhea virus 1; flavivirus; nested PCR; nucleotide sequencing; pestivirus; RT-PCR; Rift Valley fever; virion morphology; Western blot analysis

Introduction

Pestiviruses (members of the genus *Pestivirus*, the family *Flaviviridae*) are important pathogens of cattle, sheep, and pigs known to cause significant economic losses. A pestivirus infection causes clinical symptoms ranging from immune suppression to diarrhea, thrombocytopenia and infertility. In persistently infected animals the symptoms are often inapparent. In pregnant animals transplacental infection can lead to abortion, stillbirth, malformation and persistent infection of the offspring.

An epizootic of febrile illness characterized by diarrhea, abortion, fulminant neonatal mortality among the Madras red breed of sheep had occurred in 1994 in Verrapuram, Chennai, India. The epizootic was suspected as RFV-like sickness based on clinical features. RVF virus (the species *Rift Valley fever virus*, the genus *Phlebovirus*, the family *Bunyaviridae*) contains a negative single-stranded, tripartite RNA genome. It is a mosquito-borne zoonosis that causes abortion in sheep and is mainly responsible for the mortality of young sheep and goats. Pestiviruses cause clinical symptoms similar to RVF and the infection is usually asymptomatic or associated with a brief self-limited febrile illness.

Since the autopsy material (spleen) was obtained during a post-epizootic survey carried out in relation to the RVF-like sickness, the entire initial attempts were made to prove the presence of RVF virus. However, this virus could not be

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Abbreviations: BVDV-1 = Bovine viral diarrhea virus 1; BVDV-2 = Bovine viral diarrhea virus 2; i.c. = intracerebrally; MHC = major histocompatibility; MAb = monoclonal antibody; NC = nitrocellulose; p.i. = post infection; RD = rhabdomyo-scarcoma; RVF = Rift Valley fever

isolated from this material as well as mosquito pools (Joshi *et al*., 1998). The lamb spleen specimen was stored in our virus repository as unidentified virus. The present paper reports the isolation of BVDV-1, a pestivirus from this specimen.

BVDV-1 belongs to the species *Bovine viral diarrhea virus 1*, the genus *Pestivirus*, the family *Flaviviridae*. The genus *Pestivirus* includes four species; *Border disease virus*, *Bovine viral diarrhea virus 1*, *Bovine viral diarrhea virus 2*, and *Classical swine fever virus* (Hog cholera virus) (Agapov *et al*., 2004).

On the basis of CPE caused by BVDV-1 or Bovine viral diarrhea virus 2 (BVDV-2) two types, namely cytopathic (cp) and non-cytopathic (non-cp) have been identified (Meyers *et al*., 1996).

Materials and Methods

Collection and processing of specimen. During the post-epizootic survey the spleen was collected from a four-month-old male lamb. A portion of spleen was homogenized and centrifuged at 10,000 rpm for 1 hr at 4°C. The supernatant was filtered through a 0.2 µ filter and inoculated into infant Swiss albino mice by intracerebral (i.c.) route (0.02 ml/mouse). In the first passage only four of eight mice inoculated were found sick on day 4 post infection (p.i.), while the remaining mice did not show any sign of sickness within 14 days. The brains from sick mice were harvested and 10% suspensions were prepared in PBS with 0.75% bovine albumin. Two of sixteen sick mice recovered from the infection with initial virus passages but the third and onward virus passages caused sickness and death to all mice inoculated. Neither BAPS nor healthy mouse brain suspension caused illness to mice. The brains harvested from sick mice were passaged in RD, Vero and PS cells. Initially, as CPE was observed with RD cell line, this was passaged and a virus stock of a titer of 10^7 $TCID_{50}$/ml was prepared and used in further studies. Samples of cultured cells for RT-PCR were obtained by pelleting the cells at 10,000 rpm for 10 mins.

Electron microscopy. Initially, the supernatant from the third passage cultures of RD cells showing CPE was examined by transmission electron microscopy using negative staining. Later on, CPE-positive cell cultures were freeze-thawed thrice, clarified by centrifugation at 10,000 rpm for 10 mins at 4°C, and an aliquot of the supernatant was negatively stained for electron microscopy. The negative staining was done by floating a 400 mesh copper grid with carbon-coated formvar-support on the culture supernatant aliquot for 20 mins in a humid chamber. The grid was then stained with a drop of sodium phosphotungstic acid pH 6.0 and imaged under 100 KV mode of a TECNAI 12 Biotwin transmission electron microscope (FEI, Holland).

RT-PCR. Total RNA was extracted from cultured cells or infected mice brains using the Trizol reagent (Gibco-BRL) according to the manufacturer's protocol.

Brains from infected and healthy mice as well as CPE-positive infected and non-infected RD cells were tested for the presence of RVF virus by RT-PCR. RVF-specific primers defined by Swasdipan *et al*. (2001) and Sall *et al*. (2002) were used. To determine the presence of flaviviruses, flavivirus NS5 region-specific primers defined by Scaramozzino *et al*. (2001) were used. To determine the presence of alphaviruses, alphavirus-specific primers defined by Pfeffer *et al*. (1997) were used. To detect pestiviruses, pestivirus-specific diagnostic primers defined by NIHS, Cell Bank (cellbank.nihs.go.jp/cellbank/qualitycontrol/pcrprimers.htm) (Y. Takada, R. Yasuda and H. Tanabe, personal communication) were employed. The primers PestiF1 and PestiR1 were from the 5-non-coding region of polyprotein gene: PestiF1: 5'-TGCCCACAGTAG GACTAGC-3', PestiR1: 5'-ACTCCATGTGCCATGTACAG-3'. Nested primers were as follows: PestiF2: 5'-AGTCGTCAG TAGTTCGAC-3', and PestiR2: 5'-CTCTGCAGCACCCTATCA-3'. cDNAs were prepared using MMLV reverse transcriptase (Invitrogen) and the PestiR1 primer according to the manufacturer's protocol.

The PCR consisted of initial denaturation at 95°C/5 mins, 5 cycles of 94°C/30 secs, 50°C/30 secs, and 72°C/secs, 5 cycles of 95°C/secs, 48°C/30 secs, and 72°C/secs, 25 cycles of 95°C/1 min, 50°C/1 min, and 72°C/1 min, and final extension at 72°C/8 mins. The nested PCR consisted of initial denaturation at 95°C/5 mins, 35 cycles of 95°C/1 min, 50°C/1 min, and 72°C/1 min, and final extension at 72°C/8 mins.

RT-PCR products were resolved by agarose gel elctrophoresis and ethidium bromide staining.

SDS-PAGE with 4 % stacking and 12 % resolving acrylamide gels and a constant current of 6 mA for 90 mins was employed. The gels were silver stained (Kazuaki and Ebata, 1983).

Western blot analysis. Gels with proteins separated by SDS-PAGE were blotted onto nitrocellulose (NC) membranes. Blot strips were washed with 4% bovine serum albumin in PBS pH 7.2. For specific reaction a flavivirus-specific MAb (Hx-2, Gore *et al*., 1990), an anti-mouse antibody tagged with alkaline phosphatase and 5-bromo-4-chloro-3-indonyl phosphate as substrate (Sigma) were employed.

Glycosylation analysis was performed using the GLYCO-PRO Glycosylation Detection Kit from Sigma based on the periodic acid Schiff PAS method. Briefly, the proteins separated by SDS-PAGE were blotted onto a NC membrane and fixed in 50% methanol for 30 mins. The blot was then incubated with an oxidizing solution for 30 mins, then with the Schiff's reagent for 2 hrs and finally with a reducing solution. The blot was washed with distilled water and dried at room temperature.

Nucleotide sequencing. Direct sequencing of the amplified 157 bp product was performed using an ABI 3100 automated DNA sequencer and the Big Dye Terminator Kit (Applied Biosystems, USA).

Results

Virus propagation

The mice inoculated with the spleen supernatant showed sickness on day 4 p.i. When the brain suspensions from the sick mice were passaged in RD cells, CPE was observed on day 3 p.i.

Electron microscopy

Supernatants of RD cell cultures showing CPE did not contain any virus-like particles. However, lysates of these cultures prepared by 3-fold freeze-thawing showed round virus-like particles of a size range of approximately 40 to 60 nm with electron-dense core of about 30 nm in diameter and 8 nm surface projections (Fig. 1). These results were indicative of a cell associated virus. The morphological features of the virus particles were highly suggestive of a flavivirus.

RT-PCR

The PCR performed on the brains from infected and normal mice as well as normal and CPE positive RD cells with flavivirus- and alphavirus-specific primers did not show any band of expected size. The PCR with diagnostic primers for RVF virus was negative either. The PCR with pestivirus-specific primers performed on the brains from infected mice as well as CPE-positive RD cells showed the expected 280 bp band and the PCR with nested primers yielded the expected band of 157 bp (Fig. 2). Sequencing of this product showed 100% homology with the corresponding BVDV-1 sequence available in the GenBank database (Osloss virus, Acc. No. AY279527).

Western blot analysis

Western blots when probed with the flavivirus envelope protein-specific MAb HX-2 showed three bands (Fig. 3).

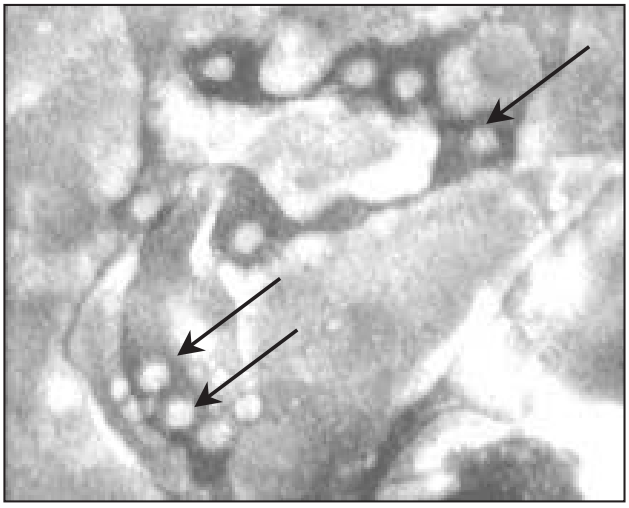

Fig. 1

Cell-associated flavivirus-like particles in RD cells

Electron microscopy, lysates of freeze-thawed cultures. Magnification 120,000x.

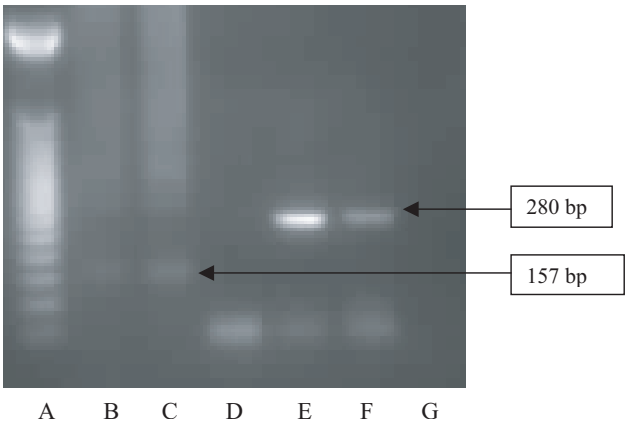

Fig. 2

RT-PCR products from infected RD cells and infected sick mouse brain

DNA size marker, 50 bp ladder (lane A); nested PCR, infected RD cells (lane B); nested PCR, infected sick mouse brain (lane C); nested PCR, uninfected mouse brain, negative control (lane D); PCR, infected RD cells (lane E); PCR, infected sick mouse brain (lane F); PCR, healthy mouse brain, negative control (lane G).

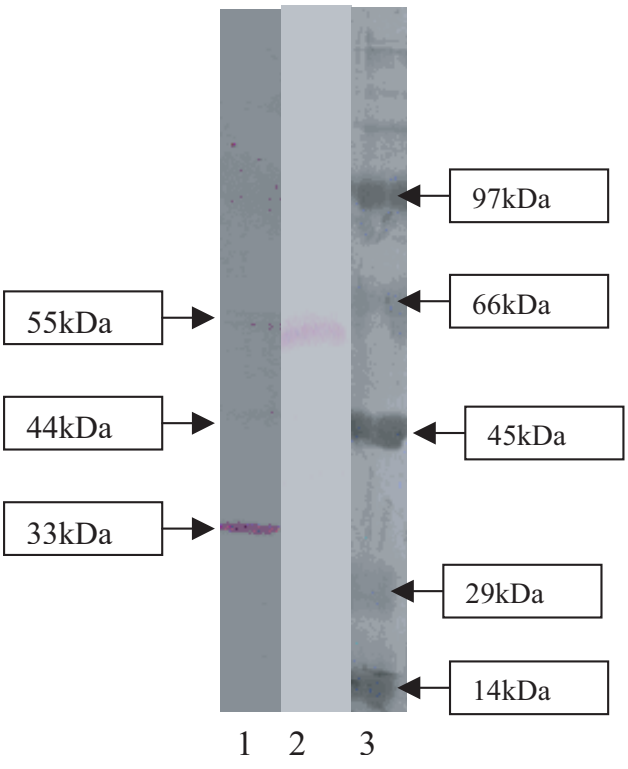

Fig. 3

Western-blot analysis of infected Vero cells with MAb HX-2

MAb specific for flavivirus envelope proteins was used. Pelleted infected Vero cell lysate (lane 1); pelleted Dengue virus-infected Vero cell lyste (lane 2); Protein markers (lane 3).

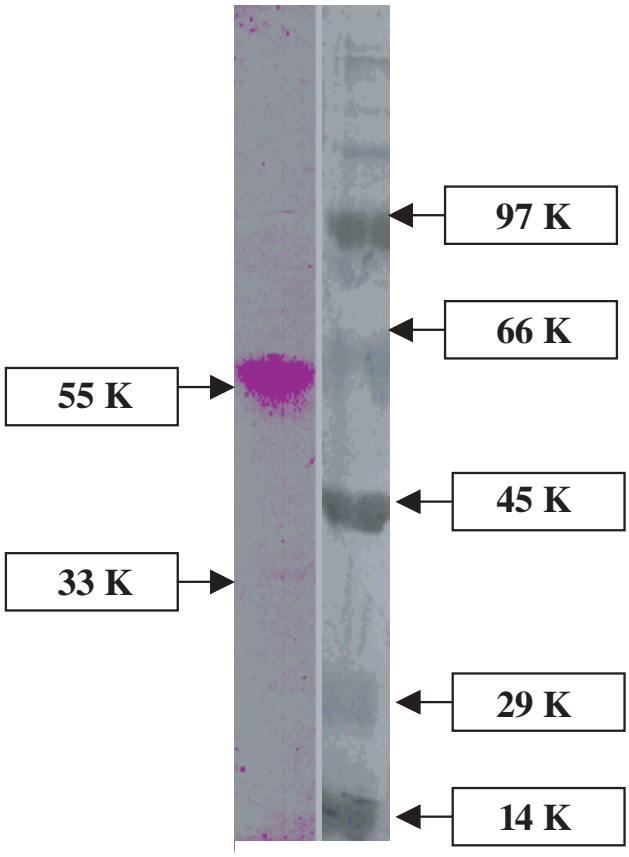

Fig. 4

Detection of glycosylated viral proteins

Western blot stained for glycosylation. Pelleted infected Vero cell lysate (lane 1); Protein markers (lane 2).

However, only the 33 K band was enough intense to prove clear reactivity while the 44 K and 55 K bands were faint and hardly interpretable. When these proteins were stained for glycosylation, the 45 K protein in contrast to the 33 K protein was found highly glycosylated (Fig. 4). This clearly suggested that MAb HX-2 reacted with the 33 K E1 protein, while presence of the E2 protein could be confirmed by the glycosylation staining. The evidence obtained by Western blot analysis and staining for glycosylation suggested the presence of 3 envelope proteins of pestiviruses, namely E^{rms}, E1 and E2.

Discussion

During *in vitro* propagation of the virus under study a delayed CPE was observed at 72 hrs p.i. in RD cells. However, during the passaging it got adapted and an early CPE was seen at 30 hrs p.i. Pestiviruses are difficult to purify because of modest growth in cell culture, inefficient release from infected cells, and association with cellular debris. Some cell-associated virus can be released from infected cells by repeated freeze-thawing (Laude, 1977). Our initial attempt to visualize virus particles by EM in the cell culture material was unsuccessful. When the attempts were made after 2- to 3-fold freeze-thawing virus particles could be seen, suggesting that virus was cell-associated.

Concerning the cytopathogenity both BVDV-1 and BVDV-2 are known to occur in two types, namely cytopathic (cp) and non-cytopathic (non-cp). The isolation of this virus was possible due to (i) its cp nature and (ii) the fact that original specimen was first inoculated in infant mice by i.c. route where MHC class I and class II immune responses were bypassed. Initial attempts to isolate the virus in Vero cells were unsuccessful but after passaging in RD cells the virus infection could be established in Vero cells

Once the virus could be visualized by electron microscopy, its identity as flavivirus and cell-associated nature were established. Enveloped spherical particles of 40 to 60 nm in diameter with an electron-dense inner core of about 30 nm in diameter were demonstrated.

An earlier report on post-epizootic survey by Joshi *et al.* (1998) has suggested the presence of Ganjam virus antibodies also in sheep sera and concluded that the epizootic might have been due to the occurrence of two viruses but had no conclusive evidence for involvement of RVF. At present, it cannot be concluded that presence of Ganjam virus in that population might have helped the conversion of non-cp to cp virus type. Further studies may elucidate this issue of great epidemiological importance.

In our study, the sequence of the part of the conserved 5'-UTR region indicated the identity of this virus. Further supportive evidence for a flavivirus was obtained when the Western blot analysis with the flavivirus envelope protein-specific MAb showed the presence of 3 envelope proteins, namely E^{rms} (45 K), E1 (33 K), and E2 (55 K). The fact that the E2 protein was highly glycosylated corresponded to the earlier reports suggesting that this protein contains four to six N-linked glycosylation sites, while the El protein contains only two to three sites (Weiland *et al*., 1990).

It should be noted that very little work has been done on the viruses of the four virus species of the genus *Pestivirus.* It has been suggested (Becher *et al.,* 1997) that transmission of BVDV-1 or BVDV-2 infection occurs by oronasal route. However, the majority of flaviviruses are transmitted by arthropods. BVDV-1 and BVDV-2 have not yet been reported from India. Their importance is based on the fact that bovine viral diarrhea causes heavy losses in the livestock in other countries where these viruses are prevalent.

The described isolation of the virus from the specimen collected from a lamb in 1994 and existence of antibodies to BVDV-1 and/or BVDV-2 in buffalos and cattle (Sudharshana *et al*., 1999) indicate that these viruses are now prevalent in India and may get established in this region involving other ruminants. Isolation of BVDV-1 from the spleen of a lamb succumbed during an epizootic of febrile illness suggests that this might be the etiological agent.

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