Characterization of two BHV-4 strains isolated in the Czech Republic

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ABSTRACT: This study describes the isolation of bovine herpesvirus 4 (BHV-4) from the respiratory tract of animals suffering from respiratory disease. DNA of new isolates, CH and Ni, was cleaved with *Bam*HI, *Eco*RI and *Hind*III in restriction enzyme analysis and the fragments were identified by co-migration with the restriction profile of the reference strain Movar 33/63 cleaved with the appropriate endonuclease. Typical profiles with polyrepetitive DNA (prDNA) fragments were detected. In order to localize the size variation within the obtained digestion fragments, Southern blot hybridization was performed. Differences between the isolates CH, Ni were localized in both the prDNAs and the unique central part of the genome and were restricted to fragment size variation.

Keywords: Bovine herpesvirus 4 (BHV-4); Restriction endonuclease analysis; Southern blot

Bovine herpesvirus 4 (BHV-4) is a member of the family *Herpesviridae* and on the basis of genomic analysis, it is classified as a gammaherpesvirus (Bublot et al., 1992). The BHV-4 genome consists of linear dsDNA of approximately 146 kbp that contains a unique central part flanked at both ends by G + C rich tandem repeats called polyrepetitive DNA (prDNA) (Bublot et al., 1990; Thiry et al., 1990).

BHV-4 strains are antigenically closely related and show very similar restriction patterns (Bublot et al., 1990). Two groups of strains have been defined, Movar 33/63 (Movar-like) and DN 599 (DN-like), based on their restriction patterns. The unique central part of the genome is very well conserved among strains with a few variations due to the presence or absence of a restriction site and fragment length variation. A study of genomic diversity of field isolates in Belgium revealed some Movar-like isolates carrying an additional *Eco*RI site (Bublot et al., 1991b). The length variations occurred in the junction regions between repeated and unique sequences and two regions V1, V2 where the left junction and V1 regions are highly polymorphic (Bublot et al., 1990, 1991a). The size of prDNA varies from one isolate to another and this variability is due to the presence of a variable number of 200 bp fragments inside the prDNA (Ehlers et al., 1985).

The BHV-4 virus is distributed worldwide and several species are susceptible to infection (Thiry et al., 1990). The virus was also isolated from hosts with a variety of clinical signs, but its role in the pathogenesis of these lesions still remains unclear (Bartha et al., 1967; Egyed, 2000). BHV-4 failed to provoke illness in experimentally infected calves, although in some cases, mild respiratory disease was observed (Castrucci et al., 1987). The virus has been isolated even from clinically healthy animals (Luther et al., 1971; Belak and Palfi, 1974; Egyed et al., 1996). BHV-4 has also been detected in natural hosts, predominantly in epithelial cells of respiratory, alimentary and urogenital tracts (Egyed et al., 1996; Lopez et al., 1996). Like other herpesviruses, BHV-4 is able to establish latency (Lopez et al., 1996; Osorio et al., 1996).

In our study, we describe the isolation and identification of BVH-4 from clinical samples of animals from two separate farms. The isolates were further

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characterized by restriction enzymes analysis and some of the variable fragments were cloned into pSPT18 plasmids and used as probes in hybridization.

MATERIAL AND METHODS

Clinical samples and virus isolation

Nasal discharges were collected from animals suffering from respiratory disease stabled at two different farms. On these farms nasal swabs from calves aged between 5–6 weeks and nasal swabs from lactating dairy cows or the lung tissue of one slaughtered cow were investigated, respectively. Clinical material was transferred to 1ml of transport medium (MEM supplemented by antibiotics: 500 IU/ml penicillin, 500 µg/ml streptomycin and 150 IU/ml nystatin) and kept at 4°C until processed.

The content of the nasal swabs were transferred to the rest of the transport medium with a syringe. Lung tissue was cut into pieces and homogenized in a mortar (in MEM) and subsequently centrifuged at 2000 × g for 10 minutes. The supernatant was then used to infect cells.

The processed nasal swabs and tissue suspensions were used to transfect semi-confluent cultures of Madin-Darby bovine kidney (MDBK) cells cultivated in Eagle's minimal essential medium (Eagle MEM) supplemented by 10% of foetal bovine serum and antibiotics (250 UI/ml penicillin, 250 µg/ml streptomycin). Cells were cultivated at 37°C and checked daily for the presence of cythopatic effect (cpe). When cpe was observed cells culture supernatant was examined by electron microscopy. Cells of cpe negative culture were subpassaged into new cultivated cells a further three times.

Direct immunofluorescence assay

The presence of the virus was determined in infected and fixed MDBK cells by a direct immunofluorescence test using the FITC-Anti-BHV-4-HIS conjugate (Riemser Arzneimittel GmbH). Infected and control monolayers were fixed with cold acetone for 10 min, washed in PBS, and incubated with the conjugate diluted 1 : 10 following the manufacturer's instructions. The results were read using the fluorescence microscope Jenaval.

Purification of DNA from viral nucleocapsids

To prepare viral DNA semi-confluent MDBK cells cultivated in Eagle MEM were inoculated with 10^3 $TCID_{50}$/ml of plaque purified virus of isolates designated CH, Ni and reference strain Movar 33/63 (CAMP V-54, Veterinary Research Institute, Brno, Czech Republic). When approximately 90% cpe was observed, the medium was harvested by centrifugation at 4500 rpm for 20 min. The supernatant was collected and ultracentrifuged at 20 000 rpm for 90 min in a Beckman SW28 rotor and the pellet was resuspended in the remaining drop of aspirated supernatant before being dounced. Subsequently, a combined DNase/RNase digestion was performed: the suspension was adjusted to 5mM $MgCl_2$, both DNase and RNase were each added to 0.1 mg/ml and the suspension was incubated at 37°C for 30 min. To purify virions the suspension was centrifuged through 15 ml of 15% sucrose at 20 000 rpm for one hour in the same rotor as above and the pellet was suspended in TNE buffer (10mM Tris-HCl, 1mM EDTA, 150mM NaCl, pH 7.5). All centrifugation steps were done at 4°C. Purified virions were mixed with 1/2 volume of lysis-buffer (75mM Tris-HCl, 25mM EDTA, 3.0% sodium-laurylsarcosinate, pH 8) and 0.1 mg/ml RNase and after incubation at 37°C for 30 min proteinase K 0.1 mg/ml was added and the suspension was further incubated at 65°C for three hours. After cooling to room temperature viral DNA was extracted with phenol/chloroform, precipitated by absolute alcohol and washed twice with 70% ethanol. After drying in vacuum the DNA samples were dissolved in TE buffer (Tris-HCl, EDTA, pH 8) and stored at –20°C until used.

Restriction endonuclease analysis

The purified viral DNA of new isolates CH, Ni and the reference strain Movar 33/63 was cleaved by restriction endonucleases. One µg of extracted DNA was digested with 20 units of *Bam*HI, *Eco*RI and *Hind*III restriction enzymes at 37°C for two hours. The viral DNA fragments obtained were separated by electrophoresis at 40 V in 0.6% agarose gel and stained with ethidium bromide. The DNA in the gel was transferred onto a nylon membrane (Hybond N, Amersham) by alkaline blotting.

Nested PCR assay

A nested PCR assay was performed with primers pairs flanking 567 bp and 260 bp fragments of the thimidine kinase gene. The specific sequences of primers have been described previously (Egyed et al., 1996). The PCR reaction were carried out in a 20 µl reaction mixture containing PCR buffer (Promega), 2mM MgCl2, 200µM of each dNTPs, 0.5µM of each primer and 2.5 IU of Tag polymerase (Promega). Two microliters of amplification mixture were used in the second round of amplification. The amplification was performed in a Unocycler (VWR). The cycling conditions were as follows: initial denaturation at 94°C for 10 min and 30 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1 min and elongation at 72°C for 90 s. A final elongation step was prolonged to 7 min and the mixture was then cooled to 10°C. PCR products were identified in a 1.5% agarose gel containing ethidium bromide at 0.5 µg per 1 ml.

Cloning of restriction fragments

Viral DNA of the BHV-4 isolate Ni was digested with *Eco*RI and *Hind*III and ligated into pSPT18 plasmid cleaved with the appropriate enzymes. Recombinant plasmids were transformed into the competent *Escherichia coli* strain DH5α. Plasmid DNA was extracted from *E. coli* using the Plasmid Midi Kit (Qiagen) and then screened for an insert by *Eco*RI and *Hind*III digestion. In order to identify inserts co-electrophoresis of inserted fragments and viral DNA digested with appropriate endonucleases was performed.

Southern blot analysis

The cloned DNA fragments were excised from plasmids by restriction enzyme digest and isolated by gel electrophoresis in low-melting temperature agarose. Hybridization probes were prepared by random priming using the Rediprime labeling system (Amersham Life Science, FRG) and [α-^{32}P]-dCTP (> 3000Ci/mM, ICN Biomedicals, FRG). After pre-annealing, hybridization was performed for 18 h at 42°C in 4 X SSPE (1 X SSPE: 0.18M sodium-chloride, 0.01M phosphate buffer, 0.001M EDTA, pH 7.4), 0.5% (w/v) non-fat dry milk, 1.0% (w/v) SDS, 0.5 mg/ml denatured calf thymus DNA, and 50% (v/v) deionized formamide. For re-hybridisation the DNA filters were heated for 60 min in 0.4N sodium-hydroxide at 45°C, followed by the pouring of boiled 0.1 X SSC (1 X SSC: 0.15M sodium-chloride, 0.015M sodium citrate, pH 7.0) containing 0.5% sodium dodecylsulfate and 0.2M Tris-HCl (pH 7.4) onto the filter and cooling down to room temperature.

RESULTS

Virus isolation and direct immunofluorescence assay

Viruses were isolated from one nasal swab sampled from a diseased calf (designated CH) and from two dairy cows (designated Ni 3, 7). Virions and nucleocapsids with morphological characteristics typical of herpesviruses were detected in the supernatants of infected cell cultures by electron microscopy. Degenerative lesions of infected MDBK cells were observed in the first passage from post-inoculation Day 8 and CPE culminated on Days 12 to 14. In the second passage, CPE became apparent on Day 2 to 3. CPE was characterized by the presence of only solitary rounded ("cytomegalic") cells of various sizes, the number of which increased slowly. No syncytia were observed, but small clusters of degenerated cells became apparent at an advanced stage of infection.

Specific positive immunofluorescence was detected in the cytoplasm and rather sporadically in the nuclei of the infected cells. No fluorescence signal was demonstrable in non-infected cells.

Restriction endonuclease analysis and PCR

Restriction profiles of CH and Ni isolates were compared and the identity of fragments was estimated by co-migration with a corresponding fragment from the restriction pattern of the reference strain Movar 33/63 cleaved by appropriate endonucleases. Cleavage of BHV-4 DNA with *Bam*HI, *Eco*RI and *Hind*III produced very similar, but not identical restriction patterns for all viruses tested (Figure 1). The number of restriction sites was the same for all viruses, and *Eco*RI and *Bam*HI yielded cleavage products that appeared as fragments with high molecular ratios (prDNA). Both CH and Ni isolates contained prDNA with one *Eco*RI and

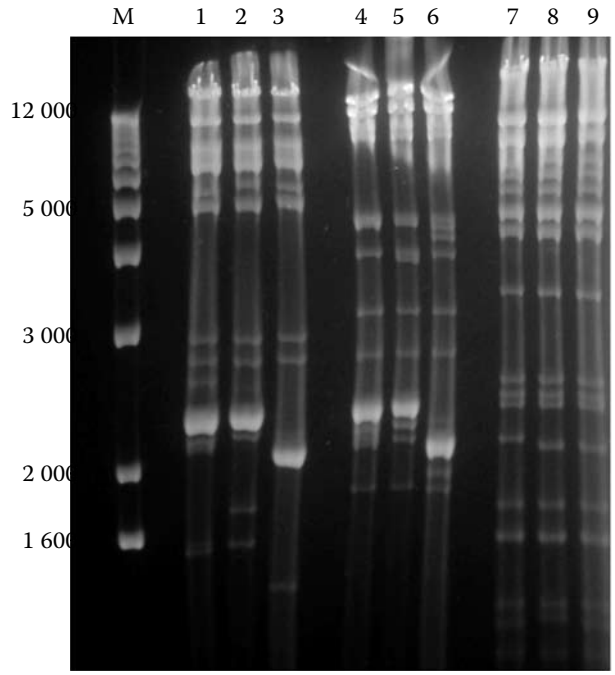

Figure 1. Restriction enzymes profile of DNA from isolates CH, Ni and reference strain Movar 33/63. Lane 1, 2, 3 *Bam*HI restriction pattern; lane 4, 5, 6 *Eco*RI restriction pattern and lane 7, 8, 9 *Hind*III restriction pattern of reference strain Movar 33/63 lane 1, 4, 7; isolate CH lanes 2, 5, 8; and isolate Ni lanes 3, 6, 9; M, molecular weight marker

*Bam*HI site and the size of prDNA varied between them; nevertheless, cleavage of viral DNA of each isolate with *Bam*HI, *Eco*RI produced prDNA of the same size. In the central part of the genome fragment size variations between isolates were localized in *Bam*HI and *Hind*III fragments, which co-migrated with I (5.4 kbp) and E (6.3 kbp) fragments of Movar 33/63, respectively. Furthermore, in the *Eco*RI restriction profile of Ni isolate, three separated fragments co-migrated near the fragment with the higher molecular ratio, representing *Eco*RI restriction fragments G (4.4 kbp), H (4.4 kbp) and I (4.35 kbp) of Movar 33/63 *Eco*RI (Figure 1).

The PCR fragments of the specific size were detected after amplification and reamplification of DNA of BHV-4 strains CH, Ni and Movar 33/63.

Analysis of recombinants and Southern blot hybridisation

The DNA purified from the new virus isolate Ni was digested with *Eco*RI and *Hind*III and cloned into pSPT18; about one hundred recombinant plasmids successfully transformed *E. coli* DH5α. The obtained recombinants were analysed and correlated with respective genomic fragments by co-electrophoresis of cleaved plasmid and genomic DNA. Fragments cloned in pVNiE22 and pVNiE50 represent two of three distinct fragments detected after cleavage of BHV-4 Ni with *Eco*RI co-migrating near the G, H, I tri-molar *Eco*RI fragment of Movar 33/63. After digestion of pVNiH48, the extracted fragment co-migrated in electrophoresis with the *Hind*III E fragment of Movar 33/63.

The clones pVNiE22, pVNiE50 and pVNiH48 were used as templates for preparing probes NiE22, NiE50 and NiH48, respectively.

The NiH48 probe strongly hybridized with K, A and E of *Bam*HI, *Hind*III and *Eco*RI restriction profiles, respectively. A weak hybridization signal was observed with the *Bam*HI I fragment. No differences were observed between *Bam*HI K and *Eco*RI A fragments; *Hind*III E fragments varied between isolates and the size variation is located in the V2 variable region of the BHV-4 genome. In order to localize the size variation of *Eco*RI fragments of BHV-4 isolate Ni Southern blot hybridizations were carried out with the NiE22 and NiE50 probes on *Eco*RI, *Bam*HI, *Hind*III patterns. Both probes hybridized with two genomic fragments and so detected junction fragments between the repeated and unique part of genome (Figure 2). In the central part, the NiE50 probe hybridized with *Bam*HI G, *Hind*III J and *Eco*RI H restriction fragments and no differences were observed between the isolates. In contrast, size variation was detected after hybridization of the ENi22 with *Eco*RI profiles, where, in the genomic unique part of Ni and CH isolates, distinct fragments were visible. These fragments are the equivalents of the *Eco*RI I left junction fragment of Movar 33/63. In *Eco*RI, the restriction profile of isolate CH I co-migrated with fragment J; on the other hand, the I fragment of Ni had a unique size. Although both NiE22 and NiE50 hybridized with prDNA, the intensity of hybridizing signals varied. The NiE22 strongly hybridized with the repeated region, but a weaker signal was detected with a fragment from the central part of genome. The opposite was true for the NiE50.

DISCUSSION

In this study, the first isolation of BHV-4 from Czech cattle herds is described. The isolates were

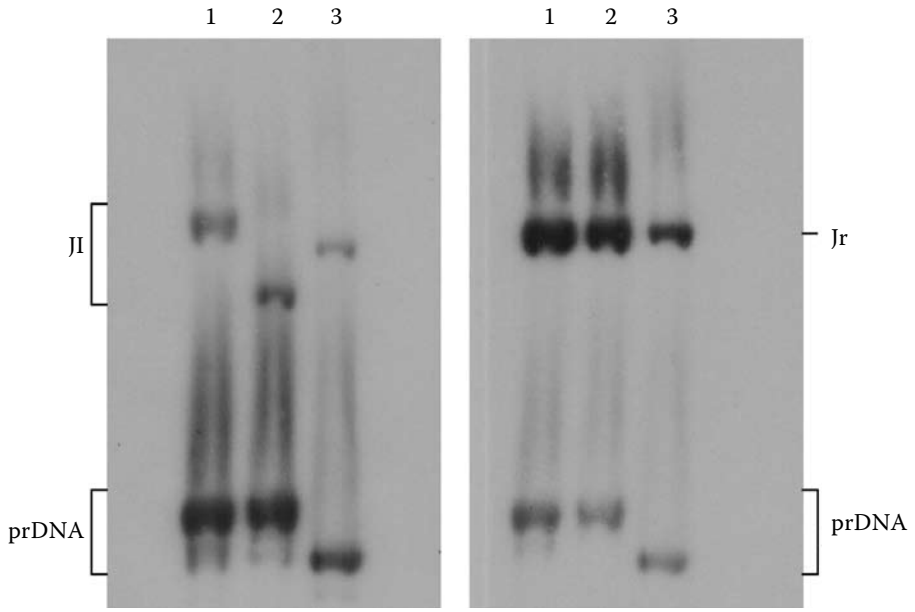

Figure 2. Southern blot hybridization of DNA of CH, Ni isolates and reference strain Movar 33/63 cleaved with *Eco*RI with the probes NiE22 and NiE50. The prDNA units, left junction (Jl) and right junction (Jr) fragments are indicated. Hybridization of A, the probe NiE22 (a) and probe NiE50 (b) with *Eco*RI digestion pattern of Movar 33/63, lane 1; isolate CH, lane 2 and Ni isolate, lane 3

characterized by restriction endonuclease analysis and Southern blot hybridization.

In natural hosts, BHV-4 can be detected in epithelial cells of the respiratory and urogenital tracts (Egyed et al., 1996). We have isolated BHV-4 from nasal swabs obtained from calves (CH isolate) and dairy cows (Ni isolates) with respiratory distress. Duplicate serum samples from calves revealed elevated antibody titre against BRSV, although the virus could not be detected in clinical samples by specific nested RT-PCR (Valentova et al., 2003). Serum samples from cows were not available. Although BHV-4 was the only virus isolated from the respiratory tract of tested animals, its role in respiratory disease pathology could not be estimated from our results. The virus was first isolated from calves suffering from respiratory disease (Bartha et al., 1967). Nowadays, its role in respiratory diseases is somewhat overlooked and BHV-4 infection is associated predominantly with reproductive tract disorders (Donofrio and van Santen, 2001; Frazier et al., 2002; Graham et al., 2005; Deim et al., 2006; Nikolin et al., 2007; Banks et al., 2008; Fabian et al., 2008). The virus has been isolated in many clinical cases (Egyed, 2000), but its causative role in the development of these lesions still remains unclear. The virus persistently infects macrophages (Donofrio and van Santen, 2001), which could further become a reservoir of BHV-4. Donofrio and colleagues studied the pathogenic role of BHV-4 in clinical metritis and revealed that BHV-4 efficiently infected endometrial stromal and epithelial cells (Donofrio et al., 2007). These authors also suggested that BHV-4 could contribute to disease by increasing the inflammation through PGE2 induced activation of lytic replication in macrophages persistently infected with BHV-4 and recruited from the bloodstream. Whether this scenario could take place in other tissues, from which BHV-4 was recovered, remains to be discovered.

The clinical material in our study was originally sampled to examine IBR infection in the herds. Electron microscopy revealed virions and nucleocapsids with morphological characteristics typical of herpesviruses and the preliminary hypothesis of BHV-1 infection was rejected by a direct immunofluorescence test with a specific FITC-Anti-BHV-4-HIS conjugate. The DNA of two new herpesvirus strains was than amplified in nested PCR specifically detecting the thimidine kinase gene of BHV-4. In order to characterize new BHV-4 isolates, CH and Ni, viral DNA was extracted and digested with restriction endonucleases *Bam*HI, *Eco*RI and *Hind*III. The restriction profiles of BHV-4 strains were similar and could be distinguished from the

restriction patterns of other bovine herpesviruses (Bublot et al., 1990, 1991b; Yamamoto et al., 2000). The restriction profiles of CH and Ni isolates revealed a typical BHV-4 restriction pattern with the presence of one hypermolar fragment after cleavage with *Bam*HI and *Eco*RI, representing polyrepetitive DNA (Ehlers et al., 1985; Bublot et al., 1990). Although different BHV-4 isolates can be present in the same herd (Bublot et al., 1991b), Ni3 and Ni7 isolates with identical restriction patterns (data not shown) were detected from clinical samples of dairy cows in our study and in a further study were handled as a single Ni isolate. Most differences between isolates are located in prDNA. In addition, three genomic regions vary in size between BHV-4 isolates, where the left junction fragment and V1 are highly variable (Bublot et al., 1990). The differences between CH and Ni isolates were localized in both prDNAs and the unique central part of the genome and were restricted to the fragment size variation. No deletion or insertion of DNA sequences was observed (Figure 1). The number and size of prDNA varied between the isolates. This variation in size seems to be due to different numbers of repetitions of a 200 bp fragment (Ehlers et al., 1985). The CH and Ni isolates possess one prDNA unit of different size and cleavage with *Bam*HI produced prDNA, which was the same size as the prDNA fragment obtained by *Eco*RI digestion. The prDNA was detected with NiE22 and NiE50 probes, which also represent junction fragments between the unique and repeated part of the BHV-4 genome. Although both NiE22 and NiE50 hybridized with prDNA, the intensity of hybridizing signals varied. NiE22 strongly hybridized with the repeated region, while a weaker signal was detected in a fragment from the central part of the genome. The opposite is true for NiE50. From these results, one can assume that the left junction fragment represented by the-NiE22 probe and that it contains a longer prDNA sequence than the right junction fragment. In our study, we were further able to identify variable left junction fragments and V2 regions between isolates by hybridization with probes NiE22 and NiH48, respectively. The V1 variable region, localized on a restriction map in the *Hind*III U fragment was not detected, because the low molecular weight fragments were not clearly visible in our digests.

The Ni isolate had a unique *Eco*RI restriction profile with three distinct fragments, instead of the one tri-molar G, H, I band of Movar 33/63. Two of these fragments were identified as the left and right junction fragments. Whilst the right junction fragments indicated that all the viruses were of an identical size, the left junction fragments varied considerably in size. The left junction fragment of CH co-migrated with the J fragment. The I fragment of Ni had a unique size and migrated as a new fragment between the right junction and J fragment. The results obtained in our study were in agreement with the findings of other authors (Bublot et al., 1991b) who demonstrated that the size of the right junction remained almost constant, while the left junction varied greatly between BHV-4 strains. On the basis of the migration pattern of digested fragments, two types of BHV-4 were previously defined (Bublot et al., 1990). Both new BHV-4 isolates have been assigned to Movar-like viruses, which have been isolated predominantly in Europe (Thiry et al., 1990; Bublot et al., 1991b). The restriction profiles of the CH isolate corresponded to the Belgian field isolates coming from a variety of clinical samples including: lochia, peritoneal fluid, lungs, nasal swabs and abomasal fluid (Bublot et al., 1991b). These authors could not detect any clear relationship between the restriction pattern and the origin of the isolate, which is consistent with our results.

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