



The establishment of PCR amplification, cloning, and sequencing of bovine herpesvirus 1 (BHV-1) glycoprotein D gene isolated in Indonesia

Dewi Noor Hidayati^{1,4}, Eko Agus Srihanto², Tri Untari¹, Michael Haryadi Wibowo¹, Koichi Akiyama³, and Widya Asmara^{1,*}

¹Department of Microbiology, Faculty of Veterinary Medicine, Gadjah Mada University, Jalan Fauna No. 2, Karangmalang, Daerah Istimewa Yogyakarta 55281, Indonesia

²Veterinary Disease Investigation Lampung (Balai Veteriner Lampung), Jalan Untung Suropati No. 2, Labuhan Ratu, Kedaton, Bandar Lampung 35142, Indonesia

³Advanced Research Support Centre (ADRES), Ehime University, 3-5-7 Tarumi, Matsuyama, Ehime 790-8566, Japan

⁴PUSVETMA (Pusat Veteriner Farma), The Ministry of Agriculture of The Republic of Indonesia, Jalan Frontage Ahmad Yani Siwalankerto No.68–70, Ketintang, Gayungan, Surabaya 60231, Indonesia

*Corresponding author: wied_as@ugm.ac.id

SUBMITTED 19 March 2019 REVISED 14 May 2019 ACCEPTED 10 June 2019

ABSTRACT Considering the increasing incidence of infectious bovine rhinotracheitis (IBR) in Indonesia, it was necessary to conduct a more in-depth study of bovine herpesvirus-1 (BHV-1) as the causative agent of IBR disease. Previous research reports indicate that the BHV-1 subtypes found in Indonesia are subtype 1.1. Currently, IBR field case detection in Indonesia still uses the serological method (ELISA), which has the potential to give false positive results and cannot explain the virus subtype. Other detection methods, such as viral isolation, take longer and require adequate resources. This study aimed to determine the BHV-1 subtypes of Indonesian isolates using molecular techniques. Nested PCR using two pairs of primers was successfully used to amplify the glycoprotein D (gD) gene. The gD gene fragment was cloned into the pGEM-T plasmid. Analysis of the gD gene sequence was subsequently carried out to determine the BHV-1 character of the Indonesian isolates. The results indicated that the isolates were different from the previous isolates, and had similarities (100%) with subtype 1.2 strain SP1777 and SM023.

KEYWORDS bovine herpesvirus-1 (BHV-1); glycoprotein D; Indonesia; infectious bovine rhinotracheitis (IBR); subtypes

1. Introduction

Infectious bovine rhinotracheitis (IBR) disease was one of the major animal diseases in Indonesia because it inflicted considerable economic losses in the livestock sector. This disease caused a decrease in livestock performance, as was indicated by weight loss, low milk productivity, abortion, and the occurrence of specific symptoms including abortus, vulvovaginitis in heifers and balanoposthitis in bulls, and encephalitis (Turin et al. 1999). The causal agent of the disease was bovine herpesvirus-1 (BHV-1) (Turin et al. 1999), which is classified in the Alphaherpesvirus subfamily and the genus of *Varicellovirus*. BHV-1 causes latency conditions for the host. Following primary infection, the virus will be transported to a neuronal body cell and establish lifelong latency. When an animal is under stress or exposed to corticosteroidal drugs (Zhu et al. 2017), the virus can be reactivated. The cattle can show either some clinical or subclinical symptoms (Rola et al. 2005). Both can transmit viruses through secretions from genital infections, artificial insemination, nasal discharge, as well as embryos.

The BHV-1 genome consists of double-stranded DNA of the length of 138 kbp (Vlček et al. 1995). The genome of the virus can evolve by three systems: mutation during replication, acquisition, and recombination with other genes (Schyns et al. 2003b). The BHV-1 genome is grouped in D-class herpesvirus together with pseudorabies (PRV), VZV, and EHV-1 (Muyilkens et al. 2007). A variation in the genome of BHV-1 that consists of mutation and recombination would be possible to emerge. The rate of synonymous nucleotide substitution per site per year was estimated to be 2 to 30 times of the host genome (Schyns et al. 2003b). The glycoprotein D (gD) gene is underlying in the US segment, which is flanked at both ends by an inverted repeat and target for vaccine development (Dummer et al. 2014). The vaccine using gD subunit is most efficacious at reducing clinical disease and virus excretion (Muyilkens et al. 2007). Furthermore, there are vaccine markers by omitting some genes that encode the glycoprotein, such as gC, gE, gI, and gG (van Engelenburg et al. 1994; Kaashoek and Van Oirschot 1996; Strube et al. 1996), and the genes that encode nucleic acid metabolism (Kit et al. 1985; Smith et al. 1994). Some vaccine pro-

TABLE 1 Some advantages and disadvantages of several types of IBR vaccine around the world.

| Vaccine type | Advantages | Disadvantages | Country | Reference |
|-----------------------|--|---|-----------------------|---|
| Killed vaccine | Induces immune response; relatively safe for hospes; more stable; easy to produce; relatively insignificant side effects | High production cost; more than one application; requires adjuvant that it may result in hypersensitivity and urticaria; there is still possibility for latency; there is not any “companion test”; susceptible to storage problems; causes bias in incomplete inactivation | USA; Australia | Ackermann and Engels (2006); Babiuk (2002); van Donkersgoed et al. (1995) |
| Modified live vaccine | Induces fast immune response; action is similar to natural infection; long immunity duration | Does not prevent latency; causes virus shedding and abortus; there is not any “companion test”; can cause virus recombination; requires a big quantity of antigen; can react to other proteins or adjuvants; its immunity period is short; does not give any local immunity | Northern Ireland; USA | Ackermann and Engels (2006); Cowley et al. (2014); Babiuk (2002) |
| Vaccine subunit | Induces good immune response | High purification cost; requires adjuvant | European Union | Babiuk (2002) |
| Vaccine marker | Is temperature resistant; does not cause any virus shedding; induces good immunity | Requires companion test in testing | European Union | Raaperi et al. (2015); Babiuk (2002) |
| Multivalent vaccine | Cannot control immunomodulation and causes side effects | Gives immunity against some disease agents in a safer way; is practical and efficient; gives immunity | - | Babiuk (2002) |
| Vaccine DNA | Very safe; the endogenously produced antigen induces good immune response in children with passive antibodies | High cost; selective application rules because of its relationship with genetic material | - | Babiuk (2002) |

ducers in the world create IBR vaccines. Table 1 summarizes some advantages and disadvantages of the existing IBR vaccines. The IBR vaccines that are commercially available include killed/inactive vaccines, the modified live virus (MLV), subunit vaccine, marker vaccine, multivalent vaccine, and DNA vaccine. The MLV is not applied for any longer because of its disadvantages, including virus latency in cattle that enables virus re-activation and virus shedding. Though there are some vaccines produced on the basis of their application, such as an intranasal application that prevents abortus in pregnant cattle, the vaccine is also not applied for any longer because its management cost is very high as a result of the screening of the pregnant cattle that should be conducted in advance. The killed vaccine is considered safer than the ML vaccine because it does not result in latency and reduces virus shedding. However, the production cost of the killed vaccine is high, while it should be applied repeatedly and requires adjuvant that may cause hypersensitivity and urticaria. The possibly incomplete inactivation process can cause a new infection. Cattle vaccinated with the killed vaccine may be re-infected by the virulent virus after a certain period of time and it causes virus shedding and spreads the infection in the cattle group (Kit et al. 1985). The application of the subunit vaccine can prevent the transmission of the disease to other animals because antigenically the subunit vaccine does not contain any live virus. It also does not cause any latency and any abortus in animals. The subunit vaccine can prevent infection symptoms and virus shedding. The disadvantages of the vaccine are that it cannot induce a good immune response in young animals, its pu-

rification cost is high, and it requires some adjuvants (Kit et al. 1985).

Based on molecular studies, BHV-1 consists of subtype 1.1; subtype 1.2a and subtype 1.2b (Metzler et al. 1985). Several years ago, BHV-1 subtypes included BHV-1.3, a cause of encephalitis in calves (Collins et al. 1993). More recently, BHV-1.3 was classified as BHV-5. A further study divided BHV-5 into subtype 5a, 5b, “non-a non-b” and c (Pidone et al. 1999). Subtype 1.1 is found predominantly in Europe and the USA (Spilki et al. 2004). Subtype 1.2 consists of two further subtypes, 1.2a and 1.2b. Subtype 1.2a is prevalent in Brazil and was present in Europe before 1970. Subtype 1.2b was reported in Australia and Europe, but not in Brazil (Jones and Chowdhury 2007). A study by Saepulloh et al. (2009) showed that isolates obtained from Java, Indonesia, turned to subtype 1.1. Until now, Indonesia has based the technology of making vaccines on whole viruses. Vaccine technology based on genetic engineering such as sub-unit vaccines, vaccine markers, or markers by removing several parts of the viral genes (especially for IBR disease) has not been investigated, as it requires large resources and supporting policies. Indonesia is still preparing itself for the application of this technology. At present, the government is planning to make IBR inactive vaccines from local isolates. Research on making inactive vaccines has been conducted by the Veterinary Disease Research Center (Balai Besar Penelitian Veteriner, Balitvet), but the local vaccine has not given any optimal protection (Sudarisman 2006).

The symptoms caused by subtype 1.1 and subtype 1.2 can show infection in both the respiratory and genital

tracts of the animal, which is different from the previous hypothesis that stated respiratory symptoms are caused more by subtype 1.1 and genital symptoms caused by subtype 1.2 (Muylkens et al. 2007). The difference between virus subtype 1.2a and subtype 1.2b is the presence or absence of abortion in cattle. One study of BHV-1 isolated from several cases in Java indicated that the subtype developed in Indonesia was subtype 1.1 (Saepulloh and Adjid 2010). By considering the increasingly widespread incidence of the disease, it was necessary to conduct a study on the molecular characteristics of the viruses, especially those coming from outside Java. It was also essential to find out the possibility of other subtypes in Indonesia as the basis for the investigation of exotic diseases in Indonesia. The difference in the subtype is useful both as study material and for the clustering of the virus's origin. At the same time, the detection of the condition using serological methods is inadequate in terms of establishing the characteristics of an isolate.

Currently, for monitoring purposes, the investigation center still uses serological methods for disease detection. The serological screening included ELISA and serum neutralization (SN) tests (Parreño et al. 2010). However, these tests have advantages and disadvantages. ELISA requires considerable time and resources. To obtain more valid results, two rounds of ELISA testing are required (especially for positive initial results), by comparing the titer within 21 days. An increase in antibody titers indicates an acute infection or reactivation. If serum antibodies decrease, it can be considered that the disease is latent (Turin et al. 1999). A serum neutralization test also requires a long testing time and vast resources (Bashir et al. 2011). The SN test processes include cell culture and antibody testing in susceptible cells. However, between these two serological methods, ELISA provides the higher sensitivity. ELISA may result not only from neutralization antibodies such as immunoglobulin but also other non-neutralization antibodies (Payment et al. 1979). Otherwise, the SN test does not depend on the type and the subtype of the viruses that develop in the field (Varela et al. 2010).

The detection of diseases using serological methods is not enough in establishing the characteristics of an isolate. PCR tests are now preferable to serological tests because they have higher sensitivity and specificity. This study aimed to provide better information of the characteristics of BHV-1 isolated from a recent field case and to describe the development of the detection methods using PCR cloning and sequencing of the BHV-1 isolate to define the subtype.

2. Materials and methods

This research was permitted by the Committee for Safe Handling of Living Modified Organism in Ehime University (permission number: H28-05) and accomplished according to the guidelines of the committee. The samples of the study were drawn from the tracheal section in field cases from Lampung, Indonesia, as previously reported (Hidayati et al. 2018). The samples were collected from

Veterinary Disease Investigation Lampung (Balai Veteriner Lampung), based on the permission letter of material transfer number 05010/PD.650/F5.H/06/2015. The preparation of the sample organ was maintained in HBSS solution (Gibco, Life Technologies, Grand Island, USA). The organ was sterilized by washing it three times using phosphate buffer saline (PBS) solution. The initial wash used a PBS mixture with antibiotics (1000 IU/mL Penicillin, 1000 µg/mL Penicillin-Streptomycin sulfate) and Kanamycin (Meiji, PT Meiji Indonesian Pharmaceutical Industries, Bangil, Indonesia). In a glass mortar, samples were crushed using a mixture of sterilized quartz sand and DMEM (Gibco, Life Technologies, Grand Island, USA). The solution containing the crushed organ was centrifuged at 1000 rpm for 5 min, and the supernatant was filtered using a Sartorius filter 0.2 µm. The aqueous filtering preparation was used in DNA extraction and viral-isolation.

2.1. Virus isolation

Primary Madin Darby Bovine Kidney (MDBK) monolayer cell free of bovine viral disease (obtained from PUSVETMA, Indonesia) was grown in Dulbecco's Modified Eagle medium (DMEM, Gibco, Life Technologies, Grand Island, USA) supplemented with 2 mM l-glutamine (Gibco, Life Technologies, Grand Island, USA), 10% fetal bovine serum (FBS, Sigma–Aldrich, St. Louis, USA), 1000 µg/mL streptomycin, 1000 IU/mL penicillin, and 250 µg/mL kanamycin (PT. Meiji Indonesian Pharmaceutical Industries, Bangil, Indonesia). Two flasks of the cell line were used. One milliliter of virus suspension was inoculated into the first flask, while the other was used as the control. The flask containing the suspension of the virus was incubated for an hour at 37°C to allow the virus to adsorb the monolayer cell. Then media containing 2% FBS was added into the flask. After one hour of incubation, the culture was stored at 37°C for 3–5 days and followed by daily observation for a cytopathogenic effect (CPE).

2.2. DNA extraction

The DNA extraction was prepared based on the manufacturer's protocol (Qiagen, cat. no. 61304, Hilden, Germany). The final nucleic acid was eluted in 0.2 mL elution buffer consisting of 0.5 mM EDTA and 10 mM Tris-Cl at pH 9.0. The concentration of the DNA in the DNA extraction was measured using a BioSpec-nano spectrophotometer at 260/280 nm (Shimadzu Biotech, Japan).

TABLE 2 The primer used in the study.

| Primer name | Sequence | PCR product length |
|-------------|------------------------------|--------------------|
| gD-F1 | GCTGTGGGAAGCGGTACG | 468 bp |
| gD-R1 | GTCTACTATGGCCTTGTGTGC | |
| gD-F2 | ACGGTCATATGGTACAAGATCGAGAGCG | 325 bp |
| gD-R2 | CAAAGGTGTACCCGCGAGCC | |

2.3. Nested PCR

PCR was carried out using two pairs of primer and two-phase PCR. The primer sequences are shown in Table 2 (Wiedmann et al. 1993). Each step consisted of 2.5 mM MgCl₂, 0.3 mM for each dNTPs, 1 U of DNA polymerase, 10 pmol forward and reverse primer, template DNA of less than 100 ng per reaction, and PCR grade water up to 50 µL. The PCR cocktail was formulated in accordance with the KAPA HiFi HotStart ReadyMix (KAPA Biosystems, Wilmington, USA) guidance. The first phase was carried out with an initial denaturation at 95°C for 3 min, denaturation at 98°C for 20 s, annealing at 53°C for 15 s in 35 cycles, extension at 72°C for 15 s, and the final extension at 72°C for 5 min. The second phase was similar, except for the annealing temperature, which was at 59.5°C for 15 s. The PCR assay followed using a Takara Thermal Cycler Dice Gradient TP600 (Takara, Shiga, Japan). The PCR product was evaluated using gel agarose at a concentration of 1.5%. The electrophoresis ran on 100 A. The agarose was soaked in ethidium bromide for about 15 min before UV transillumination visualization.

2.4. PCR cloning and sequencing

The second round PCR product was added with 10x A-attachment (Toyobo, Osaka, Japan) and was purified using the Fast gene gel/PCR extraction kit (Nippon Genetics, Tokyo, Japan). The DNA fragment was then ligated into plasmid pGEM-T (Promega, Madison, USA) by T4-ligase enzyme (Promega, Madison, USA). The plasmid was transformed into competent *E. coli* C3040 (NEB® stable, High efficiency, New England BioLabs, Ipswich, USA) and incubated overnight at 37°C. After incubation, the white and smooth colony was picked up with a sterilized toothpick and transferred into LB broth media containing 100 µL ampicillin per mL and incubated at 37°C overnight with constant shaking. After preparation by chemistry lysis (Sambrook et al. 1989), the plasmid was confirmed using PCR amplification with the designated

primers. The cloned plasmid was purified using the Fast Gene plasmid mini kit (Nippon Genetics, Tokyo, Japan) and the purity level was measured using a BioSpec-nano spectrophotometer (Shimadzu, Kyoto, Japan). The high concentration plasmid was digested using ScaI (Takara Bio, cat. no. 1084A, USA,) for 60 min at 37°C, and 20 min at 80°C. DNA sequencing was carried out using a Applied Biosystems 3130 Genetic Analyzer (Applied Biosystems, Foster City, USA) and BigDye Terminator V3.1 cycle sequencing kit at the annealing temperature of 50°C with the M13 primer method. The BLAST software was used to confirm the similarity of the sample with the alphaherpesvirus. The sequence data were analyzed using Seq Man from the DNASTAR Lasergene software (version 7). The alignment was made using reference genes retrieved from a gene bank with accession numbers AJ004801.1, KU198480.1, JX898220.1, KY215944, KM258882.1, and KM258883.1. The nucleotide sequence was deposited in the DNA Data Bank of Japan (DDBJ) with accession number LC425527.

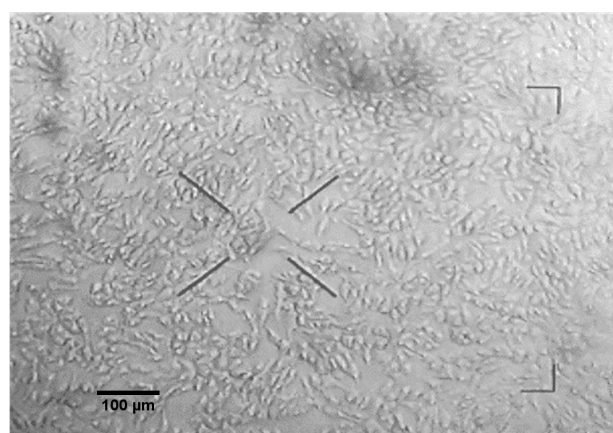
2.5. Sequence analysis and in silico restriction enzyme analysis

Analysis of the in silico restriction enzyme was carried out using TaqI and AluI provided by the SeqBuilder program (DNASTAR Lasergene ver 7). TaqI will cut (5') TC-GA (3') sequences, and AluI will cut (5') AG-CT (3') sequences. Sequence analysis and the construction of a phylogenetic tree were performed using the MegAlign program (DNASTAR Lasergene ver 7).

3. Results

3.1. Cytopathogenic effect (CPE) of virus culture

According to Nandi et al. (2009), the CPE of specific BHV-1 is characterized by a grape-like cluster. The cytopathogenic effect was observed on day three after inoculation. The more vacuoles found and followed by cell



(a)



(b)

FIGURE 1 (a) Sample W5 after three days of observation. The CPE was observed as a grape-like cluster. Pointer indicates cleft around monolayer. (b) The control of the MDBK cell line after three days of observation. The pictures were taken using an inverted microscope (Nikon Eclipse, TE 2000-U, Japan) at the same magnification (10x).

rupture. The visualization of the CPE is shown in Figure 1a. The grape-like cluster of the globular cell engirdled around the cleft in the monolayer, which resulted in a very different visualization of control cells that were not inoculated by the virus (Figure 1b).

3.2. PCR cloning and sequencing

The sample showed positive amplification of the glycoprotein D gene, which was made using the nested PCR method. Cloning and sequencing procedures followed, in order to obtain readable sequencing results considering that some bands were very thin (Hidayati et al. 2018). The PCR product of the second phase of amplification was successful, ligated in the pGEM-T. The white colony of the plasmid was spread evenly on the LB plate, as show in Figure 2. Ten colonies were picked up from the plate to the culture in the LB broth media to multiply the plasmids. After incubation overnight and purification (Fast Gene, Nippon Genetics, Japan), the plasmids were run in gel agarose electrophoresis to confirm the positive result using the second primer. The cloned plasmids (no. 9 and 10, Figure 3) were about 325 bp (positive). This recombinant plasmid

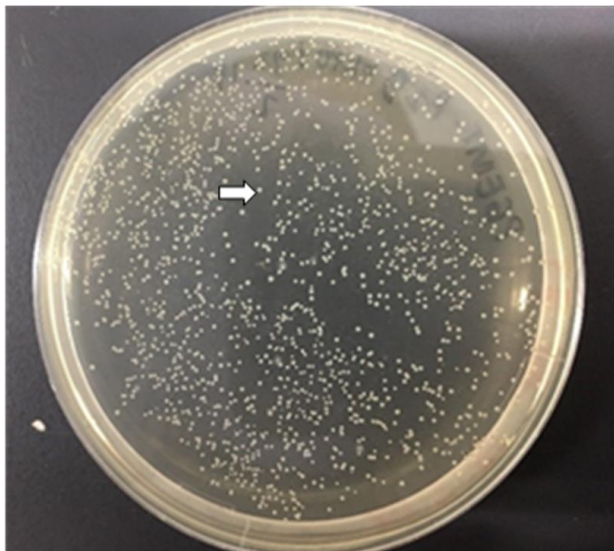


FIGURE 2 The colony of the transformant after 18 hours of incubation.

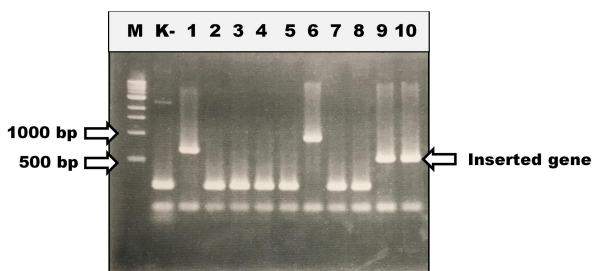


FIGURE 3 The confirmation of the inserted gene by gel agarose electrophoresis. The length of the inserted gene is about 325 bp of the targeted gene plus approximately 141 bp of pGEM-T multiple cloning region, resulted of about 466 bp).

was purified using the Fast Gene (Nippon Genetics, Japan) plasmid mini kit and was cut with the ScaI restriction enzyme to obtain a linear band. The sample sequence and reference gene were aligned using MegAlign (DNASTAR Lasergene ver. 7). The electropherogram result of the cloned fragment is shown in Figure 4. The nucleotide phylogenetic tree was reconstructed using DNASTAR Lasergene, as shown in Figure 5. The Bootstrap 1000x was found out using the neighbor-joining method. The branch lengths were measured using the Kimura method.

3.3. Sequence analysis

The region of gD (nt 118129 – 118446 based on ORF of ref. no. KU198480.1) consisted of about 318 nt. The G+C content was 64%. The alignment result showed SNPs that distinguished BHV-1.1 and BHV-1.2 at four sites (nt 118258, 118281, 118422, 118427 of ORF ref. no. KU198480.1) (Table 3). Only a transition mutation occurred (A-G and T-C). The resulting alignment of the sequence showed a high similarity between BHV-1.1, BHV-1.2, and the sample. It confirmed the mean ± SD identities of 98.70% ± 0 (BHV-1.1 vs. sample) and 100% ± 0 (BHV-1.2 vs. sample). The calculation of the synonymous and nonsynonymous ratio (dn/ds) was 0.34 (dn/ds<1).

3.4. In silico restriction enzyme analysis

Restriction enzyme analysis using in silico AluI and TaqI showed a number of different intersection points between subtype 1.1 and subtype 1.2, as presented in Table 4. Subtype 1.1 showed two points of intersection with AluI (nt 126 and 163 of the gD sample), while TaqI showed four intersection points (nt 21, 198, 228, and 290 of the gD sample). Subtype 1.2 showed three points (nt 126, 163, and 295 of the gD sample) of intersection using AluI and five points using TaqI (nt 21, 198, 228, 290, and 297 of the gD sample). The sample showed an intersection map of the restriction enzyme that was the same as that of subtype 1.2.

4. Discussion

Viral isolation aims to determine the presence of a virus in a specimen. It assumes viral positive if any particular CPE is shown in the monolayer of a susceptible cell. In this study, the CPE was observed on day three, as indicated by retractile and globular cells to corners and the formation of a grape-like cluster (Nandi et al. 2009). Cells

TABLE 3 Nucleotide polymorphism analysis.

| Position along sequence based on ORF of ref. no. KU198480.1 | Nucleotide comparison to subtype 1.1 | Polymorphism |
|---|--------------------------------------|--------------|
| 118258 | T → C | Transition |
| 118281 | G → A | Transition |
| 118422 | A → G | Transition |
| 118427 | G → A | Transition |

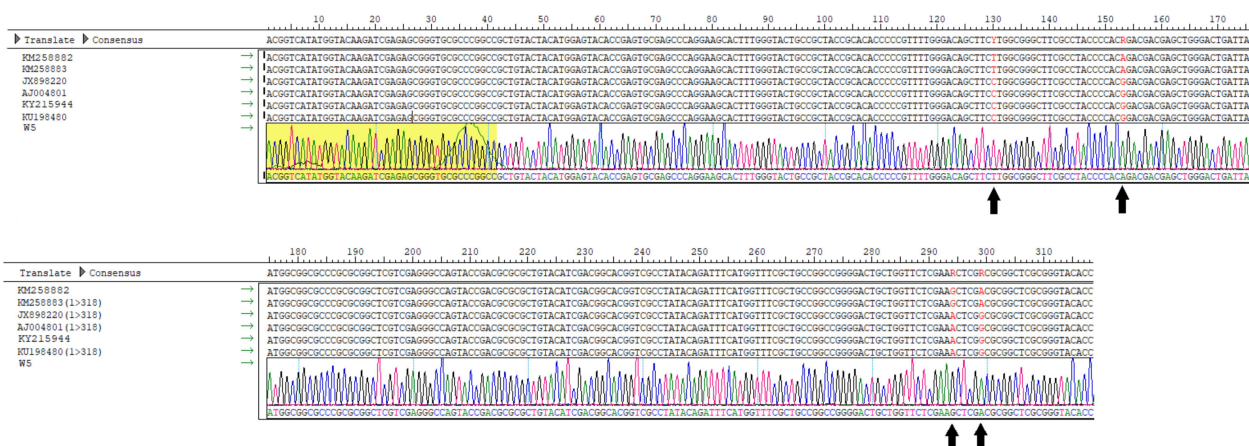


FIGURE 4 Analysis of electropherogram which indicated the point of mutation (arrows). The fragment consists of about 325 bp. The yellow background states the process of an initial sequence of dye terminator.

will experience rupture over time, which is consistent with Weiblen et al. (1992). The presence of the globular cells that subsequently burst is a sign of cell damage because of virus replication. Other CPE occurred with the formation of more plaque. The nucleocapsids in the cytoplasm are transported to the Golgi apparatus that small vacuoles form, which contains enveloped virions and is transported to the plasma membrane for the exocytosis process. The development of apoptosis and necrosis give rise to the death of the cell (Muylkens et al. 2007).

The fusion of the virus initiates apoptosis, which induces the decrease of the CD4+ of T cells. Therefore, BHV-1 infection disrupts the immune response and causes a BRDC infection (Jones and Chowdhury 2007). The MDBK control cells still seemed to be intact. The MDBK cells were susceptible to BHV-1 infection. They can express glycoprotein D of the BHV-1 and resistant to the interference of other heterologous viruses such as HHV-1 (human herpesvirus-1) and SuHV-1 (suid herpesvirus-1). BHV-1 enters the cells through natural bonding between the glycoprotein B and glycoprotein C with the heparin sulfate receptor followed by the bond between the glycoprotein D and specific cellular receptor and causes virus fusion with the plasma membrane. Through this mechanism, the virus penetrates the susceptible cells. Glycoprotein D plays an essential role as a receptor-mediated agent for the viral antigen to enter the cells. Glycoprotein D is also a crucial gene in cell-to-cell spread. BHV-1 can intracellularly enable cell-to-cell spread, causing an inability of the antibody to prevent the spread of the virus (Babiuk et al. 1996). The CPE of the sample was found at the first passage on the MDBK cell line. For detection purposes, the specimen

was considered to be negative after three times passages. In the cells infected by a herpes virus, virus replication takes place in the cell nucleus. The intranuclear formation can be observed, which enlarges the presence of an inclusion body of Cowdry type A (inclusion body, observable with eosinophil staining). The nucleus produces nucleocapsids for virion and will be discharged through nuclear pores (nucleus membrane holes) into the cytoplasm. The nucleus experiences pyknosis, and the cell cytoplasm decreases followed by karyorrhexis (Studdert 2008).

This study aimed to develop robust detecting methods of field case based on the amplification of the gD gene with an excellent quality of molecular sequence data. The research process is described in Figure 6. The nested PCR (nPCR) method used a pair of primers (Rola et al. 2005). Nested PCR can overcome a low quality of DNA (Aswad and Katzourakis 2014). This method also offers much more sensitive detection than viral isolation (Babiuk et al. 1996), where nested PCR is able to detect 100–1000 fold more sensitively than viral isolation (Masri et al. 1996). An amplified gene of 325 bp was observed, with no false positive result. The fragment was ligated into the pGEM-

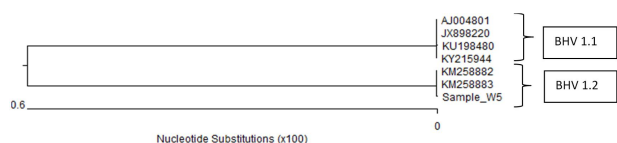


FIGURE 5 The result of the phylogenetic tree of the sample (W5) and the reference genes. Sample W5 is grouped in BHV-1.2.

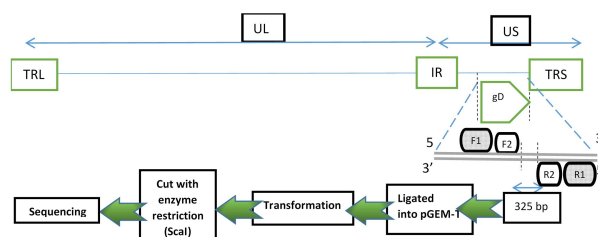


FIGURE 6 The whole genome of BHV-1. The genome is classified into two parts of Unique Long (UL) segment of the length of about 106 kb and Unique Short (US) segment of the length of about 10 kb. The US is enclosed by the internal repeat (IR) and terminal repeat (TR) short that are reverted to each other. The UL also enclosed by the IR and terminal repeat long (TRL) and often observed in a single orientation. The glycoprotein D (gD) gene was in the US region. The amplification of 325 bp of gD was made using two pairs of primer (nested PCR). The amplified gene was inserted into pGEM-T and cloned into a competent cell. The plasmid was cut using the ScaI restriction enzyme before being subjected to sequencing.

TABLE 4 The results of restriction enzyme analysis using in silico AluI and TaqI.

| Group | Sequence | gD restriction enzyme | |
|-------------|-----------|-----------------------|-------------------------------|
| | | AluI | TaqI |
| Subtype-1.1 | AJ004801 | 2 (nt 126, 163) | 4 (nt 21, 198, 228, 290) |
| | KU198480 | 2 (nt 126, 163) | 4 (nt 21, 198, 228, 290) |
| | JX8982201 | 2 (nt 126, 163) | 4 (nt 21, 198, 228, 290) |
| | KY2159441 | 2 (nt 126, 163) | 4 (nt 21, 198, 228, 290) |
| Subtype-1.2 | KM258882 | 3 (nt 126, 163, 295) | 5 (nt 21, 198, 228, 290, 297) |
| | KM258883 | 3 (nt 126, 163, 295) | 5 (nt 21, 198, 228, 290, 297) |
| Samples | W/5 | 3 (nt 126, 163, 295) | 5 (nt 21, 198, 228, 290, 297) |

T and cloned to competent *E. coli* C3040. The molecular cloning of the specific plasmid could control the enhancement quality of the DNA.

We used the gD gene sequence with a primer design by Wiedmann et al. (1993). Subsequently, the cloning result was cut using the ScaI restriction enzyme. The ScaI restriction enzyme enables natural DNA linearization by cutting the plasmid DNA on a point AGT-ACT. Based on our experiences during the study, the plasmid that has been linearized using the ScaI enzyme is more accessible to sequence and gives excellent results, possibly due to the high GC content in the genome sequence of BHV-1. The GC content caused stable bonding within its nucleotide. The GC content of the sequence alignment was 64%, similar to suid herpesvirus and human herpesvirus (70%) (Dummer et al. 2014). Davison and Clements (2010) stated that the genome of BHV-1 was 125–240 kbp and had a nucleotide composition of GC that varied from 32 to 74%.

The sequence had a high similarity with BHV-1.2 strain SM023 (ref. no. KM258882.1) and BHV-1.2 strain SP1777 (KM258883.1). The mutation along this sequence occurred at four points of mutation, as shown in Table 3. The transition changed the purine nucleotide (A to G) and pyrimidine nucleotide (T to C). The transition more frequently takes place along the sequence as compared to the transversion, but the transversion is more capable in determining the difference in the genetic distance compared with the transition. The result of synonymous and nonsynonymous ratio indicated that this sequence has a negative selection, meaning that the sequence has experienced purification selection (Traesel et al. 2014).

This study also used the restriction enzyme prediction AluI and TaqI to proof the similarity of the sample and reference genes. Restriction enzyme analysis, particularly AluI and TaqI, was used to confirm the specificity of the sequence (Rola et al. 2005). Previously, restriction enzyme analysis site was used to differentiate the type and subtype of BHV-1 (Metzler et al. 1985; Spilki et al. 2004). Previous researchers also used this method to define the subtype of BHV-1 in Java, Indonesia (Saepulloh and Adjid 2010). It resulted in two fragments: 231 and 133 bp by TaqI, and 322 and 124 bp by AluI. They classified samples from Java in subtype 1.1. This study used a recent example

from Lampung, and revealed a different result. Previous research by Noor et al. (2019) resulted in a mixed allele using the AluI and TaqI restriction enzymes. The restriction endonuclease analysis (REA) methods could have instability because the genomic changes of the virus inside the host body, resulting from various tissue sources. However, the BHV-1 genome has four isomeric forms, generated from a flip-flop direction during DNA replication (Schyns et al. 2003a). It showed three points of intersection with AluI and five intersection points with TaqI. This study also revealed that the similarity between the sample and reference gene was 98.7–100%; the restriction enzyme mapping of the samples was similar to that of the BHV-1.2 reference gene. However, antigenically, the BHV-1.1 subtype had a high similarity with subtype 1.2 (98.7–99.8%), as stated by Esteves et al. (2008). The genetic distance of a sequence to other sequences can be measured using evolutionary distances reconstructed by variability within the nucleotide sequence (Turin et al. 1999). The phylogenetic tree clearly showed that the sample was grouped in the same branch as subtype 1.2.

5. Conclusions

Based on molecular analysis using SNP and in silico REA using AluI and TaqI, the BHV-1 of an Indonesian isolate found in a recent case was very similar to subtype 1.2.

Acknowledgments

The authors would like to extend their gratitude to the Director of the Advanced Research Support Centre (ADRES), Ehime University, Japan, the Director of Animal Health, the Ministry of Agriculture of the Republic of Indonesia for promoting data of the research; the Head of Veterinary Disease Investigation Lampung for the samples; and the Head of PUSVETMA for facilitating the cell culture. This study was supported by the Japan Student Service Organization.

Authors' contributions

DNH established the objectives of the study, designed, planned, EAS supported the molecular analysis, TU,

MHW, AK, and WA and supervised the experiments and corrected the manuscript. All of the authors read and approved the final manuscript.

Competing interests

The authors state that they have no competing interest.

References

- Ackermann M, Engels M. 2006. Pro and contra IBR-eradication. *Vet Microbiol.* 113(3-4):293–302. doi:10.1016/j.vetmic.2005.11.043.
- Aswad A, Katzourakis A. 2014. The first endogenous herpesvirus, identified in the tarsier genome, and novel sequences from primate rhadinoviruses and lymphocryptoviruses. *PLoS Genetics.* 10(6):e1004332. doi:10.1371/journal.pgen.1004332.
- Babiuk L, van Drunen Littel-van den Hurk S, Tikoo S. 1996. Immunology of bovine herpesvirus 1 infection. *Vet Microbiol.* 53(1–2):31–42. doi:10.1016/S0378-1135(96)01232-1.
- Babiuk LA. 2002. Vaccination: A management tool in veterinary medicine. *Vet J.* 164(3):188–201. doi:10.1053/tvj.2001.0663.
- Bashir S, Singh R, Sharma B, Yadav SK. 2011. Development of a sandwich ELISA for the detection of bovine herpesvirus type 1. *Asian Pac J Trop Med.* 4(5):363–366. doi:10.1016/s1995-7645(11)60104-1.
- Collins JK, Ayers VK, Whetstone CA, van Drunen Littel-van den Hurk S. 1993. Antigenic differences between the major glycoproteins of bovine herpesvirus type 1.1 and bovine encephalitis herpesvirus type 1.3. *J Gen Virol.* 74(8):1509–1517. doi:10.1099/0022-1317-74-8-1509.
- Cowley DJB, Graham DA, Guelbenzu M, Doherty ML, More SJ. 2014. Aspects of bovine herpesvirus 1 and bovine viral diarrhoea virus herd-level seroprevalence and vaccination in dairy and beef herds in Northern Ireland. *Ir Vet J.* 67(1):18. doi:10.1186/2046-0481-67-18.
- Davison AJ, Clements JB. 2010. Herpesviruses: general properties. In: Mahy BW, ter Meulen V, Borriello SP, Murray PR, Funke G, Kaufmann SH, Steward MW, Merz WG, Hay RJ, Cox F, Wakelin D, Gillespie SH, Despommier DD, Borriello SP, Murray PR, Funke G, editors. *Topley & Wilson's microbiology and microbial infections.* Chichester: John Wiley & Sons. doi:10.1002/9780470688618.taw0231.
- Dummer LA, Leite FPL, van Drunen Littel-van den Hurk S. 2014. Bovine herpesvirus glycoprotein D: a review of its structural characteristics and applications in vaccinology. *Vet Res.* 45(111):1–12. doi:10.1186/s13567-014-0111-x.
- Esteves P, Dellagostin O, Pinto L, Silva A, Spilki F, Ciacci-Zanella J, Hübner S, Puentes R, Maisonnave J, Franco A, Rijsewijk F, Batista H, Teixeira T, Dezen D, Oliveira A, David C, Arns C, Roehe P. 2008. Phylogenetic comparison of the carboxy-terminal region of glycoprotein C (gC) of bovine herpesviruses (BoHV) 1.1, 1.2 and 5 from South America (SA). *Virus Res.* 131(1):16–22. doi:10.1016/j.virusres.2007.08.004.
- Hidayati DN, Untari T, Wibowo MH, Akiyama K, Asmara W. 2018. Cloning and sequencing gB, gD, and gM genes to perform the genetic variability of bovine herpesvirus-1 from Indonesia. *Vet World.* 11(9):1255–1261. doi:10.14202/vetworld.2018.1255-1261.
- Jones C, Chowdhury S. 2007. A review of the biology of bovine herpesvirus type 1 (BHV-1), its role as a cofactor in the bovine respiratory disease complex and development of improved vaccines. *Anim Health Res Rev.* 8(2):187–205. doi:10.1017/s146625230700134x.
- Kaashoek MJ, Van Oirschot JT. 1996. Early immunity induced by a live gE-negative bovine herpesvirus 1 marker vaccine. *Vet Microbiol.* 53(1-2):191–197. doi:10.1016/s0378-1135(96)01247-3.
- Kit S, Qavi H, Gaines JD, Billingsley P, McConnell S. 1985. Thymidine kinase-negative bovine herpesvirus type 1 mutant is stable and highly attenuated in calves. *Arch Virol.* 86(1-2):63–83. doi:10.1007/bf01314114.
- Masri SA, Olson W, Nguyen PT, Prins S, Deregt D. 1996. Rapid detection of bovine herpesvirus 1 in the semen of infected bulls by a nested polymerase chain reaction assay. *Can J Vet Res.* 60(2):100–107.
- Metzler AE, Matile H, Gassmann U, Engels M, Wyler R. 1985. European isolates of bovine herpesvirus 1: A comparison of restriction endonuclease sites, polypeptides, and reactivity with monoclonal antibodies. *Arch Virol.* 85(1-2):57–69. doi:10.1007/bf01317006.
- Muylkens B, Thiry J, Kirten P, Schynts F, Thiry E. 2007. Bovine herpesvirus 1 infection and infectious bovine rhinotracheitis. *Vet Res.* 38(2):181–209. doi:10.1051/vetres:2006059.
- Nandi S, Kumar M, Manohar M, Chauhan RS. 2009. Bovine herpes virus infections in cattle. *Anim Health Res Rev.* 10(1):85–98. doi:10.1017/s1466252309990028.
- Noor HD, Tri U, Haryadi WM, Widya A, Koichi A. 2019. Dna sequence variability analysis of the gD and the UL36 genes of bovine herpesvirus-1 isolated from field cases in Indonesia. *J Appl Anim Res.* 47(1):206–211. doi:10.1080/09712119.2019.1600521.
- Parreño V, López MV, Rodriguez D, Vena MM, Izuel M, Filippi J, Romera A, Faverin C, Bellinzoni R, Fernandez F, Marangunich L. 2010. Development and statistical validation of a guinea pig model for vaccine potency testing against infectious bovine rhinotracheitis (IBR) virus. *Vaccine.* 28(13):2539–2549. doi:10.1016/j.vaccine.2010.01.035.
- Payment P, Assaf R, Trudel M, Marois P. 1979. Enzyme-linked immunosorbent assay for serology of infectious bovine rhinotracheitis virus infections. *J Clin Microbiol.* 10(5):633–636.
- Pidone CL, Galosi CM, Echeverria MG, Nosetto EO, Etcheverrigaray ME. 1999. Restriction endonuclease analysis of BHV-1 and BHV-5 strains isolated in

- Argentina. *J Vet Med Ser B*. 46(7):453–456. doi:10.1046/j.1439-0450.1999.00257.x.
- Raaperi K, Orro T, Viltrop A. 2015. Effect of vaccination against bovine herpesvirus 1 with inactivated gE-negative marker vaccines on the health of dairy cattle herds. *Prev Vet Med*. 118(4):467–476. doi:10.1016/j.prevetmed.2015.01.014.
- Rola J, Larska M, Polak MP. 2005. Detection of bovine herpesvirus 1 from an outbreak of infectious bovine rhinotracheitis. *Bull Vet Inst Pulawy*. 49:267–271.
- Saepulloh M, Adjid RMA. 2010. Isolasi dan identifikasi bovine herpesvirus-1 pada sapi perah dan potong di Indonesia [Isolation and identification of bovine herpesvirus-1 in Indonesian dairy and beef cattle]. *Jurnal Ilmu Ternak dan Veteriner*. 19(2):376–387.
- Saepulloh M, Wibawan IWT, Sajuthi D, Setiyaningsih S. 2009. Karakterisasi molekuler bovine herpesvirus type 1 isolat Indonesia [Molecular characterization of Indonesian isolate of bovine herpesvirus type 1]. *Jurnal Ilmu Ternak dan Veteriner*. 14(1):66–74.
- Sambrook J, Fritsch EF, Maniatis T. 1989. *Molecular cloning: a laboratory manual*. 2nd edition. New York: Cold Spring Harbor Laboratory Press.
- Schynts F, McVoy MA, Meurens F, Detry B, Epstein AL, Thiry E. 2003a. The structures of bovine herpesvirus 1 virion and concatemeric DNA: implications for cleavage and packaging of herpesvirus genomes. *Virology*. 314(1):326–335. doi:10.1016/s0042-6822(03)00437-9.
- Schynts F, Meurens F, Detry B, Vanderplassen A, Thiry E. 2003b. Rise and survival of bovine herpesvirus 1 recombinants after primary infection and reactivation from latency. *J Virol*. 77(23):12535–12542. doi:10.1128/jvi.77.23.12535-12542.2003.
- Smith GA, Young PL, Rodwell BJ, Kelly MA, Storie GJ, Farrah CA, Mattick JS. 1994. Development and trial of a bovine herpesvirus 1-thymidine kinase deletion virus as a vaccine. *Aust Vet J*. 71(3):65–70. doi:10.1111/j.1751-0813.1994.tb03329.x.
- Spilki FR, Esteves PA, de Lima M, Franco AC, Chiminazzo C, Flores EF, Weiblen R, Driemeier D, Roehe PM. 2004. Comparative pathogenicity of bovine herpesvirus 1 (BHV-1) subtypes 1 (BHV-1.1) and 2a (BHV-1.2a). *Pesqui Vet Bras*. 24(1):43–49. doi:10.1590/s0100-736x2004000100010.
- Strube W, Auer S, Block W, Heinen E, Kretzdorn D, Rodenbach C, Schmeer N. 1996. A gE deleted infectious bovine rhinotracheitis marker vaccine for use in improved bovine herpesvirus 1 control programs. *Vet Microbiol*. 53(1-2):181–189. doi:10.1016/s0378-1135(96)01246-1.
- Studdert MJ. 2008. Bovine herpesviruses. In: Mahy B, van Regenmortel M, editors. *Desk encyclopedia of animal and bacterial virology*. Oxford: Academic Press. p. 65–71.
- Sudarisman. 2006. Tingkat efikasi berbagai vaksin IBR inaktif yang dibuat menggunakan virus isolat lokal pada sapi perah di Kabupaten Bandung yang diuji dengan uji serum netralisasi. *Jurnal Veteriner*. 7(4):139–147.
- Traesel CK, e Silva MS, Weiss M, Spilki FR, Weiblen R, Flores EF. 2014. Genetic diversity of 3' region of glycoprotein D gene of bovine herpesvirus 1 and 5. *Virus Genes*. 48(3):438–447. doi:10.1007/s11262-014-1040-5.
- Turin L, Russo S, Poli G. 1999. BHV-1: New molecular approaches to control a common and widespread infection. *Mol Med*. 5(5):261–284. doi:10.1007/bf03402063.
- van Donkersgoed J, Guenther C, Evans BN, Potter AA, Harland RJ. 1995. Effects of various vaccination protocols on passive and active immunity to *Pasteurella haemolytica* and *Haemophilus somnus* in beef calves. *Can Vet J*. 36(7):424–429.
- van Engelenburg FAC, Kaashoek MJ, Rijsewijk FAM, van den Burg L, Moerman A, Gielkens ALJ, van Oirschot JT. 1994. A glycoprotein E deletion mutant of bovine herpesvirus 1 is avirulent in calves. *J Gen Virol*. 75(9):2311–2318. doi:10.1099/0022-1317-75-9-2311.
- Varela A, Holz C, Cibulski S, Teixeira T, Antunes D, Franco A, Roehe L, Oliveira M, Campos F, Dezen D, Cenci A, Brito W, Roehe P. 2010. Neutralizing antibodies to bovine herpesvirus types 1 (BoHV-1) and 5 (BoHV-5) and its subtypes. *Vet Microbiol*. 142(3-4):254–260. doi:10.1016/j.vetmic.2009.10.016.
- Vlček Č, Beneš V, Lu Z, Kutish GF, Pačes V, Rock D, Letchworth GJ, Schwytzer M. 1995. Nucleotide sequence analysis of a 30-kb region of the bovine herpesvirus 1 genome which exhibits a colinear gene arrangement with the UL21 to UL4 genes of herpes simplex virus. *Virology*. 210(1):100–108. doi:10.1006/viro.1995.1321.
- Weiblen R, Kreutz LC, Canabarro TF, Schuch LF, Rebelatto MC. 1992. Isolation of bovine herpesvirus 1 from preputial swabs and semen of bulls with balanoposthitis. *J Vet Diagn Invest*. 4(3):341–343. doi:10.1177/104063879200400321.
- Wiedmann M, Brandon R, Wagner P, Dubovi EJ, Batt CA. 1993. Detection of bovine herpesvirus-1 in bovine semen by a nested PCR assay. *J Virol Methods*. 44(1):129–139. doi:10.1016/0166-0934(93)90015-j.
- Zhu L, Thompson J, Ma F, Eudy J, Jones C. 2017. Effects of the synthetic corticosteroid dexamethasone on bovine herpesvirus-1 productive infection. *Virology*. 505:71–79. doi:10.1016/j.virol.2017.02.012.