DNA Sequence variability analysis of the gD and the UL36 genes of Bovine herpesvirus-1 isolated from field cases in Indonesia

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1. Introduction

Since the first outbreaks of infectious bovine rhinotracheitis (IBR) in 1950 in Colorado and in 1953 in California (Schroeder and Moys, 1954; Miller, 1955; Yates, 1982), this disease has continued to spread worldwide. The first clinical case of IBR disease in Indonesia was reported in the Lampung region (Marfiatingin-sih, 1982) in 1981, the disease has persisted in this country since then.

The classification of BHV-1 into various subtypes has changed as studies of the virus have provided further details on the viral genome. BHV-1 subtypes 1.1 and 1.2 are very closely related and are classified based on specific symptoms in infected cattle (Muylken et al., 2007). Molecular methods, including enzymatic restriction fragment length polymorphism (RFLP) (Metzler, Schudel, et al., 1985; Rocha, et al., 1998; Takiuchi et al., 2005; Kamiyoshi et al., 2008) subtype-specific monoclonal antibodies (Metzler, Matile, et al., 1985), restriction endonuclease mapping (Brake and Studdert, 1985; Engels, et al., 1986), and characterization using single nucleotide polymorphism (SNPs) (Fulton et al., 2016; Chase et al., 2017) has been used to differentiate types and subtypes.

The most extensively characterized gene is that encoding glycoprotein D (gD located in US, also known as US6); gD has functions related to viral immunity and antigenicity (Collins, et al., 1993). UL36 gene also well-studied recently. UL36 gene was located in UL and forming tegument structure. The protein encoded by UL36 exhibits strong divergence among the various BHV-1 subtypes (d’Offay et al., 2016). In different subtypes of BHV-1, the upstream regions of the UL36 gene harbours insertions/deletions (indels) relative to its counterparts, and these differences are responsible for diversity among BHV-1.2b isolates (d’Offay et al., 2016). Herpesviruses may evolve faster than the host (Thiry et al., 2006), the herpesviral evolutionary rate estimated of $3 \times 10^9$ per amino acid per year (McGeoch et al., 2000).

The research described here had two related aims, both performed using Indonesian field isolates of BHV-1. The first goal was to characterize the molecular basis of the obvious subtype-specific differences in the sequences of middle third of the gD gene (a region known to contains SNPs) and in the downstream end of the UL36 gene (a region known to contain indels). The second goal was to compare the evolutionary process based on the sequence variations detected in gD and UL36.

2. Material and methods

2.1. Source of DNA samples and ethical approval

The samples consisted of nasal swabs and tracheal sections, and were obtained from The Animal Disease Investigation Centre (ADIC) in Lampung and Bukittinggi, Indonesia based on a letter of approval for material transfer No. 05010/PD.650/F5.H/06/2015 and No. 22022/PK.310/F4/02/2016. This study was approved by The Committee for Safe Handling of Living Modified Organism in Ehime University (Permission number: H28-05) and carried out according to the guidelines of the committee.
2.2. Preparation and amplification of DNA

The samples from nasal swabs were prepared per OIE instructions (OIE 2010). The samples from tracheal organ were prepared as described previously (Hidayati et al. 2018). DNA purification using a QIAamp DSP DNA Mini Kit (Qiagen, Hilden, Germany); the resulting nucleic acid was recovered in 200 µL elution buffer consisting of 10 mM Tris-Cl and 0.5 mM EDTA, pH 9.0. DNA fragments were amplified by nested PCR (Vilcek et al. 1994). The sequences of the gD primers were gD-1F 5′ACCCAGTGCGACCCCAGGA3′; gD-1R 5′GGTTCGGCTCTGCCCTCG3′; gD-2F 5′ACCCGACACCCCGTGTGG3′; gD-2R 5′TCGGTCTGGGGGCCC3′; gUL36-1F 5′TAGCGCGCCACCGGCTATA CG3′; gUL36-1R 5′TCTGGACGGCACGAAAGA; AGG3′; gUL36-2F 5′TAGACGACGAGATTGGCGA3′; gUL36-2R 5′GGCATGGGAC TCCGTGTT3′.

The PCR reaction mixtures in each round were generated using KAPA HiFi HotStart ReadyMix (KAPA Biosystems, Woburn, MA) and consisted of 2.5 mM MgCl2, 0.3 mM each of dNTPs, 1 U of DNA polymerase, 10 pmol each of the forward and reverse primers, and less than 100 ng template DNA per reaction, with the balance of the volume consisting of PCR-grade water. First-round amplification of the gD fragment was performed: initial denaturation at 95°C for 3 min; 35 cycles of denaturation at 98°C/20 sec, annealing at 55°C/15 sec, extension at 72°C/5 min; and a final extension at 72°C/5 min. Second-round amplification of the gD fragment was performed according to the same programme but with annealing at 59°C. First-round amplification of the UL36 fragment was performed: initial denaturation at 95°C for 3 min; 35 cycles of denaturation at 98°C/20 sec, annealing at 60.7°C/15 sec, extension at 72°C/5 sec; and a final extension at 72°C/5. Second-round amplification of the UL36 fragment was performed according to the same programme but with annealing at 59°C. 3. Results and discussion

3.1. Amplification of gD and UL36 fragments

The sequences were deposited in DDBJ (DNA Data Bank of Japan), and the accession numbers are LC318523-LC318532 and LC368822-LC368831. The gD primers were designed to yield a 511-bp amplicon; the UL36 primers were designed to yield a 211-, 184-, or 157-bp amplicon (Figure 1).

3.2. Sequence analysis of the gD and UL36 fragments

The conclusion of the sequence variability of gD and gUL36 fragments summarized in Table S1.

The resulting alignment of the gD fragments among BHV-1.1, BHV-1.2, and the study samples revealed strong sequence similarity, with mean ± SD identities of 98.04 ± 0.32% (BHV-1.1 vs. samples), 98.13 ± 0.42% (BHV-1.2 vs. samples), and 98.34 ± 0.42% (among samples). Specifically, 4 separate point mutations were detected in this region (observed at nucleotide residues 462, 631, 666, and 912). Two of the SNPs (nt 631 and 666) were predicted to result in changes in the amino acid sequence (aa 211 and 222) of the gD glycoprotein when comparing BHV-1.1 and BHV-1.2 (Figure 2(a)). The reassortment of the SNPs, especially at positions in the gD protein, suggests that homologous recombination has occurred within the gD sequence. The calculated synonymous and nonsynonymous ratio (dn/ds) was 0.22 (dn/ds < 1). This value indicates negative selection (H0 = dn < ds), meaning that these sequences have been subjected to purifying selection (Traesel et al. 2014). A previous paper (Saepullah and Adjid 2010) using Alul and Taql restriction enzymes confirming that the Indonesian isolates grouped with BHV-1.1. In this study, this region contains variations that included Alul and Taql sites, as well as palindromic and inverted sequences (TGGAGGTCCGAGCCAGCT), indicating that the samples should be assigned to subtype BHV-1.2. Notably, other work has revealed that the US region (within which gD is located) exhibits a high degree of sequence diversity.

The sequences analyzed were 211, 184, and 157 bp in length. The sequences were deposited in DDBJ (DNA Data Bank of Japan), and the accession numbers are LC318523-LC318532 and LC368822-LC368831. The gD primers were designed to yield a 511-bp amplicon; the UL36 primers were designed to yield a 211-, 184-, or 157-bp amplicon (Figure 1).
Figure 1. DNA sequence alignment of the UL36 partial open reading frame. The indel region is indicated by a 27-nt insertion (at a copy number of 2–5 presented as boxes outlined with black or red, and the background of blue, yellow, purple, and grey). The differentiation of the BHV-1.2 allele vs. BHV-1.1 allele is indicated by some degeneracy due to point mutation of C to T in the indel (red block letter, in sample B/1; B/31; B/32, and L/33). The dashes indicate spaces introduced to provide alignment among fragments of different lengths (due to the presence/absence of the indel).
Figure 2. (a) Sequence alignment of deduced amino acid (aa) sequence of the gD protein, extending from aa 137–306 of the full-length protein. Arrows indicate the positions of aa residues corresponding to the SNPs. Blue and red backgrounds indicate amino acids that differ between BHV-1.1 and BHV-1.2 (respectively). (b) Sequence alignment of the deduced aa sequence of the UL36 protein extending from aa 2428–2496 of the full-length protein. The arrow indicates the position of the aa residue that differs between BHV-1.1 and BHV-1.2. The red-outlined box indicates the sequence region corresponding to the indel. The black box indicates the sequences that are unique to BHV-1.2 as a result of the indel and the changed amino acid residue. Green, blue, yellow, and purple backgrounds indicate the positions of the repeated 9-aa peptide sequence encoded by the indel.
and Sharma 2012), although the possible function of this inversion remains unknown (Hammerschmidt et al. 1988). Several laboratories have hypothesized that this inversion plays a role in viral recombination, yielding different isomeric forms of the virus (Sheldrick and Berthelot 1975; Dutch et al. 1992; Schynts and Sharma 2012), although the possible function of this inversion remains unknown (Hammerschmidt et al. 1988). Several laboratories have hypothesized that this inversion plays a role in viral recombination, yielding different isomeric forms of the virus (Sheldrick and Berthelot 1975; Dutch et al. 1992; Schynts and Sharma 2012), although the possible function of this inversion remains unknown (Hammerschmidt et al. 1988).

An alignment of the UL36 fragments from the Indonesian samples (n = 10) with the corresponding sequences from the reference genomes (n = 8) suggested a shift in the position of the deleted region without changing the length of the missing nucleotides. The experimental samples showed mean ± SD identities of 95.28 ± 0.05% (BHV-1.1 vs. samples), 95.39 ± 0.09% (BHV-1.2 vs. samples) and 93.24 ± 0.12% (among samples). The indel in the Indonesian samples (B/1, B/31, B/32 and L/33) corresponded to a loss of 27 nt and exhibiting occasional degeneracy due to point mutations (C to T at nt 7610 and 7662 relative to accession no. AJ004801), while samples (L/5, L/6, L/9, L/10, P-252 and P-357) present of 27 nt (with a copy number 2–5) relative to accession no. AJ004801 (nt 7625–7651) (Figure 1). The 27-nt indel repeat in the BHV-1.1 samples is predicted to encode the peptide DAYPPAPAH (Figure 2(b)). In the BHV-1.2 samples (B/1, B/31, B/32, and L/33), the first of the aforementioned nucleotide substitutions (at nt 20 of the first repeat) results in an amino acid substitution of Pro to Leu, yielding the peptide DAYPPAH (where the underlined L indicates the altered aa sequence compared to that encoded by BHV-1.1). In contrast, the second substitution (at nt 18 of the second repeat) converts a GCC codon to GCT; because of the degeneracy of the code, both codons correspond to Ala, and the deduced peptide is unaltered compared to those encoded by the BHV-1.1 strains.

### 3.3. Phylogenogram variability of gD and UL36 fragments

Phylogenetic trees for the protein sequences are shown in Figure 3. The tree derived from the gD amino acid sequences (predicted from amplified gD fragments) (Figure 3(a)) suggested that all 10 of the novel Indonesian samples formed a clade separate from the BHV-1.1 reference strain. Specifically, the gD peptides encoded by the gD fragments from these samples were most similar to the BHV-1.2 reference strains, i.e. BHV-1.2 strain B589 from Australia (KM258881) and BHV-1.2 strain K-22 from the USA (KM258880). The gD peptide encoded by sample L/33 was identical to those encoded by BHV-1.2 reference strains SM023 (an American isolate) and SP1777 (a European isolate). Karlin et al. (1994) suggested that divergence at the genomic and protein levels can differ, given that the DNA sequence emphasizes sequence specificity. However, most studies employing phylogenetic tree construction are based on protein sequence comparisons (Karlin et al. 1994).

In contrast, the tree derived from the UL36 amino acid sequences (predicted from amplified UL36 fragments) (Figure 3(b)) exhibited a branching pattern distinct from that obtained with the gD peptides. Specifically, some samples that were affiliated with the BHV-1.2 group in the gD peptide-based tree (marked in Figure 3(a) with red stars) were instead affiliated with the BHV-1.1 group in the UL36 peptide-derived tree (Figure 3(b)). Notably, the UL36-based tree showed that samples L/5, L/6, L/9, L/10, P-252, and P-375 sorted with BHV-1.1 isolate 216 II (accession no. KY215944; a strain from India) rather than with isolate NVSL, Cooper strain and complete strain (accession no. JX898220, KU1984801, and AJ004801 respectively; from the USA and Europe). Genetic divergence at gD (US6) and UL36 appeared to differ. The indel in UL36 permitted differentiation of the novel isolates into two groups, while the SNPs in gD suggested that the new viruses formed a single group (based on protein sequence). These findings are supported by those of Karlin et al. (1994), who highlighted the evolutionary divergence of US compared to that of UL. In particular, the tegument protein (UL36) appears to have evolved more rapidly (as inferred from aa sequences) than proteins encoded by the US region (e.g. gD) (Karlin et al. 1994). In the present study, we observed a mixture of point mutations affecting palindromic and inverted sequences in a fragment of the gD ORF, which is located in the US region. However, the fragment of the UL36 ORF examined in this study contains a transposon (Robinson et al. 2008) that may affect the structure and/or function of the UL36 tegument protein (Möhl et al. 2010), with resulting effects on cellular phenotypes (Casacuberta and González 2013).
4. Conclusions

All 10 novel Indonesian field isolates of BHV-1 were classified as members of the BHV-1.2 subtype based on the deduced amino acid sequence of the protein encoded by an amplified fragment of the gD ORF. In contrast, the sequence of an amplified fragment of the UL36 ORF suggested that the novel strains be grouped differently than indicated by the gD sequence analysis. Given that the variation in UL36 was higher than that in gD, analysis of the UL36 sequence may be suited for detecting diversity among BHV-1 isolates. It concludes that the gD sequence analysis was suited for rough classification of BHV-1 subtype, the UL36 sequence permitted detection of BHV-1 subtype polymorphisms.

Disclosure statement

No potential conflict of interest was reported by the authors.

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