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Identification and Characterization of Bovine Viral Diarrhea Virus from Indonesian Cattle

(IDENTIFIKASI DAN KARAKTERISASI VIRUS BOVINE VIRAL DIARRHEA DARI SAPI INDONESIA)

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ABSTRACT

Bovine viral diarrhea virus (BVDV) is an important viral disease, which a ubiquitous pathogen of cattle with worldwide economic importance and due to its misdiagnose with other viruses. The goal of the current study was to identify and characterize of BVDV by reverse transcriptase polymerase chain reaction (RT-PCR) and followed by sequence genome analyses. Blood, feces, and semen samples were collected from 588 selected cattle from animals suffering from diarrhea and respiratory manifestation. RT-PCR results showed that the 69 (11.74%) samples were positive to BVDV. Further molecular characterization was conducted only with 17 PCR positive samples. The results indicated the 17 Indonesian BVD virus isolates were belonging to the genotype-1 of BVDV (BVDV-1) based on sequence analysis and a phylogenetic relationship between Indonesian BVDV isolates and BVDV in the world. This finding is the first report of BVD-1 circulated in Indonesian cattle.

Keywords : BVDV, RT-PCR, genotype-1.

ABSTRAK

Bovine viral diarrhea virus (BVDV) adalah salah satu penyakit virus penting dan pathogen pada sapi dan mempunyai dampak ekonomi yang luas dan sering terdiagnosis dengan virus lainnya. Tujuan penelitian ini untuk mengidentifikasi dan mengkarakterisasi BVDV dengan uji *reverse transcriptase polymerase chain reaction* (RT-PCR) yang dilanjutkan dengan analisis sekuen genomnya. Sampel darah, feses, dan semen telah dikoleksi dari 588 ekor sapi yang menunjukkan gejala diare dan gangguan pernafasan. Hasil uji RT-PCR menunjukkan bahwa 69 (11,74%) sampel yang diuji positif terhadap BVDV. Karakterisasi molekuler dengan analisis sekuen dan kekerabatan filogenetik dilakukan pada 17 sampel yang positif, menunjukkan bahwa isolat tersebut termasuk dalam genotype-1 (BVDV–1) ketika dibandingkan dengan isolat lokal dan BVDV yang ada di dunia. Temuan ini merupakan laporan pertama tentang adanya peredaran virus BVDV-1 di Indonesia.

Kata-kata kunci : BVDV, RT-PCR, genotipe-1.

INTRODUCTION

Bovine viral diarrhea virus (BVDV) is one of an economically important viral pathogen of cattle, belongs to the genus *Pestivirus* of the family *Flaviviridae* (Belknap *et al.,* 2000; Ghazy *et al.,* 2007; Ghazi *et al.,* 2008). The viral genome is a single-stranded (+) RNA, 12.5 kb in length, without a poly(A) tail (Jalali *et al.,* 2004). The virus consists of two different genotypes, namely BVDV-1 and BVDV-2. The disease is associated with several clinical symptoms, including mild diarrhea, respiratory disease, congenital malformations, reproductive disorders and mucosal disease (Baker 1987). Infection of bovine fetuses with BVDV-2 during the first trimester gestation may result of persistent infection to the calf or immune tolerance (Charleston *et al.,* 2001; Givens *et al.,* 2003). Serum samples from these animals fail to react in most conventional serological tests and these animals shed large amounts of virus in all body secretions and excretions over prolonged periods (Ozkul *et al.,* 2002).

More over, the prevalence of BVD has been reported on the basis of the detection of antibody against BVDV (Daliri *et al.,* 2007), and the reactors varied from 20-90% (Houe, 1995). In Indonesia, the bovine virral diarhea – mucosal disease (BVD-MD) has been broken out in 1988. In this out break Bali breed cattle, Brahman Cross, Sahiwal, Local Ongole breed cattle and Buffaloes of all ages and both sexes were affected (Soesilo, 1989). Clinical BVD in cattle had been reported (Adjid, 2004). The prevalence of antibody against BVDV in cattle in Indonesia varied from 28%-77% (Sudarisman, 2009). This indicated that the BVDV has been occurred in Indonesia. However, no reported in viral detection.

Reverse transcriptase (RT)-PCR has become an increasingly popular procedure for detecting BVDV and other pestiviruses (Andre *et al.,* 1995). Two limiting factors that affect successful performance of RT-PCR are nucleic acid purification methods and the oligonucleotide-specific primers used (Givens *et al.,* 2003). Other important considerations include the thermocycling parameters and the reagents used (Daliri *et al.,* 2007). Similar to RNA hybridization, RT-PCR performance can also be affected by BVDV genome variability (Alansari *et al.,* 1993). All existing BVDV RT-PCR protocols require separate RT reactions and PCRs, which may increase the potential contamination. Most of the methods using total RNA extraction from cell-cultured, and only a few from the original specimens (Hamel *et al.,* 1995). None of these methods use total RNA extraction directly from blood, feces, and semen specimens. Instead, total RNA obtained from the purified lymphocytes was used, because the available RNA extraction methods fail to consistently eliminate PCR-inhibiting compounds present in blood (Alansari *et al.,* 1993). Recent refinements in PCR and nucleic acid purification techniques have provided opportunities for developing more rapid, sensitive and specific BVDV detection assays. By using the cationic surfactant tetradecyltrime thylammonium oxalate Catrimox-14 [Cat-14]), RT-PCR quality RNA can be extracted directly from whole blood, feses and semen in one hour (Andre *et al.,* 1995). In addition, recently published protocols combine the reagents for both RT and PCR in one tube and RT-PCR can thus be performed by a single, uninterrupted thermal cycling program (Andre *et al.,* 1995). The current study aimed to identify and characterize BVD, using RT-PCR and direct viral RNAs extraction from lymphocytes, feces and semen samples from infected and apparently healthy cattle.

RESEARCH AND METHODS

Samples

The present investigation were carried out at the Artificial Insemination Centre (AIC) Lembang (West Java); dairy and beef cattle farms in Sumedang and in Pangalengan (West Java); and beef cattle in Ungaran (Central Java). Blood samples were collected through a jugular vein puncture from cattle. Cattle suffered from diarrhea and respiratory symptoms, were sampled and blood was collected in anticoagulant EDTA. A similar volume of histofaque (Sigma, USA) was added to the whole blood sample, centrifuged at 1.500 rpm for 15 minutes to separate the buffy coat (Quinn *et al.,* 1994). Semen samples were obtained from an artificial insemination center. Semen was diluted in a Tris-buffered-fructose-glycerol-yolk extender up to 30 million to 80 million spermatozoa per mL, and the mixture was divided into 200 μL aliquots in insemination tubes. The semen samples were transported in liquid nitrogen and were stored at minus 70°C until use. The feces were directly collected from the cattle used the glove and placed into the plastic bag and recorded.

Nested RT-PCR

The external primers for primary RT-PCR, 5'-AAGATCCACCCTTATGAGC-3' and 5'-AAGAAGCCATCATCACCCACA-3', were derived from nucleotides 10,385 to 10,404 and 11,528 to 11,547, respectively (Collett *et al.,* 1988). The nested PCR primers for secondary PCR, 5'-TGGAGATCTTTCACACAATAGC-3'(BVDV-1 specific), 5'-GGGAACCTAAGAA CTAAATC-3' (BVDV-2 specific), and 5'-GCTGTTTCACCCAGTTAGTACAT-3', were derived from nucleotides 10,758 to 10,779, 10,514 to 10,533, and 11,096 to 11,117, respectively. BVD virus RNAs were obtained either by extraction from the blood, feces, and semen using a commercial RNA extraction kit (Qiagen, USA) and RT-PCR was performed. Reverse transcription was carried out at 37°C for 30 minutes, followed by denaturation at 94°C for three minutes. The primary PCR reactions were cycled 25 times at 94°C for 20 s, 50°C for 30 s and 72°C for 30 s, with a final extension

step of 72°C for 15 minutes. The products were then used as a template in a secondary PCR for 40 cycles. This was performed in the same manner as the primary PCR. The amplified products were electrophoresed on a 2% agarose gel and stained with ethidium bromide (Gilbert *et al.,* 1997).

Sequencing

Sequencing using the ABI PRISM sequencer engine 3130 models, the process of sequencing using the Big Dye terminator v3.1. cycle sequencing kit (Part No. 4337455, Applied Biosystems, USA) based on the recommendation of the manufacturer's kit. Primers used were primer pair (forward and reverse) with a concentration of 5-10 pmol/mL. While the concentration of DNA required for sequencing is 3-10 ng/mL with a total volume of 20mL.

Sequence Analysis and Phyllogenetic Tree

The ABI PRISM sequencer 3130 was used for sequencing. The process of analyzing sequences of DNA sequencing and amino acid translation were done by using the BioEdit software Version 7.0.5 (Hall, 1999). Alignment sequences using the ClustalW software version 1.83 (Thompson *et al.,* 1994). The similarity or homology sequences between BVDV isolates were analyzed by using the Basic Alignment Search Tools (BLAST) from the National Center for Biotechnology Information (NCBI). While analysis of phylogenetic relationship between sequences performed with the program Molecular Evolutionary Genetic Analysis (MEGA) software version 4.0. (Kumar *et al.,* 2004), the construction design phylogenetic tree using the Unweight Pair Group Methods with arithmetic mean (UPGMA) based on Kimura-2 parameter to 2000 replication. The percentage of amino acid sequence similarities and nucleotide using

BigDye terminator v3.1 cycle sequencing kit (Part No. 4337455, Applied Biosystems, USA) based on the recommendation of the manufacturer's kit . Primers used were primer pair (forward and reverse) with a concentration of 5-10 pmol/mL. While the concentration of DNA required for sequencing is 3-10 ng/mL with a total volume of 20mL.

RESULTS AND DISCUSSION

Detection of BVDV

A total of 537 samples of blood and feces of Friesian Holstain cattle (FH) and Ongole breed cattle (PO) was collected. The samples consisted of 170 blood samples from FH cattle in Pengalengan (West Java), 207 blood samples of FH cattle from Jakarta, 45 fecal samples of FH cattle from Lembang (West Java), 13 faecal samples of PO cattle from Bogor (West Java), and 102 blood samples of FH cattle from Sumedang (West Java). In addition, 51 submitted semen and feces samples of Limousin cattle, FH cattle, Bali cattle, Madura cattle, and Brahman-Angus crossbred cattle from West Java and Central Java. The result indicated that 46 537 samples (8.57%) were BVDV positive, and 23 of 51 (45.09%) submission samples were positive BVDV using PCR, as presented in Table 1.

Purification of BVDV Samples

From 69 positive samples using PCR, 17 samples were selected for molecular characterization purposes using sequencing. Those samples consisted of seven blood samples (WJ10, WJ19, WJ20, WJ60, WJ57, WJ170, and WJ167) from West Java; three blood samples (J123, J130, and J121) from Jakarta ; three feces samples (CJ066, CJ008, and CJ009) from Central Java ; and four samples of semen (CJ014, CJ016, CJ003, and CJ005) from Central Java (Figure 1).

Characterization of BVDV

A total of 17 samples successfully sequenced and compared with BVDV virus subtype 1 and subtype 2 from GeneBank, namely BVDV-1 strains Bega, BVDV-1 NADL, BVDV-2 CH693 (Chile strain), BVDV-2 CH470 (Chile strain), and BVDV-2 CH640 (Chile strain). The results of phylogenetic tree indicated that BVDV from Indonesian isolates were more closely related to BVDV subtype 1 (BVDV-1) compared with BVDV subtype 2 (BVDV-2). Therefore all BVDV isolates from Indonesia included in BVDV subtype 1 (BVDV-1) (Figure 2).

Table 1. Detection of BVDV RNA in semen, blood, and faeces samples from cattle at West Java, CentralJava and Jakarta.

No	Source of Animal	Breed of cattle	Samples	Total	Total Positive BVDV
1.	Pengalengan (West Java)	FH	Blood	170	20
2.	Jakarta	FH	Blood	207	10
3.	Lembang (West Java)	FH	Feces	45	0
4.	Bogor (West Java)	PO	Feces	13	6
5.	Sumedang (West Java)	FH	Blood	102	10
		Sub Total		537	46
		Percentage			8.57
6.	Sample submission:				
	a. West Java	Limousin	Semen	3	3
		FH	Feces	19	10
	b. Central Java	Bali Cattle	Semen	3	3
		Madura Cattle	Semen	2	2
	Brahman-Angus	Feces		14	4
	Limousin	Feces		10	1
		Sub Total		51	23
		Percentage			45.09
		Total		588	69
		Percentage			11.74

FH: Friesian Holstain cattle
PO: Ongole Crossbreed cattle

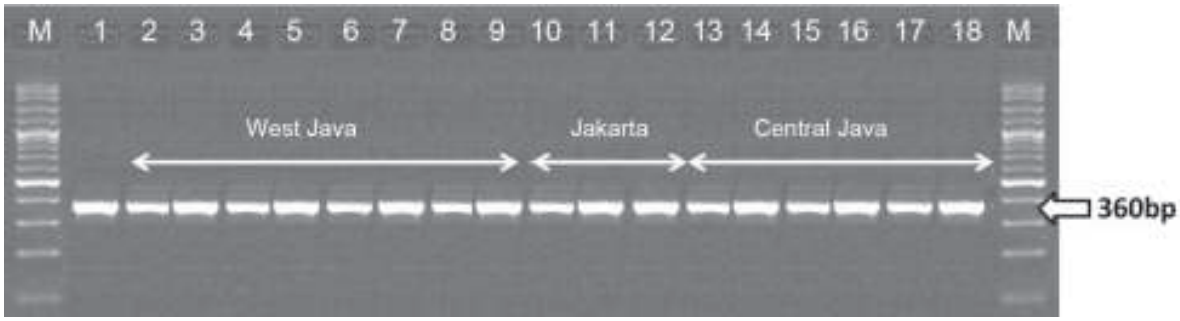

Figure 1. Detection of Bovine Viral Diarrhea Virus in semen, blood and feces of cattle from West Java, Central Java and Jakarta. The fragment length of positive samples is 360 bp. Lane M: molecular weigh 100bp; Lane 1: BVDV-1 NADL strain (positive control); Line 2 - 9: Blood samples WJ10, WJ19, WJ19, WJ20, WJ60, WJ57, WJ170, and WJ167; Line 9-12: Blood samples J123, J130, and J121; Line 13-15: Faeces samples CJ066, CJ008, and CJ009; Line 16-18: semen samples CJ014, CJ016, CJ003, and CJ005.

Infectious Bovine Viral Diarrhea Virus has spread throughout the world and caused economic loss for cattle breeding industry. There are two genotypes BVDV can be distinguished, namely BVDV-1 and BVDV-2. Both have similarities in the clinical cause of disease that can lead to failure in diagnosing a disease based on clinical symptoms (Charleston *et al.,* 2001; Given *et al.,* 2003). Conventional serologic test often fail to diagnose and determine the serotype BVDV (Daliri *et al.,* 2007). Therefore, PCR techniques for identification of BVDV and followed by sequencing of the PCR products were used to determine the genotype BVDV in this study.

Sequence analysis showed that all 17 samples tested were classified as BVDV genotype 1 (BVDV-1). None of the field samples had a close relationship to BVDV genotype 2 (BVDV-2). The result concluded that BVDV-1

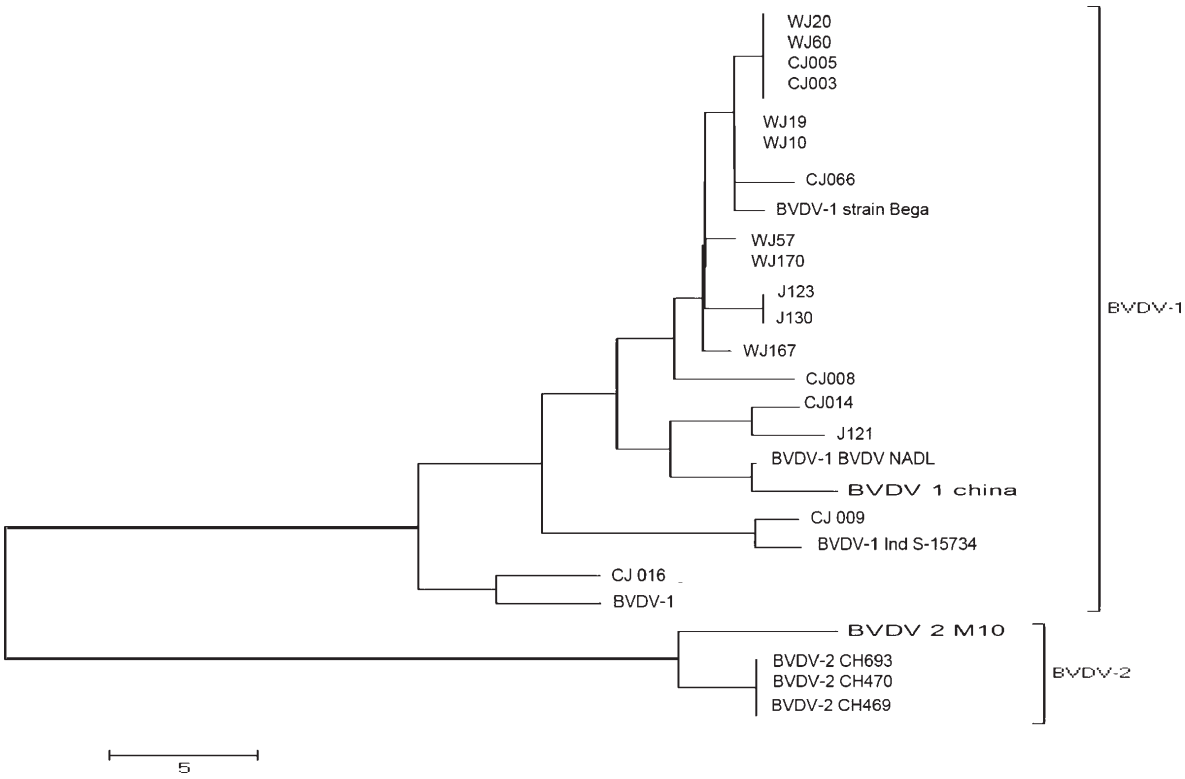

Figure 2. Phylogenetic trees of Bovine Viral Diarrhea Virus Indonesian isolates compared to BVDV in GeneBank based on the amino acid sequences. The tree was generated by the neighbor-joining method. The lengths of the branches in the tree inferred by the neighbor-joining method reflect phylogenetic distances. BVDV-2 was used as the out group.

circulating in the cattle population in Indonesia. The phylogenetic analysis showed that Indonesian isolates of BVDV-1 were divided into four subtypes. BVDV-1a was composed of isolates West Java (WJ20, WJ60, WJ19, WJ10, WJ170, and WJ167), Central Java (CJ005, CJ003, and CJ063), Jakarta (J123 and J130); and BVDV-1 strain Bega isolates. Subtype BVDV-1b consists of isolate from Central Java (CJ014), Jakarta (J121), and including BVDV-1 and BVDV-1 NADL China. Subtype BVDV-1c consists of BVDV isolate from Central Java (CJ009) and included BVD-1 S-15734 isolate from India, and subtype BVDV-1d consists of BVDV isolate from Central Java (CJ016) and isolate BVDV-1 MD74 from Australia.

The sequence analysis indicated that only BVDV-1 is circulated in Indonesia. This may derive from India, China, and Australia through modified live BVDV-1 vaccine strain used. It is logic if only BVDV-1 virus can be isolated as the vaccine used in Indonesia is modified live BVDV-1 vaccines strains as well. The question may arrise what strain contained in the vaccine circulated in Indonesia? If many strain circulated in Indonesia such as Australian strain, Indian strain or Chinese strain that presented in this sequence result, there is a tendency to have an antigenic shift or drift among the strain, and it will produce a new strain which will produce a different virulence. Hence, further study should be conducted. However, this is the first finding of BVDV-1 circulated in Indonesian cattle.

Taylor and Rodwell (2001) reported that BVDV-1 is generally used for vaccine production, whereas BVDV-2 has been isolated primarily from fetal bovine serum. BVDV-2 isolates were very common in persistent infection (PI) status of the cattle derived from a group of animals vaccinated against BVDV, and in died animals due to hemorrhagic syndrome (a form of acute BVDV). In addition, the information regarding persistent infection in cattle and Bovine Viral Diarrhea-Mucosal Disease (BVD-MD) cases in Indonesia were not available at present. Further studies were needed to determine the clinical and pathologic characteristics of BVDV-2. In

addition, BVD vaccines circulating in Indonesia was imported and only containing BVDV-1 strains. For the prevention of cattle from both genotypes BVDV infection, it was necessary to develop an effective vaccine BVDV using both genotype namely BVDV-1 and BVDV-2 strains .

CONCLUSSION

Based on the results of sequence analysis and the phylogenetic relationship between BVDV isolates from Indonesia were compared with BVDV isolates in the world, all 17 isolates of BVDV from West Java, Central Java, and Jakarta belong to the BVDV-1 (genotype 1).

SUGGESTION

Since little information is currently available regarding PI calves and BVD-MD cases in Indonesia, further studies are required to investigate the pathological and clinical characteristics of BVDV-2 isolates. In addition, for the prevention of cattle from two genotypes of BVDV infection, it is necessary to develop effective BVDV vaccine using both BVDV-1 and BVDV-2 strains.

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