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Isolation and Characterization of a Field Strain of Bovine Ephemeral Fever Virus in China

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Abstract: The infected blood was collected from dairy cattle showing similar Bovine Ephemera Fever (BEF) clinical signs in Taizhou area of Zhejiang province. The blood frozen-thawed 3 times was concentrated decuple by centrifugation, then the virus had 7 blind passages in suckling mice by an intracerebral inoculation route and 10 passages in BHK21 cells. The suckling mice died regularly 3-4 days after inoculation from the 3rd passage with 100% mortality rate. In BHK21 cells, the CPE occurred from the secondary passage and 80% cells approximately emerged pathological changes post-infection from the 4th passage. The virus isolated was proved to be Bovine Ephemera Fever Virus (BEFV) by RT-PCR, Transmission Electron Microscopy (TEM), Virus Neutralization (VN) test and IFA test. The virus isolated was designated JT02L. The homologies of amino acid sequence of the G gene compared with JB76H (Beijing strain), Taiwan strain and BB7721 (Australia's strain) all were above 90%.

Key words: BEFV, characterization, field strain, isolation, TEM, China

INTRODUCTION

BEF was 1st found in East Africa in 1867 year, then spreaded rapidly in many countries in Africa, Asia and Oceania along with the cattle industry progress, so the disease has existed for more than a century after being found in the world (Yeruham *et al*., 2003; Hsieh *et al*., 2005; Walker, 2005). In China, the similar disease was 1st recorded about in 1949, but the 1st strain of BEFV was not isolated from a cattle during a major epidemic until 1976 year and the disease was confirmed in China (Hong, 2001).

The inoculation of healthy susceptible cattle with infected blood was the most reliable assay system and was considered to be the most sensitive system available to conserve BEFV. In 1967, Vander westhuzen isolated the virus at 1st from a cattle by intracerebral inoculation of suckling mice with crude white cell fractions and Japanese and Australian researchers also isolated BEFV using the same method. After that the virus was adapted successfully to grow in BHK21 cell (Yin and Liu, 1997). In 1976, Chinese researchers isolated BEFV by inoculation of both suckling mice brain and BHK21 cell during an outbreak of BEF (Hong, 2001).

However, it wasn't reported about the isolation of BEFV systematically in recent 30 years. In this research, an epidemical field strain of BEFV in China was isolated and characterized, which was great helpful for the study on vaccine of BEF.

The field strains isolated from different areas may be more effective to be used as vaccine strain in the epidemic region than the current vaccine for controlling BEF.

MATERIALS AND METHODS

The infected blood was collected from BEF affected dairy cattle herd of Taizhou area of Zhejiang province in 2002 year and conserved in -75°C. Experimental cattle were purchased from BEF-free cattle herds. SPF suckling mice were purchased from experimental animal base, Lanzhou Biological Product Research Institute. BHK21 cell and sera to Rabies Virus (RV) were kept in the State Key Laboratory of Veterinary Etiological Biology, Lanzhou Veterinary Research Institute in China; JB76H strain and positive serum to BEFV were kindly provided by Prof. Kuizhang Yuan at Harbin Veterinary Research Institute in China.

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Experiment of infecting cattle: Two-years-old cattle, which had been free to BEFV by VN test, were inoculated with the infected dairy cattle's blood from Taizhou by vein at a dose of 2 mL cattle^{-1}. The temperatures of the cattle were detected before inoculation and after infection every 24 h. At the same time, the cattle were observed carefully and all symptoms were noted.

Virus isolation by intracerebral inoculation of suckling mice: The 20 mL infected blood conserved in -75°C was frozen and thawed 3 times and centrifuged at 10000 r min^{-1} for 30 min, from which the supernatant containing virus particles was obtained and centrifuged at 45000 r min^{-1} for 3 h again. At last, the sedimentation, resuspended with 1 mL cell maintenance medium (containing 2% fetal bovine serum, 100 units mL^{-1} Penicillin, 100 μg mL^{-1} Streptomycin and 100 μg mL^{-1} Kanamycin sulfate), was filtrated through a 0.2 μm filter and inoculated in suckling mice's brains at dosage of 20 μL mouse^{-1}. The brains of dead mice post-infection were collected and grinded with cell maintenance medium to 10% (w v^{-1}) tissue homogenate. The homogenate with being frozen-thawed 3 times was centrifuged for 30 min at 10000 r min^{-1} and then the supernatant was filtrated and inoculated suckling mice's brains again and seven blind passages were performed as the same method. The control mice only inoculated cell maintenance medium at dosage of 20 μL mouse^{-1} were kept every blind passage.

Virus isolation in BHK21 cell: Confluent BHK21 cell monolayers in 25 cm^2 flasks were infected with the supernatant of the 7th passage virus in suckling mice brain (2 mL flask^{-1}) and incubated at 37°C for 1 h, then cell maintenance medium was added into the flasks (10 mL flask^{-1}). The cultures were maintained at 37°C for 5 days and observed every 24 h interval. If no CPE within 5 days, the cultures were frozen and thawed 3 times and inoculated BHK21 cells again till 10 blind passages (Wang *et al*., 2007; Tan *et al*., 2008).

Characterization of the virus by RT-PCR amplification: The total RNAs were prepared from infected BHK-21 cells using RNA extracting Kit (Collins *et al*., 1992; Guan *et al*., 2003). Then the cDNA was synthesized using approximately 1 μg of the total RNA with primer F (5' CCT CAC AAT GTT CAA GGT CCT C 3'). The cDNA was amplified by *Taq* polymerase with primers F (5' CCT CAC AAT GTT CAA GGT CCT C 3') and B (5' GCA GGA ACA TGA TTG CCC TGT T 3') in order to obtain BEFV G gene. After the initial denaturation at 94°C for 5 min, the amplification proceeded through a total of 35 cycles consisting of denaturation at 94°C (40 sec), annealing at 52°C (1 min), primer extension at 72°C (160 sec) and the final extension of 10 min at 72°C. The PCR products were identified by agarose gel electrophoresis and purified with a PCR Clean-Up Kit and cloned into pMD18-T vector. The sequence of the positive plasmid was analyzed and compared with JB76H (Beijing strain), Taiwan strain and BB7721 (Australian strain).

Observation of the virus by an electron microscopy: Preparations of the supernatant from suckling mice brain and CPE-producing BHK21 cells were mounted on grids previously rendered hydrophilic by grow-discharge and were negatively stained in 2% phosphotungstic acid and observed under a transmission electron microscope (Halpin *et al*., 2000; Gard *et al*., 2007).

Characterization of the virus by VN test: VN test was carried out with the virus isolate in the BHK21 cell culture according to micro-neutralization test for bovine ephemeral fever published by Ministry of Agriculture of the People's Republic of China in 2002 (Wakeley *et al*., 2004; Xu *et al*., 2004). JB76H strain was as positive control.

Immunofluorescent Antibody (IFA) test: When CPE was detected, the cell culture supernatant was used to be inoculated at 16 well glass chamber slides paved with BHK21 cell monolayer. The chamber slides were incubated at 37°C with 5% CO_2. When CPE was observed in these wells, the supernatant was removed and the cell sheet was fixed in cold acetone for 20 min at -20°C and then stained by an IFA technique (Chua *et al*., 2002). Antiserum to BEFV was used as the primary antibody, then a fluorescein isothiocyanate-labelled goat anti-bovine IgG was used to stain the preparations. Uninfected cells, overlaid with antiserum to BEFV and stained as described above, were used as an indicators. The specific immunofluorescence reaction was examined with a fluorescence microscope.

RESULTS

Experiment of infecting cattle: The temperatures of the cattle were 38.0-38.5°C before infection and 38.4-39.0°C within 120 h post-infection. But 144 h after infection, it was up to 39.4° and the symptoms of nasal mucus and tears were observed. Total 168 h post-inoculation, the cattle began to reject food and water and their temperatures rise to 41.0°C. After the serious symptoms above continued for 24 h, the temperatures dropped below 39.0°C and the cattle got well 3 days later.

Virus isolation by inoculation of suckling mice brains: All the suckling mice inoculated the infected blood died during 6-10 days post infection in the 1st blind passage and the secondary passage mice died on the 5th or 6th

Fig. 1: Syndromes in suckling mice caused by the virus derived from blood of affected cattle, a) control suckling mouse, b-c) the suckling mice presenting paralysis signs in hind legs after inoculation with the infected blood, d-e) the suckling mice died owing to being inoculated with the infected blood

day after inoculation. From the 3rd to the last passage, all the mice inoculated died regularly on the 3rd or 4th day after infection. The suckling mice often showed paralysis and stiffness in hind legs on the 2nd to 3rd day after inoculation and died during 12-24 h later (Fig. 1). On the contrary, all the control suckling mice were alive and fine.

Virus isolation in BHK21 cell: The BHK21 cells, inoculated with the 7th passage of the virus-infected suckling mice brain, had no CPE within 5 days post inoculation. In the 2nd and 3rd passages of BHK21 cell monolayer, CPE was seen 48 h after infection and approximately 50-70% cells emerged CPE 48 h. From the 4th passage to tenth passage, CPE was observed 24 h post-infection and 80% cell cultures showed CPE, so the cultures were harvested (Fig. 2).

Characterization of the virus by RT-PCR amplification: The aim gene obtained by RT-PCR was about 1965 bp in size, which included BEFV G gene by sequence analysis. The homologies of the nucleotide acid sequence on G gene of the virus isolate compared with JB76H (Beijing strain), Taiwan strain and BB7721 (Australian strain) were 96, 98.6 and 89.3%, respectively. The identities of speculated amino acid sequence of G gene of the virus isolate with JB76H, Taiwan strain and BB7721 were 97.2, 97.9 and 92.9%, respectively. All the data above indicated the virus isolate was BEFV and was designated JT02L.

Electron microscopic examination: The virus particles were observed from infected suckling mice brains and

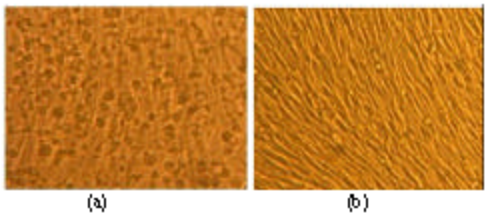

Fig. 2: CPE in BHK21 cells inoculated with the virus-infected suckling mice brains, a) BHK21 cells showing CPE, b) normal BHK21 cells

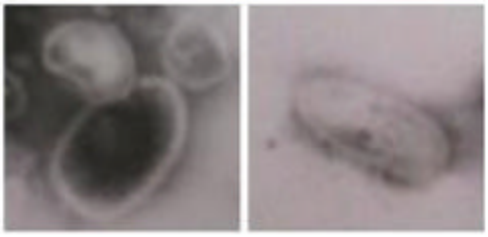

Fig. 3: Electron micrograph of the viruses isolate. Original magnification: ×100000

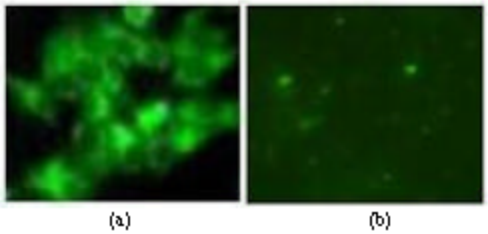

Fig. 4: The results of IFA test, a) the specific fluorescence was produced in the BHK21 cells infected with the virus isolate, b) there was no specific fluorescence in BHK21 cells uninfected as control

BHK21 cells, which showed bullet-like or semi-olive in outline shape and had spikes on the surface of the envelope protein and were identical to BEFV's shapes as reported (Yin and Liu, 1997) (Fig. 3).

Micro-neutralization test: The titers of the virus isolate were 10^{6} $^{1}TCID_{50}$ mL^{-1}. The standard positive serum to BEFV with 1:64 dilution could neutralize completely the virus isolate being same as JB76H strain.

IFA test: Infected cells showed very intense fluorescence throughout the cell sheet (Fig. 4). At the same time, the IFA specificity was confirmed by the absence of fluorescent staining in the uninfected control cells.

DISCUSSION

In this research, the infected blood was collected from cattle herd during a BEF outbreak in Taizhou of Zhejiang province in 2002 year and conserved in -75°C for 5 years and used to infect healthy cattle successfully. Then a field strain of the virus was isolated by inoculation of suckling mice brain and blind passages in BHK21 cells in 2007 year. The results of RT-PCR, electron microscopic examination, VN test and IFA test all showed the virus is just BEFV.

Other members of Rhabdoviridae, namely Berrimah virus and Kimberley virus, haven't been reported in China (Zakrzewski *et al*., 1992) and wasn't used to carry out cross-reaction in VN test. This test indicated that BEFV conserved in -75°C for 5 years was able to survive.

It has been the 1st detailed report that a field virus of BEFV was isolated and characterized in China since the standard strain JB76H was isolated in 1976. In this research, the G gene of the virus isolated was amplified (Bai *et al*., 1987; Sambrook *et al*., 2002; Walker *et al*., 1992; Dhillon *et al*., 2000).

By analysis of the G gene sequence, the homologies of the nucleotide acid and deduced amino acid sequence were all above 90% compared with JB76H strain, Taiwan strain and BB7721 strain, respectively, which indicated that BEFV was high conservative between different epidemic periods and areas.

At present, BEF has existed about 60 years in China and has many outbreaks in 25 provinces in these years.

At present, this disease has many prevalences in Zhejiang, Fujiang, Jiangshu and Guangdong provinces in the south and east of China although no major outbreaks have been reported since 1991 (Hong, 2001). The epidemic period becomes short, occurring a time every 3-5 years, since 20 years past, while it is 1-2 years in Guangdong province.

In China, an inactivated vaccine from JB76H strain cultured on cell BHK21 has been applied to defend BEF (Yin and Liu, 1997). But because of the loss of antigenicity of BEFV during domestication (Cybinski *et al*., 1990, 1992; Walker *et al*., 1991, 1994; Kongsuwan *et al*., 1998), the vaccine couldn't stimulate a high enough antibody titer to preserve cattle herd from being infected or stop epidemic of BEF completely.

After the virus isolate have been obtained, the next work is to research why the antigenicity of the virus was attenuated during the domestication. We will amplify the genes of the different passages viruses obtained from suckling mice brains and BHK21 cells respectively and conclude the variational rule of nucleotide acid and deduced amino acid between every passage viruses by sequence analysis. On the other hand, we will make default or mutate some genes of the virus through the reverse genetics system to analyze the variation of antigenicity (Mishra *et al*., 2007; Yu *et al*., 2007). At last, the principle of loss of viral antigenicity will be clarified at molecular level, which will be great useful to exterminate BEF both in theory and practice.

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