

Original Research Article

Genetic Variability of Bovine Viral Diarrhea Virus in Persistently Infected Cattle in Central Java, Indonesia

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Abstract

Fifteen bovine viral diarrhea virus (BVDV) positive samples collected from persistently infected (PI) dairy cattle in Central Java, Indonesia, were used to detect the genetic characterization of BVDV based on the 5'-Untranslated Region (5'UTR) of the viral genome. Through phylogenetic and nucleotide sequence analysis of the 5'UTR regions of the samples investigated, it was determined that all the 15 IP-BVDV field positive samples had the BVDV-1 genotype. No evidence of BVDV-2 genotype was found in our recent collected blood samples. The presence of the genetic variability among the BVDV-1 circulating in the dairy cattle population in Central Java, Indonesia was not proven. As a conclusion, further research involving higher number of BVDV-1 positive samples from different region of Indonesia is urgently needed.

Keywords: Bovine viral diarrhea virus, 5'-Untranslated Region, genetic variability, phylogenetic analysis, genotype

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INTRODUCTION

Bovine viral diarrhea virus (BVDV) is a common pathogen of cattle that causes major economic losses to dairy industry worldwide. The virus is a member of Flaviridae family along with flavivirus and hepatitis C groups (Francki et al., 1991). A wide range of clinical manifestations from subclinical to fatal disease occur in association with BVDV infection but mainly infects the digestive and reproductive tracts of cattle. The reproductive consequences of BVDV infections range from conception failure, early embryonic death, abortions, stillbirths, congenital malformations, stunted weak calves and the birth of persistently infected (PI) calf (calves) (Baker, 1995). Persistently infected calves are immunotolerant and the most dangerous reservoir of BVDV, serving as BVDV carrier for their entire lives and continuously transmit the diseases by direct contact to susceptible and unvaccinated herd mates.

The genome of BVDV contains a single-stranded positive-RNA, which is approximately 12.3 kb in length, and is flanked at either end by 5' and 3' untranslated regions (5'UTR, 3'UTR) (Meyers and Thiel, 1996). In the

early genetic characterization, two genotypes of BVDV (BVDV-1 and BVDV-2) are recognized in most countries, causing acute and persistent infections with similar clinical manifestations. Acute infection with some highly virulent BVDV-2 has been associated with a severe hemorrhagic syndrome with high mortality of cattle (Ridpath et al., 1994). Later analysis revealed at least 12 BVDV-1 sub-genotypes (Vilcek et al., 2001) and 2 BVDV-2 sub-genotypes (Nagai et al., 2008). The existence of sub-genotypes is a reflection of the BVDV genetic diversity that exists in nature. This diversity found throughout the world and continues to expand, as new populations are being surveyed (Barros et al., 2006). The characterization of the circulating sub-genotypes in cattle from a particular region can contribute to a better understanding of the epidemiology and pathogenesis of BVDV infections and indicate the importance of genetic monitoring in BVDV control.

In Indonesia, bovine viral diarrhea is considered to be a strategic disease. However, up to date there is still no national program for control and eradicating of BVDV.

BVDV serology is the only effort that has been regularly done for screening the pathogen exposure of potential breeding. Even though, as the sole diagnostic technique, BVDV serology has many caveats. The objective of this study is to detect the genetic characterization of recent BVDV isolates from PI dairy cattle in Central Java, Indonesia, based on the 5'-UTR coding region.

MATERIALS AND METHODS

Samples

One hundred and sixty five sera samples were collected from the herds that historically have low reproductive performance and never been vaccinated in Central Java, Indonesia. To confirm persistent infection, blood samples from all positive BVDV cattle were re-collected and re-tested after 4 week interval.

Antigen Capture Elisa (ACE)

The serum samples were assayed individually by Antigen Capture Elisa (IDEXX herdcheck BVDV Antigen Test Kit) performed to the manufacturer's specification for the presence of protein E^{ns} (gp 44-48) glycoprotein. Positive control, negative control and all serum samples were first diluted 1:1 using dilution buffer. Fifty microliters (μ l) of each diluted positive control, negative control and serum samples were pipetted into wells followed by 120 min incubation at room temperature. After incubation, the wells was washed three to five times using 300 μ l washed buffer. After washing, 100 μ l of anti-bovine HRP conjugated were dropped into each wells and incubated for 30 minutes at room temperature. After incubation, the plate were washed as previously described to remove the excess conjugate. For color development, 100 μ l of 3,3',5,5' tetramethylbenzidine (TMB) substrate were added into each well. After incubation in the dark for 10 min, the reaction was terminated by adding 100 μ l of stop solution into each well. The absorbance at 450 nm was monitored in ELISA reader.

RNA extraction and RT-nested PCR

Viral RNA was extracted from 200 μ l sera samples and positive control isolate using a high pure RNA isolation kit (Roche Life Science, Mannheim, Germany) as described by the manufacturer. The reverse transcriptase polymerase chain reaction (RT-PCR) reactions were performed in one-step using the Superscript III one step RT-PCR system with Platinum Taq polymerase (Invitrogen, Carlsbad, USA) according to manufacturer's specification, in PEQLAB Primus 25 advanced Thermal Cycler (Biotechnologie GmbH, Germany). Genetically

conserved regions in the 5'UTR of the BVDV genome (Vilcek et al., 1994) were specifically amplified using pairs of primers 324 and 326 (Table 1). Thermal conditions were as follows: 50°C for 30 min of reverse transcription, 94°C for 3 min of initial denaturing followed by 30 cycles of 94°C for 1 min, 52°C for 1 min, 72°C for 1 min and then final elongation at 72°C for 10 min.

The genotyping in this study was done using a nested PCR. In the first RT-PCR amplification, the Superscript III one step RT-PCR system with Platinum Taq polymerase (Invitrogen, Carlsbad, USA) and primer set A (Table 2) was used. The PCR mixtures were then subjected to the following cycling condition: 42°C for 1 h of reverse transcription, 94°C for 3 min of initial denaturing followed by 30 cycles of 94°C for 30 s, 50°C for 45 s, 72°C for 1 min and then one cycle of final elongation at 72°C for 10 min. In the PCR second amplification, a 198 bp DNA product from the first amplification was used as a template in the second round RT-nested PCR. The PCR reaction was prepared similarly to the first amplification but without RT enzyme and primer set B (Table 2). Thermal conditions for second amplification were as follows: 94°C for 2 min of initial denaturing followed by 30 cycles of 94°C for 30 s, 50°C for 45 s and 72°C for 1 min followed by final elongation at 72°C for 7 min.

For subgenotyping, the 5'UTR of the BVDV genome product (288 bp) from this stage was used as a template in the second round RT-nested PCR that was done using the same conditions but different primers pairs for each reaction (1 AF/ABR;1BF/ABR) (Table 1). All the PCR products were separated on a 1.5% agarose gel, stained after electrophoresis with ethidium bromide and visualised using ultraviolet transillumination. Two isolates contain Singer strain and 890 strain were respectively used as BVDV type 1 and BVDV type 2 control positive.

For sequencing, PCR products were purified using High Pure PCR Product Purification Kit (Roche Life Science, Mannheim, Germany). Forward and reverse 5'-UTR sequences for each sampel were aligned and used in phylogenetic analysis. The sequences were compared to other previously published sequences. The sequence identities of nucleotide, as well as the estimation of the evolutionary divergence between sequences were analysed using Bioedit and Mega6 software, respectively (Tamura et al., 2013). The same tool was used to perform Neighbor-Joining analysis.

RESULTS

Using both ACE and RT-PCR techniques, 15 out of 165 dairy cattles were positive for PI-BVDV (9.09%). This result were confirmed using RT-PCR test showing specific product of expected 288 bp size visible on the 1.5% agarose gel (Figure 1). Blood samples from PI-BVDV positive animals were re-taken and retested one month apart for confirming the virus persistence.

Table 1. Oligonucleotide primers used in this study

Primers	Genotype	Sequence (5'-3')	Product	References
324 326	BVDV	5' ATG CCC WTA GTA GGAQ CTA GCA 5' TCA ACT CCA TGT GCC ATG TAC 3'	288 bp	Vilcek et al. (1994)
1 AF 1 BF	BVDV-1a BVDV-1b	5' TCG ACG CCT TRR CAT GAA GGT 3' 5' TCG ACG TTT GGA GGACAA GC 3'	169 bp 169 bp	Ridpath and Bolin (1998)
1 ABR	BVDV 1a and 1b	5' CCA TGT GCC ATG TAC AG 3'		

Table 2. Oligonucleotide primers for genotyping used in this study

Primers	Genotype	Sequence (5'-3')	Product	References
Sense Set A	BVDV-1 and BVDV-2	5' GTA GTC GTC AGT GGT TCG 3'	198 bp	Collett et al. (1988)
Antisense Set A (first amplification)		5' GCC ATG TAC AGC AGA GAT 3'		
Sense Set B Antisense Set B (second amplification)	BVDV-2	5' CGA CAC TCC ATT AGT TGA GG 3' 5' GTC CAT AAC GCC ACG AAT AG 3'	105 bp	Ridpath and Bolin (1995)

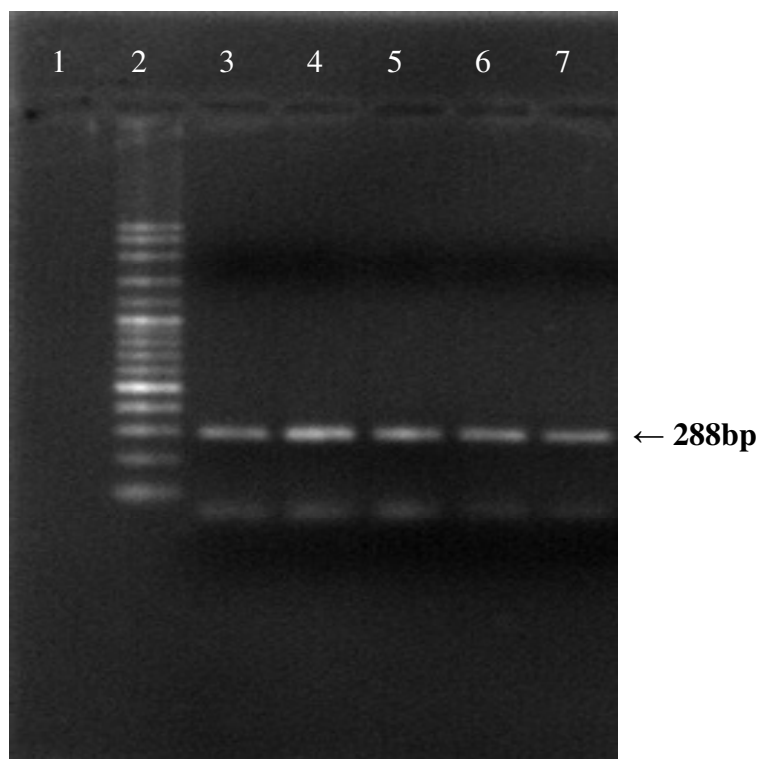


Figure 1. PI-BVDV detection by RT-PCR conventional technique. (lane 1: negative control, lane 2: Vivantis DNA marker plus 100 bp, lane 3: 890 strain isolate, lane 4 Singer strain isolate, lane 5-7 PI-BVDV positive field samples).

Using the oligonucleotide primers pairs listed in Table 2, after the first amplification, the DNA bands from all field

samples and positive control either BVDV-1 and BVDV-2 were clearly visible at 198 bp (Figure 2). After the second

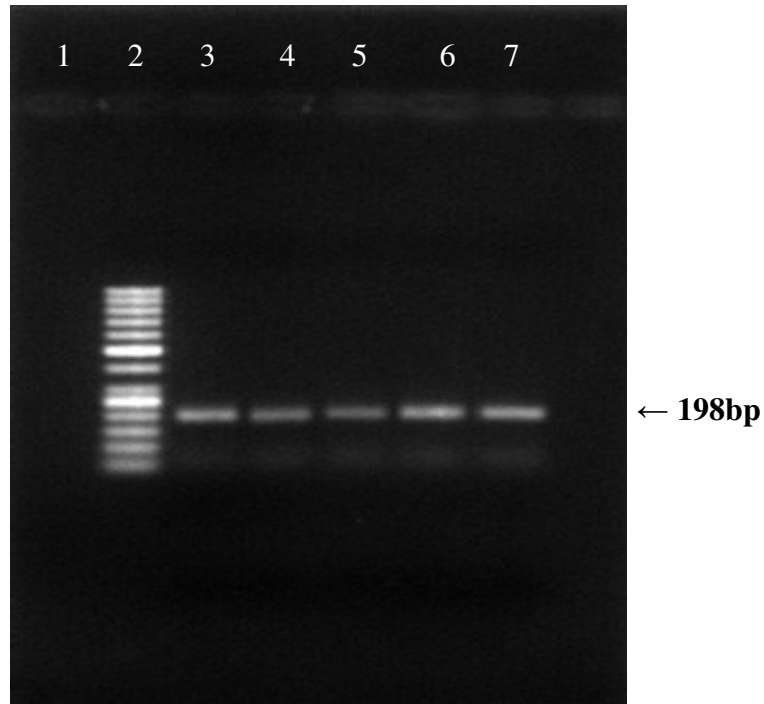


Figure 2. First PCR amplification of RNA extracted from positive PI-BVDV samples. Lane 1: negative control, lane 2: Vivantis DNA marker 50 bp, lane 3: Singer strain BVDV-1 positive control, lane 4: 890 strain BVDV-2 positive control, lane 5-7: BVDV-1 positive field samples.

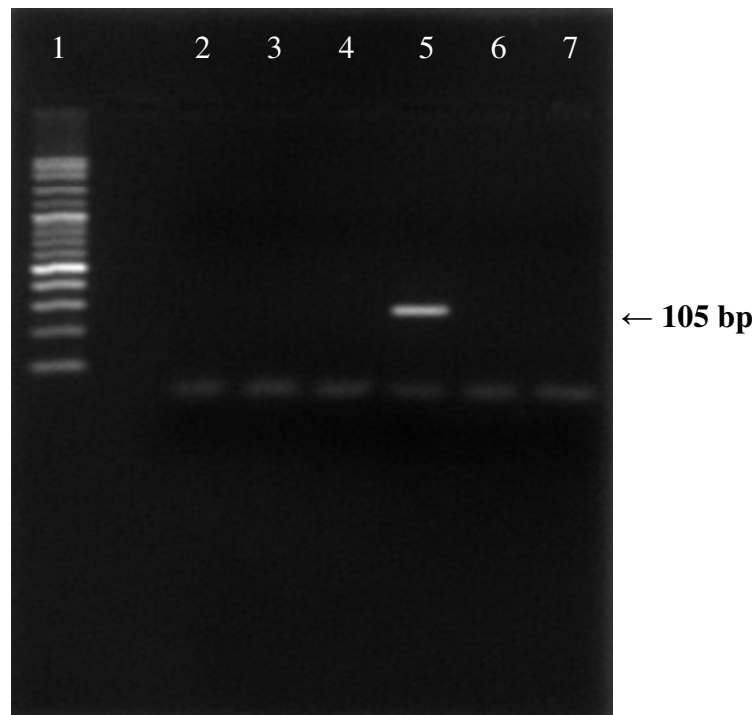


Figure 3. Second PCR amplification of RNA extracted from positive PI-BVDV samples. Lane 1: Vivantis DNA marker 50 bp, lane 2: negative control, lane 3: Singer strain BVDV-1 positive control, lane 4, 7-8: BVDV-1 positive field samples, lane 5: 890 strain BVDV-2 positive control.

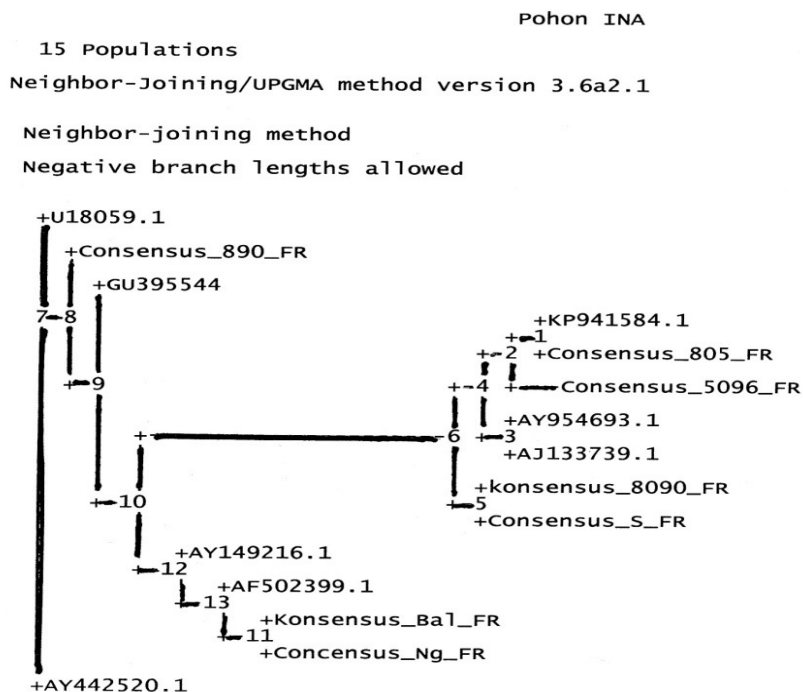


Figure 4. Phylogenetic tree of the 5' untranslated regions (UTR) of BVDV strains and isolates. The tree was generated from comparative alignment of sequences from 288bp of the 5'UTR of the BVDV genome.

amplification, the BVDV genotype 1 were no longer visible but BVDV genotype 2 clearly amplified at 105 bp (Figure 3). Based on the result, the genetic typing of viral RNA revealed that only BVDV genotype 1 were present and BVDV genotype 2 was absent in our collection of samples (Figure 3). Using the specific primers pairs listed in Table 1 the existence of subgenotype of BVDV-1 could not be detected. In the present study, however, based on the phylogenetic analysis, 2 samples (805_FR and 5096_FR) were in the same clade with published sequence KP941584 (Figure 4). KP941584 is considered to be BVDV-1a (Workman et al. 2015).

DISCUSSION

Bovine viral diarrhea virus (BVDV) infections are endemic in cattle industry in most part of the world. High prevalence combine with the negative effects on reproduction health conditions in affected herds result in significant economic impact to the cattle production all over the world (Grooms, 2004). In this study, attempt to identify that BVD was likely to be present on the herd(s) was done by collecting samples from unvaccinated herd(s) and among the cows with the history of poor conception rates. According to Sayers (2004), poor conception rate is one of an indication that the herd may have BVD. The adverse effect on conception has been

attributed to fertilization failure (Baker, 1995). In most herds, infertility is the most common and costly because it can go undiagnosed (Navarre, 2006). Among other infectious diseases, bovine viral diarrhea (BVD) is one of the most important infectious diseases which associated with infertility and abortion (Murray, 1991). Finding the PI-BVDV animal(s) in a herd is necessary for implementation of BVDV control program. The PI animal is considered the main source of BVDV infection, as it constantly eliminates high titers of virus in the secretions and excretions throughout it's entirely life (Lindberg and Houe, 2005). Regardless of their prevalence, PI animals are the main source of infection within the herd. In close confinement housing operation a PI animal can infect up 90% of the herd regardless of their prevalence (Hoe, 1999).

In this study we focused on the genetic typing of BVDV positive samples collected from persistently infected cattle. The oligonucleotide primers used for the first amplification or the second amplification were respectively specific for a 198 bp and 105 bp fragment of the p80 BVDV gene. The results revealed that all the 15 IP-BVDV field positive samples had BVDV-1 genotype (Figures 2 and 3). No evidence of BVDV-2 genotype was found in our recent collected blood samples suggesting either a very low prevalence or its absence. According to Pellerin et al. (1994), the BVDV-1 genotype is spread worldwide while the BVDV-2 genotype occur mainly in

the USA and Canada (Ridpath, 1994), and at a lower rate in other European countries (Ridpath, 1994). BVDV strains are further divided into sub-genotypes. Two sub-genotypes of both BVDV-1 (BVDV 1a and BVDV 1b) and BVDV-2 (BVDV 2a and BVDV 2b) have been reported (Evermann and Ridpath, 2002). Several evidence suggested the consequences of antigenic differences between BVDV-1 subgenotypes including comparative neutralization titers (Fulton et al., 2003); vaccination breaks (Van Campen and Woodard, 1997) and the selective response of persistently infected animals toward viruses outside the subgenotype but not to viruses within the same subgenotype (Fulton et al., 2003). All of the facts suggesting the importance of having several sub-genotypes of BVDV in the vaccines for a better protection.

The genetic diversity of the IP-BVDV field positive samples in this present study was done by determining the nucleotide sequences of the 5'UTR. Using the primers pair in Table 2, we failed to prove the presence of the genetic variability among the BVDV-1 circulating in the dairy cattle population in Central Java, Indonesia. The result of this study were make sense since the majority of dairy cattle used in this study were originally coming from Australia in which the BVDV-1c sub-genotype is predominant (Mahony et al., 2005). However, based on the phylogenetic analysis (Figure 4) two IP-BVDV positive samples in this study (805_FR and 5096_FR) sharing highest similarity (99% homology) with the representative subgenotype BVDV-1a KP941584 isolates which are currently circulating in Kansas, USA (Workman et al., 2015). The discrepancy of the research result probably due to the low number of samples analysed, it is impossible to determine the prevalence of BVDV-1 subtypes circulating in Central Java, Indonesia. In consequence, further research involving higher number of BVDV-1 positive samples from different region of Indonesia is urgently needed.

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