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Detection of *Bovine Herpes Virus* –I Infection Causing Infectious Bovine Rhinotracheitis in Imported Cattle: Serology and Molecular Method

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Abstract

Infectious Bovine Rhinotracheitis (IBR) is a disease in cattle caused by *Bovine Herpesvirus 1* (BHV-1). IBR disease in cattle is spread worldwide, is highly infectious, causing respiratory, reproductive, and neurological disorders. This disease is also economically disadvantageous in international trade. Traffic of cattle from abroad is still quite high, with a prevalence rate in the country of origin, Australia, of 15-96%. Subclinical infections often occur and also cause latent infections, so antigen and antibody detection is required to determine the animal's health status. This study aims to detect BHV-1 antibodies and antigens in imported cattle. Samples were taken from 25 imported cattle that entered through the port of Tanjung Intan Cilacap with two samplings. The first and second collection was carried out with an interval of 1 week. Samples taken included serum samples and nasal swabs from cows showing clinical symptoms of IBR. The sample is then tested with *Enzimes-Linked Immunosorbent Assay* (ELISA) antibodies and *real-time polymerase chain reaction* (PCR) 5% did not detect BHV-1 antigen. Samples with seronegative results, 88% detected BHV-1 antigen. The conclusion of this study was that 40% of the samples detected BHV-1 based on serology and real time PCR.

Keywords: *Bovine Herpesvirus*; Elisa; Real-Time PCR

Introduction

Infectious Bovine Rhinotracheitis (IBR) is an infectious and contagious disease in domestic cattle and wild cattle caused by a virus*Bovine Herpes Virus Type-1* (BHV-1). BHV-1 was first identified in dairy cows in California, USA, in 1953, but this virus remains a globally important pathogen and has a significant impact on health and well-being (Raaperi *et al*., 2014). BHV-1 can cause lifelong latent infection and can reactivate when an animal is stressed or exposed to corticosteroid drugs (Zhu *et al*., 2017).

According to *Office International des Epizooties* (OIE), IBR disease is one of the main problems in cattle farming, which has the potential to endanger international trade. Cattle traffic from abroad is still quite high. Indonesia is currently still importing cattle from Australia. The prevalence rate in Australia is 15-96% (AFFA., 2000). According to Sudarisman (2003), in Indonesia positive reactions to IBR serology do not only occur in imported animals but also in native Indonesian livestock. Serologically, IBR disease has existed in dairy cattle, beef cattle, and buffalo from several provinces in Indonesia with a prevalence of 5-72.9% (Sarosa, 1985). According to Naipospos (2014), all of UPT Breeding within the Directorate General of Health and Human Services (except BPTUHPT Bali Cattle) showed positive antibody titers ranging from 4-76%.

One of the duties of the Agricultural Quarantine Agency is to prevent the entry and spread of Quarantine Animal Pests (HPHK). Quarantine measures for importing beef cattle from abroad to

the territory of the Republic of Indonesia must be carried out through strict handling and inspection of large ruminants. IBR disease cannot be detected from clinical symptoms alone. The diagnosis of IBR disease has been developed in various ways, namely by virus isolation and identification, serological tests, immunoassay examinations, and detection of genetic material through molecular biology techniques. Diagnosis of BHV-1 infection can be difficult for many reasons. Subclinical disease is common, and BHV-1 can cause latent infection.

The ELISA test is a fast, inexpensive, and particular way to detect antibodies to BHV-1 (Riegel*et al.,*1987). The weakness of this test is that new antibodies can be seen through serological tests approximately 12 days after infection (Rodistits*et al.,*2006). The PCR test can confirm the diagnosis in the early stages of the disease, where the antigen has entered the body but no antibody titers have yet been formed. Animals in a state of latent infection can show positive antibodies (OIE, 2018). It is necessary to carry out serological and molecular tests to ensure the presence of antibodies and BHV-1 antigen in the sample.

This study was conducted to detect BHV-1 antibodies and antigens in the imported cattle. It was hoped that reports on the presence of IBR in imported beef cattle can be used as policy material in importing cattle.

Materials and methods

This research was approved and declared to meet ethical requirements for research on animals by the Research Ethics Commission of the Faculty of Veterinary Medicine, Gadjah Mada University, Yogyakarta, with number 0079/EC-FKH/Int./2020.

Sample

Samples were taken from 25 imported cattle that entered through the port of Tanjung Intan Cilacap with two samplings. The first and second collection was carried out with an interval of 1 week. Samples taken included serum samples and nasal swabs from cows showing clinical symptoms, namely runny nose, conjunctivitis, emaciation, and lethargy.

Materials and tools

The material used is the IBR ELISA Kit (IDEXX), which consists of a microplate that has been coated with inactive BHV-1 antigen, positive control, negative control, conjugate, diluent (2% BSA PBS), tetramethylbenzidine (TMB) substrate, reaction stop solution. (H solution$_2SO_4$), washing solution (0.05% Tween-PBS). Materials for real-time PCR include the QIAamp DNA Mini Kit (Qiagen Cat. No. 51304) consisting of QIAamp Mini Spin Columns, Collection Tubes (2 ml), Buffer AL, Buffer ATL, Buffer AW1, Buffer AW2, Buffer AE, QIAGEN® Protease, Solvent Protease, Proteinase K and*Selection Guide*. SensiFast Probe Lo-Rox Kit_terdiri dari RNase Free Water, 2X Sensifast Probe Mix, Primer Forward gBF 20 µM, Primer Reverse gBR 20 µM, dan Probe IBR 5 µM. Ethanol 96-100%,

Primer dan Probe yang digunakan sebagai berikut :Primer gB-F: 5'-TGT-GGA-CCT-AAA-CCT-CAC GGT-3' (position 57499–57519 GenBank®, accession AJ004801), Primer gB-R: 5'-GTA-GTC-GAG CAG-ACC-CGT-GTC-3' (position 57595–57575 GenBank®, accession AJ004801), TaqMan Probe: 5'- FAM-AGG-ACC-GCG-AGT-TCT-TGC-CGC-TAMRA-3' (position 57525–57545 GenBank®, accession AJ004801) . The equipment used in this study included micropipettes, analytical balances, centrifuges, incubators, ELISA readers (ELISA readers), and *Biosafety Cabinet Class II. PCR Work station,* Thermocyler, Heating Block, and PCR tube 0,2 ml

Time and Place of Research

The research was conducted from June 2020 to January 2021. Samples were taken in the form of serum and nasal swabs from imported male and female cattle that entered through the Quarantine Station.

Cilacap Class I Agriculture in June 2020. Serum sampling was carried out two times with an interval of 1 week. ELISA and Real-Time PCR tests were carried out at the Wates Veterinary Center.

Testing with ELISA

The ELISA test for IBR used in this study was the IBR gE ELISA Kit (IDEXX). All reagents and samples were removed at room temperature. The

washing buffer was prepared with a 1:10 dilution. 75 µl buffer diluent was added to all wells. Sample serum, positive and negative control serum, as much as 25 µl were added according to the layout. The microplate was covered and incubated for 60 minutes at 37°C^{O}C. The microplate was washed three times. 100 µl of conjugate was filled into the microplate, then closed and set for 60 minutes at 37^{O}C. The microplate was rewashed 3 times. 100 µl of TMB substrate was loaded into the microplate, then covered, incubated for 15 minutes at room temperature, and placed in a dark place. The stop solution was filled in as much as 100 µl., then read using an ELISA reader (ELISA reader) at a wavelength (λ) 450 nm. The ELISA result assessment is obtained from the formula below:

S/P = (sample OD – negative control OD), (OD control positive – OD control negative)

PCR Real-Time Testing

Sample Preparation. Sample preparation was carried out in the BSC *Class* II. Specimen forms nose, which is suspected to contain the BHV-1 virus. Sample *swab* in viral transport media was vortexed, then the *swab* pressed against the tube wall and *swab* then thrown away. The swab sample in transport medium sterile viral/PBS solution was centrifuged at 1,000 rpm for 10 minutes; then, the supernatant was taken. **DNA extraction according** to the QIAamp DNA Mini Kit DNA Extraction Protocol (QIAGEN, Cat. No. 51304). **Setup*Wash Buffer* 1 (WB 1).** 25 ml of 96-100% ethanol was added to 19 ml*Wash Buffer1* (WB1), available in the kit. WB1, added to ethanol, is stored at room temperature.

Preparation*Lysate.* A mixture of lysis buffer and proteinase *K* prepared according to the amount of sample to be extracted into a 2-ml tube (if the model is below 10) or 15-ml (if the example above 10) the composition is Lysis Buffer 200 µl plus*Proteinase K* 25 µl for one-time reaction/ sample.

The tube contains a mixture of lysis buffer and and*Proteinase K* transferred to space 2 µl Xeno DNA control was added to a box containing a variety of Lysis Buffer and proteinase *K.* Or add as much Xeno DNA as the number of samples (eg ten samples = 20 µl Xeno DNA), directly into a tube containing a mixture of Lysis Buffer and*Proteinase K*, then an aliquot of 227 µl mixed solution (Lysis Buffer +*Proteinase K* + Xeno DNA) as many as the number of samples to be extracted. 200 µl of each prepared piece was added to the solution mixture above. Positive and negative controls were designed by adding 200 µl IBR positive control cells and 200 µl IBR negative control cells. Incubation was carried out at 56ºC for 10 minutes, then immediately proceeded to the Binding and Washing stages.

Binding and Washing Stage

Ethanol 96-100% A total of 200 µl was added to the tube*Lysate* to obtain a final concentration of 37% ethanol, then closed the lid and mixed by vortexing for 15 seconds. The tube is centrifuged briefly to remove the melt from the inside of the cap. The mixture (including the residue) is carefully infused *QIAamp spin column* (in a 2 mL collection tube) without wetting the rim, then centrifuged at 8000 rpm for 1 minute. The QIA spin column was placed in a clean 2 mL collection tube, and the filtrate tube was discarded. The QIAamp spin column was opened, and AW1 500 µL Buffer was added without wetting the rim, then centrifuged at 8000 rpm for 1 minute. The QIA spin column was placed in a clean 2 mL collection tube, and the filtrate tube was discarded. The QIAamp spin column was opened, and 500 µL AW2 Buffer was added without wetting the rim, then centrifuged at full speed (13,000 rpm) for 3 minutes. The QIA spin column is placed in a clean 2 mL collection tube, and discard the tube containing the filtrate and centrifuged at full speed (13,000 rpm) for 1 minute, then proceeded to step*elution*.

Level elution. Place the QIAamp spin column in a 1.5 mL microcentrifugation tube and discard the filtrate collection tube. The QIAamp spin column is then opened and 50 µL AE Buffer or distilled water was added, incubated at room temperature for 1 minute, then centrifuged at 8000 rpm for 1 minute. This step was repeated again, then the DNA was stored in a -20 freezer or direct use.

Master Mix Reagent Preparation

Master mix using **SensiFast Probe Lo-Rox Kit** with the composition of 1 reaction: RNase Free Water 0.8 µl, 2X Sensifast Probe Mix 10.0

µl, Primer Forward gBF 20 µM 0.4 µl, Primer Reverse gBR 20 µM 0.4 µl, Probe IBR 5 µM 0.4 µl. Each reaction (1 tube of optical PCR/optical 96-well plate) was carried out in aliquots of 12 µl, and then 8 µl of each DNA template was added for the sample tested, positive control cells, and negative control cells. A positive control can use positive control cells or IBR/BHV-1 DNA control. Close the PCR tube or seal plate with an optical adhesive cover and spin on a benchtop centrifuge with a 96-well plate adapter to lower all reagents to the bottom of the well at 1,500 rpm for 1 minute.

Realtime PCR reaction.

The tube or 96-well plate is run in a real-time PCR thermocycler with the following reaction conditions: 1 X: 50^{O}C for 2 minutes; 1 X: 95^{O}C for 5 minutes; 45X (cycle); 95^{O}C for 15 seconds; 60^{O}C for 45 seconds. The results were analyzed using the software available on the machine.

Interpretation of IBR/BHV-1 Real-Time PCR Results

The threshold setting for real-time PCR IBR/BHV-1 is done before determining the interpretation. The cycle threshold (Ct) is set automatically (Auto Ct) to reduce operator subjectivity. The detection limit for Real-time PCR IBR/BHV-1 based on the adjustment of the Ct value of the IBR/BHV-1 DNA control can be concluded as follows: Positive (Ct<40), Dubius (40<Ct<45), Negative (CT = 45).

Data analysis

Detection results by ELISA test and real-time PCR in this study were analyzed descriptively.

Table 1. Test Results with Antibody ELISA and real-time PCR

No	Kode Sample	Hasil ELISA I	Hasil ELISA II	Hasil Real-Time PCR
1	590	Positive	Positive	Positive
2	631	Positive	Positive	Negative
3	787	Positive	Positive	Positive
4	907	Positive	Positive	Positive
5	989	Positive	Positive	Positive
6	1019	Positive	Positive	Positive
7	1094	Positive	Positive	Positive
8	1162	Positive	Positive	Negative
9	1170	Positive	Positive	Positive
10	1285	Positive	Positive	Negative
11	1321	Positive	Positive	Negative
12	1342	Negative	Negative	Positive
13	1396	Positive	Positive	Positive
14	1429	Positive	Positive	Negative
15	1432	Positive	Positive	Positive
16	1433	Negative	Negative	Positive
17	1607	Negative	Negative	Positive
18	1659	Negative	Negative	Positive
19	1690	Negative	Negative	Positive
20	1707	Negative	Negative	Negative
21	1720	Negative	Negative	Positive
22	1821	Positive	Positive	Positive
23	1869	Negative	Negative	Positive
24	2063	Positive	Positive	Negative
25	2381	Negative	Negative	Positive
Result Prosentage : Positive (+)		64 %	64 %	72 %
	Negative(-)	36 %	36 %	28 %

Results and Discussion

The results of the ELISA test from the first and second collection of 25 serum samples obtained seropositive results for 16 samples and nine samples for seronegative. Meanwhile, 25 nasal swab samples examined by Real-Time PCR obtained 18 positive and seven negative results (Table 2).

ELISA test results based on the table above showed 64% seropositive and 36% seronegative. The high IBR seropositivity in the examined samples is directly proportional to the high prevalence rate in the country of origin, Australia, which is 15-96% (AFFA., 2000). According to Sudarisman (2003), positive reactions to IBR serology do not only occur in imported animals but also in native Indonesian livestock. Serologically, this disease has existed in dairy cattle, beef cattle, and buffaloes from several provinces in Indonesia, with a prevalence of 5-72.9% (Sarosa, 1985). In fact, according to Naipospos (2014), all UPT Breeding within the Directorate General of Health and Human Services (except BPTUHPT Bali Cattle) showed positive antibody titers (range 4-76%).

The samples examined in this study came from cattle that were not vaccinated because the import entry document did not state that the cattle had been vaccinated in the country of origin. Antibody

detected can occur because the cow has been exposed to the IBR virus through natural infection during maintenance/ fattening in the stable, and latent infection can also occur. The immune response can be detected 12 days after the animal is infected with BHV-1 and lasts for approximately 14 months. The increase in immune titers continues to increase up to 10 months after infection (Rodistits*et al.,*2006). This immune response can last a lifetime, although it may be below the detection limits of some tests after several years (Mechor*et al*., 1987). BHV-1 can persist in infected animals in a latent state in sensory neurons, for example, in the trigeminal or sacral ganglia. Viruses can be reactivated and cause viruses shedding (re-excretion) without showing clinical symptoms. And animals have positive antibodies. (OIE, 2018). There are several ways that can activate and accelerate the virus reactivation process in a latent state, including if the cattle are stressed, the cages are too crowded, transportation, and administration of corticosteroids (Muylkens*et al*., 2007).

Imported cattle can come from several feedlots in the area of origin, so that if there is an infection on the ship, transmission will easily occur. Direct transmission through breathing of the BHV-1 virus is very easy from one livestock to another or from one group to another. The virus can be spread through nasal secretions or droplets containing the virus (Mars *et al*., 2000).

New infection and re-excretion of virus from cows with latent infection can occur during the cow's journey of approximately ten days on board. With conditions of poor density and air circulation in the ship, cows become stressed and exhausted during transportation. The incubation period after infection occurs for 2-3 days, followed by discharge from the nose, drooling, fever, lack of appetite, and depression. Within a few days, the discharge from the nose and eyes turned mucopurulent. Most infections are very mild or subclinical (Van Oirschot et *al*., 1993). A comparison of OD/titer values in the ELISA I test and ELISA II test showed that ten samples experienced an increase in OD, and 15 samples experienced a decrease in OD. An increase in antibody titer indicates infection or reactivation. Serum antibody titer decreased; it can be assumed that the disease is latent (Turin et al. 1999). According to OIE (2018), A seroconversion from negative to positive or fourfold or a higher increase in antibody titers is considered to prove acute infection

Method of *real-time polymerase chain reaction* (PCR) in this study aimed to detect the presence of genetic material of the glycoprotein C (gC) marker gene from the BHV-1 virus. The real-time PCR method is an alternative test that is very precise for the identification of the BHV-1 virus in the above samples. Real-time PCR results are expected to be read faster with a higher level of sensitivity and specificity than conventional IBR PCR. PCR results targeting the gC region of BHV-1 are in line with several previous studies (Afshar and Eaglesome, 1990; Van Engelenburg et al., 1993). The GC code for the nucleotide sequence is shown to be present in all BHV-1 strains and is

highly specific. Glycoprotein C is a glycoprotein on the surface of the main envelope that plays a role in viral virulence. Glycoprotein C is also required for viral entry by binding to target cell heparin sulfate receptors (Van Engelenburg et al., 1993)

Real-Time PCR testing has several advantages over conventional PCR methods. Real-Time PCR testing uses only one pair of primers, which provides sensitivity close to or equal to the nested PCR method, with a much lower risk of contamination. Target amplification and detection are carried out simultaneously. There is no post-amplification PCR product handling, which significantly reduces the risk of contamination (Anonymous., 2008). Based on the Real-Time PCR test, 72% positive results and 28% negative results were obtained from 25 samples. Limit of detection for IBR/BHV-1 Real-time PCR based on adjustment of IBR/BHV-1 DNA control Ct values. From several optimization tests, the PCR results can be interpreted as follows: Positive (Ct<40), Dubius (40<Ct<45), and Negative (Ct=45).

Comparison of the ELISA test and real-time PCR showed that ten samples detected antibodies in the serum samples and also the BHV-1 antigen obtained from a bovine nose swab sample. At the same time, 1 sample did not show the presence of both. This is possibly due to BHV-1 infection with previous exposure to the ten samples while there was no infection and no prior exposure to 1 sample. Eight seronegative samples showed the presence of BHV-1 antigen on the nasal swab, which indicated the possibility of a new infection or the beginning of an infection so that the immune response had not appeared. Other studies have also reported detecting BHV-1 in seronegative cow samples. Dekaet *al.*(2005) examined 24 semen samples, 12 each from seropositive bulls and seronegative., Rocha *et al*. (1998) also reported the detection of BHV-1 DNA by PCR from seronegative cows. So, there is not always a correlation between antibody status and virus excretion because BHV-1 DNA detection by PCR was also obtained from seronegative cows. Six seropositive samples did not show BHV-1 virus from the nasal swab samples. This may be due to prior exposure and may be related to the fact that the herpes virus may persist for life or Latent infection with periodic reactivation and shedding of the virus may occur intermittently or continuously (Rola*et al.,*2005)

Conclusion

Based on the ELISA test and real-time PCR showed that 40% reaction to BHV-1 antibodies and antigen from serum and nasal swabs samples of imported beef cattle.

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