Restriction Endonuclease Analysis of Bovine Herpesvirus Type 1 Isolates from Calves with Fatal Encephalitis: Comparison with Vaccine Virus

Motohiro HORIUCHI, Noriko YAMAZAKI, Hidefumi FURUOKA, Takane MATSUMI, Michio NAKAGAWA, Naotaka SHIGURO, and Morikazu SHINAGAWA
Departments of Veterinary Public Health and Veterinary Pathology, Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Hokkaido 080, Japan

(Received 4 October 1994/Accepted 22 March 1995)

ABSTRACT. Meningo-encephalitis in feedlot cattle sporadically occurred in the Tokachi area in northern Japan. The calves had been vaccinated intranasally with a mixed live-vaccine (infectious bovine rhinotracheitis virus, bovine viral diarrhea-mucosal disease virus, and parainfluenza 3 virus) for which intramuscular inoculation was indicated. Two additional live vaccines, bovine adenovirus type 7 and bovine respiratory syncytial virus, had been inoculated simultaneously. Eleven isolates of bovine herpesvirus type 1 were plaque-purified from two brains with fatal encephalitis; their viral DNAs were examined by restriction endonuclease analysis (REA) using PstI and HindIII. The REA patterns of the virus clones were almost identical to those of the vaccine strain 758-43, suggesting that the isolates from this outbreak of fatal encephalitis originated in the abnormally administered vaccine.

 slate: BHV-1, meningoencephalitis, restriction endonuclease analysis.


Bovine herpesvirus type 1 (BHV-1) causes respiratory and genital diseases in cattle, the syndromes being known as infectious bovine rhinotracheitis (IBR) and infectious pustular vulvar vaginitis (IPV)/balanoposthitis, respectively. Less commonly, BHV-1 is also associated with a variety of syndromes such as encephalitis, conjunctivitis, abortion, enteritis, and fatal systemic infection of neonatal calves [5].

Restriction endonuclease analysis (REA) of BHV-1 DNA suggests that certain restriction fragment-migrating patterns may be associated with particular clinical syndromes [4]. Although there exists partly contradictory results [13], BHV-1 isolates can be classified into 3 main groups according to REA patterns and clinical manifestations: group one, BHV-1.1 or IBR virus, causes respiratory tract disease; group two, BHV-1.2 or IPV virus, causes genital disease [4, 9, 11]; and group three, BHV-1.3 or bovine encephalitis herpesvirus, which was recently renamed from BHV-1.3 to BHV-5 [16], exhibits neuropathogenic potential [2, 3]. REA of BHV-1 has been used as a diagnostic technique for the identification of field isolates associated with particular symptoms and for the identification of vaccine strains of BHV-1, especially where they may be associated with disease [20].

In 1992, a fatal meningoencephalitis of calves sporadically occurred in one farm, in the Tokachi area in northern Japan. We isolated BHV-1 from the brains of those calves and compared the REA patterns with those of the vaccine strain.

Brains of two calves affected with encephalitis, in which intranuclear inclusion bodies were observed by histopathological examination, were used for virus isolation. Ten percent of the brain homogenates were inoculated into Madin-Darby bovine kidney (MDBK) cells, and the cells were observed for cytopathic effect (CPE). For viral plaque purification, diluted brain homogenates were inoculated to MDBK cells, and the cells were overlayed with Eagle's minimal essential medium containing 2% fetal calf serum and 0.7% agarose. Four days later, the cells were stained with neutral red and virus plaques were isolated by using a Pasteur pipette under a microscope. The plaque-purified viruses were propagated once in MDBK cells, and the resulting viruses were used as seed viruses.

Viral DNA was obtained from cell free virions. Culture fluid of virus-infected cells (about 800 cm² of monolayer cells) were clarified by low-speed centrifugation. Virions were pelleted by centrifugation at 100,000 × g for 2 hr, and the pellet was treated with proteinase K [100 µg/ml proteinase K, 0.01 M Tris-HCl (pH 8.0), 0.01 M EDTA, 0.1 M NaCl, 1.0% SDS] at 56°C. After proteinase K digestion, the solution was extracted with an equal volume of a 1:1 mixture of phenol and chloroform, and then viral DNA was precipitated by ethanol. Viral DNA was digested with restriction endonucleases either PstI or HindIII. PstI and HindIII digests were electrophoresed on 0.7% and 1.0% agarose gels, respectively.

The herd of 100 feeder cattle showed an estimated 5% mortality by the fatal meningoencephalitis. The owners had vaccinated twice their animals intranasally with vaccines that were intended to be administered intramuscularly, at one and three weeks old. The live vaccines used were for adenovirus type 7, bovine respiratory syncytial virus, and a combined vaccine to IBR, bovine virus diarrhea-mucosal disease and parainfluenza 3 viruses. Disease symptoms began within two weeks after the second vaccination. From two calves with this fatal encephalitis, we used homogenized brain tissue to inoculate to MDBK cells. Those cells showing viral CPE were assayed for the presence of BHV-1 antigen using a monoclonal antibody against BHV-1 glycoprotein IV [15] in an indirect fluorescent assay, and all proved positive (data not shown). This prevalence of the virus in the brain homogenates led us to try direct plaque purification of BHV-1 from brain homogenates. In this way, we isolated eleven BHV-1 clones (Table 1).

REA patterns from the eleven plaque-purified isolates are shown in Figs. 1 (calf K) and 2 (calf Pr2202). Also shown in Figs. 1 and 2 are control REA patterns of the unrelated IBR Los Angeles strain and of strain 758-43.
Table 1. Isolation of BHV-1 from cattle affected with fatal encephalitis

<table>
<thead>
<tr>
<th>Calf</th>
<th>Sex</th>
<th>Agea)</th>
<th>Diagnosis</th>
<th>Isolated from</th>
<th>Plaque-purified isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>K</td>
<td>Male</td>
<td>1.5</td>
<td>Encephalitis</td>
<td>Brain</td>
<td>KA, KB, KC, KD, KE, KF</td>
</tr>
<tr>
<td>Pr2202</td>
<td>Male</td>
<td>1.5</td>
<td>Encephalitis</td>
<td>Brain</td>
<td>PA, PB, PC, PD, PE</td>
</tr>
</tbody>
</table>

a) Months old.

Fig. 1. Restriction endonuclease digest patterns of BHV-1 isolates from calf K. (a) PstI pattern, (b) HindIII pattern. Comparison to strain 758-43; arrows and arrowheads indicate additional and missing fragments, respectively.

which was the strain used to vaccinate the herd. The isolates from calf K were almost indistinguishable from each other by REA patterns. Four of six plaque-purified isolates and the unpurified isolate from the calf were identical to strain 758-43 in PstI and HindIII patterns [Figs. 1a and b; KB, KC, KE, KF and K (unclone)]. Of the remaining two isolates, KA differed from strain 758-43 in the HindIII pattern and KD differed in both the PstI and HindIII patterns, as shown by arrows. The REA patterns of the isolates from calf Pr2202 were also extremely similar to those of strain 758-43 (Figs. 2a and b). Two of the five isolates, PA and PE, had PstI patterns that were indistinguishable from strain 758-43. The remaining three, PB, PC, and PD, differed from strain 758-43 by one fragment in both the PstI and HindIII patterns.

Comparison of strain 758-43 with the Los Angeles strain revealed several fragments that differed in their migration with both PstI and HindIII digestions. The Los Angeles strain shows a typical HindIII pattern for BHV-1.1 [10]. Fragment J in the Los Angeles strain had no apparent counterpart in the HindIII pattern of strain 758-43 (Figs. 1b and 2b, indicated by the arrow with the capital letter). The strain 758-43 was attenuated by serial passage in swine testis (ST) cells. When the HindIII pattern of the prototype strain 758 was compared with that of the strain 758-43, the former possessed the J fragment, while the latter did not (data not shown). This indicates that the J fragment was lost during attenuation. Furthermore, we could not find any report describing the lack of the J fragment in the HindIII pattern of BHV-1.1 [4, 6, 8, 11–13]. These facts suggest that the apparent lack of the J
fragment is unique to the strain 758–43, and thus the isolates in this study probably originated in the vaccinal virus.

The strain 758–43 is reported to propagate $10^5$-times higher than the prototype strain 758 in ST cells at 30°C [7]. Therefore, we examined the growth property of the isolates in ST cells (Table 2). The ST cells seeded at $8 \times 10^5$ cells per cm$^2$-bottle were challenged with the viruses at a multiplicity of infection of 0.5. The supernatants were harvested at the indicated times and virus titers were determined by plaque assay using MDBK cells. At 7 days post infection, the strain 758–43 propagated about 4-times higher than the strain 758 and the isolates. This result may indicate that the isolates are not identical to the strain 758–43. However, one should be careful in considering the result because no data that described on the growth property of re-isolated 758–43 virus from cattle experimentally challenged with strain 758–43 has been presented in the original report [7].

Attenuated live vaccines are widely used to control BHV-1 infection in cattle, but some problems associated with vaccination have been reported, e.g., abortion after intramuscular vaccination [14], and vaccine-induced epizootics confirmed by REA [20]. BHV-1 genome easily changes after only one passage in vivo [19], in contrast to its stability after serial passages in vitro [13, 19]. For that reason, we analyzed several clones that were directly plaque-purified from brain homogenates. The HindIII pattern of the vaccine strain and the isolates appeared to resemble that reported for BHV-1.1, but not that of BHV-5 [3]. Some of the isolates exhibited REA patterns that were identical to the vaccine strain, while others differed slightly, suggesting that the isolates originated in the vaccine, and that the genome of the vaccine virus had changed within the affected calves. Another possibility is that the vaccine inoculum might have contained heterologous populations. However, because we did not analyze plaque-purified clones of strain 758–43 from the same lot of combination vaccine used for the herd, we could not

Table 2. Propagation of isolates in ST cells at 30°C

<table>
<thead>
<tr>
<th>Virus</th>
<th>Virus titer (PFU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 day</td>
</tr>
<tr>
<td>758–43</td>
<td>$4.0 \times 10^4$</td>
</tr>
<tr>
<td>758</td>
<td>$2.7 \times 10^4$</td>
</tr>
<tr>
<td>KF</td>
<td>$5.3 \times 10^5$</td>
</tr>
<tr>
<td>PB</td>
<td>$6.0 \times 10^4$</td>
</tr>
</tbody>
</table>
assess this possibility. It is also conceivable that a BHV-1 virus whose REA pattern is very similar to that of the vaccinal virus might exist in field and that such a virus caused the meningo-encephalitis.

Although intranasal inoculation have been used for IBR live-vaccine in U.S., however, with regard to the 758–43 strain of which the instruction mentions intramuscular inoculation for vaccination, it remains unclear whether the irregular route of inoculation provoked this case. BHV-1 may spread from the oro-nasal cavity to the brain through the trigeminal nerve [1], however, our pathological findings suggested that the virus invaded the brain through the olfactory nerve rather than the trigeminal nerve (Furuoka et al., submitted for publication). Hence, microbes in the nasal cavity or other live vaccines inoculated simultaneously, might be implicated in the spread of the isolate through the olfactory nerve. Ecological and animal management factors, as well as the habits of individual animals, should be considered carefully to understand the cause of this case.

Although the facts that strain 758–43 revealed a unique REA pattern and that the REA patterns of the isolates were almost identical to that of strain 758–43 strongly suggest that the isolates originated in the vaccine, the discrepancy between the REA pattern and the growth property in ST cells confuses the situation. Therefore, to distinguish the vaccine virus clearly from wild isolates, markers which vaccine virus specifically possesses or loses, and which can be detected by simple methods such as enzyme-linked immunosorbent assay or polymerase chain reaction will be required. A suid herpesvirus type 1 vaccine that has a deleted glycoprotein I gene is successfully used as a marker vaccine because of the lack of the immunoresponse against glycoprotein I [17]. Recently, a glycoprotein E deletion mutant of BHV-1 is reported to be avirulent and highly immunogenic [18]. The genetically-modified BHV-1 is a good candidate for a live BHV-1 marker vaccine that is clearly distinguishable from wild-type strain by the marker.

ACKNOWLEDGEMENTS. We thank Dr. K. Okazaki (Department of Veterinary Microbiology, Tokyo University of Agriculture and Technology) for providing the monoclonal antibody. We also thank Dr. S. Osame (Department of Internal Medicine, Obihiro University of Agriculture and Veterinary Medicine) for his helpful comments.

REFERENCES