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REGULAR ARTICLES

Molecular and phylogenetic characterization of bovine coronavirus virus isolated from dairy cattle in Central Region, Thailand

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Abstract Bovine coronavirus (BCoV) is involved mainly in enteric infections in cattle. This study reports the first molecular detection of BCoV in a diarrhea outbreak in dairy cows in the Central Region, Thailand. BCoV was molecularly detected from bloody diarrheic cattle feces by using nested PCR. Agarose gel electrophoresis of three diarrheic fecal samples yielded from the 25 samples desired amplicons that were 488 base pairs and sequencing substantiated that have BCoV. The sequence alignment indicated that nucleotide and amino acid sequences, the three TWD isolated in Thailand, were more quite homologous to each other (amino acid at position 39 of TWD1, TWD3 was proline, but TWD2 was serine) and closely related to OK-0514-3strain (virulent respiratory strain; RBCoV).The amino acid sequencing identities among TWD1, TWD2,TWD3, and OK-0514-3 strain were 96.0 to 96.6%, those at which T3I, H65N, D87G, H127Y, andQ136R were changed. In addition, the phylogenetic tree of the hypervariable region S1subunit spike glycoprotein BCoV gene was composed of three major clades by using the 54 sequences generated and showed that the evolutionally distance, TWD1, TWD2, and TWD3 were the isolated group together and most similar to OK-0514-3 strain (98.2 to 98.5% similarity). Further study will develop ELISA assay for serologic detection of winter dysentery disease.

Keywords Bovine coronavirus · Dairy cattle · Nested PCR · Winter dysentery disease

Introduction

Bovine coronavirus (BCoV) is a single-stranded, non-segmented, positive sense RNA genome of 27 to 32 kb. BCV virion is enveloped and pleomorphic to spherical in shape about 80–200 nm in diameter. It is classified in the order *Nidovirales*, family *Coronaviridae*, subfamily *Coronavirinae*, genus *Betacoronavirus* (Group 2 Coronavirus) subgroup 2A (Graham et al. 2013). Its genome includes 13 open reading frames (ORFs) flanked by 5′ and 3′ untranslated regions. The genome of BCoV contains of five structural proteins and non-structural proteins. Five major structural proteins are encoded within the genomic RNA such as hemagglutinin-esterase (HE) protein (ORF3), spike (S) glycoprotein (ORF4), small membrane (E) protein (ORF8), transmembrane (M) protein (ORF9), and nucleocapsid (N) protein (ORF10) (Chouljenko et al. 2001; Masters 2006).

The S protein is a 180-kDa glycoprotein, length 4038 bp (nn 2152–6243), type 1 viral fusion protein on the viral surface, playing an important role in induction of neutralizing antibodies and cleaved at the amino acid position 768–769 in two subunits: S1 subunit and S2 subunit (Yoo and Deregt 2001). The S protein forms club-shaped structure which has amino-terminal S1 receptor binding unit that is the bulbous part, whereas the carboxyl-terminal S2 membrane fusion unit is the stalk part (Bosch et al. 2003). Moreover, S1 subunit has the downstream hypervariable region (HVR) that is different in each strain, but S2 subunit is conserved among strains (Weiss and Martin 2005).

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Table 1 Primer sequences along with expected PCR product size and the thermal cycling condition

Primer name	Primer sequence		Product size (bp)	Reference	
S1HS	(5′)-CTATACCCAATGGTAGGA-(3′)		885	Brandao et al. (2003)	
S1HA	(5′)-CTGAAACACGACCGCTAT-(3′)				
S1N	(5′)-GTTTCTGTTAGCAGGTTTAA-(3′)		488		
S1NA	(5′)-ATATTACACCTATCCCCTTG-(3′)				
Thermal cycling condition					
	Pre-denaturation	Denaturation	Primer annealing	Primer extension	Final extension
Outer primers	94 °C; 180 s	94 °C; 60 s ×35 cycles	53.4 °C; 90 s	72 °C; 60 s	72 °C; 600 s
Internal primers	94 °C; 180 s	94 °C; 60 s ×25 cycles	58.4 °C; 90 s	72 °C; 60 s	72 °C; 600 s

BCoV is the cause of winter dysentery disease, that is a hemorrhagic enteritis with anorexia, dehydration, emaciation, and suffering severely from decreasing production in adult dairy cattle. Moreover, BCoV causes watery diarrhea in newborn calves whose range ages from 1 week to 3 months (Blowey and Weaver 2011). BCoV infection is a serious cause of economic losses to the dairy industry around the world (Saif 2004). Besides, the maximum decrease in milk production ranges around 10% and may last for 1–2 weeks, after that milk production levels are regained in mild epidemics of BCoV, but in severe epidemics, milk production decreases to 30% and continues up to 1 month (Radostits et al. 2007).

BCoV was first reported by Mebus in USA (Mebus et al. 1972, 1973), which is the Mebus strain, and by Kanno, T. in Asia (Kanno et al.2007),which is the Kakegawa strain; Aiumlamai et al. (1992) reported only prevalence of bovine coronavirus antibody in bulk tank milk samples at 93% in Muaklek area, Saraburi Province, Thailand, but there are no reports of bovine coronavirus molecular diagnosis in cattle. This study reveals the molecular and phylogenetic characterization of bovine coronavirus by molecular methodology in dairy cattle in Thailand.

Materials and methods

Sample collection

Twenty-five Holstein-Friesian cattle in six farms that had clinically suspected cattle in Central Region, Thailand, were collected whole bloods and feces. Some cattle showed clinical signs such as lethargy, bloody diarrhea, watery diarrhea, milk production decreasing, and death in some cows. All samples were stored at −80 °C until processing.

RNA extraction and cDNA synthesis

RNA were extracted from whole bloods and feces of all cattle using FavorPrep™ Viral Nucleic Acid Extraction Kit I (Favorgen®) according to the manufacturer's instructions; then 11 ul of each RNA was added into a PCR tube containing of 4 ul of 5× reaction buffer, 2 ul of Random hexamer primer, 2 ul of 10 mM dNTP, 0.5 ul of RNase Inhibitor, and 1 ul of Thermo Scientific RevertAid™ Reverse Transcriptase; mix gently and spin down; then incubated 10 min at 25 °C followed by 60 min at 42 °C and terminate the reaction by heating at 70 °C for 10 min in a thermal cycler (Bio-Rad T100™, Bio-Rad®).

PCR primers and conditions

Two pairs of primers were followed Brandao et al. (2003) that conserved regions flanking the hypervariable region of the S gene (GenBank accession no. M31053): outer primers (sense S1HS 5′-CTATACCCAATGGTAGGA-3′ and anti-sense S1HA 5′-CTGAAACACGACCGCTAT-3′) with a predicted 885-bp-long product (nn 1204 to 2088 from the S gene) and internal primers (sense S1N 5′-GTTTCTGTTAGCAG GTTTAA-3′ and anti-sense S1NA 5′-ATATTACACCTATC CCCTTG-3′) with a predicted 488-bp-long product (nn 1329 to 1816 from the S gene) (Brandao et al. 2003).The expected PCR product size alongside the thermal cycling conditions of the primers was given in Table 1. PCR reactions were set up into 20-ul volume containing 18 ul PCR master mix (2.5 units Taq DNA polymerase (Invitrogen™) in 1× PCR-$MgCl_2$ buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP),0.5 uM of each primer, 2 ul of the DNA template ,and total volume was made up to 20 ul. DNA from the whole bloods as well as from the feces of both calves and cows was used. The expected PCR products

Table 2 GenBank accession numbers of reference strains of subgroup 2A genus *Betacoronavirus*, country of origin used in neighbor-joining analysis of spike glycoprotein sequences

Strain	Year	Country	Strain origin	GenBank accession no.
Mebus	1972	USA	Enteric	U00735
LY-138	1965	USA	Enteric	AF058942
ENT	1998	USA	Enteric	AF391541
LSU-94LSS-051-2	1994	USA	Respiratory	AF058943
OK-0514-3	1996	USA	Respiratory	AF058944
L9	1991	USA	Vaccine strain	M64667
182NS	2000	USA	Respiratory	DQ320764
220NS	1998	USA	Respiratory	DQ320762
232NS	2000	USA	Respiratory	DQ320763
Norden vaccine	1991	USA	Vaccine strain	M64668
F15	1979	France	Enteric	D00731
Quebec	1972	Canada	Winter Dysentery	AF220295
BCQ7373	1992	Canada	Winter Dysentery	AF239306
BCQ1523	1989	Canada	Enteric	AF239307
BCQ2590	1992	Canada	Winter Dysentery	AF239317
BCQ3994	2001	Canada	Respiratory	AF339836
BCO44175	2000	Canada	Respiratory	AF239309
BCO43277	2000	Canada	Respiratory	AF239308
BCQ571	1989	Canada	Enteric	AH010363
BCQ9	1989	Canada	Enteric	U06091
BCQ20	1989	Canada	Enteric	U06092
BCQ2070	1989	Canada	Enteric	U06090
BR-UEL1	2004	Brazil	Enteric	DQ479421
BR-UEL2	2004	Brazil	Enteric	DQ479422
BR-UEL3	2004	Brazil	Enteric	DQ479423
Kakegawa	1980	Japan	Winter Dysentery	DQ479424
KCD1	2004	South Korea	Enteric	DQ389632
KCD2	2004	South Korea	Enteric	DQ389633
KCD4	2004	South Korea	Enteric	DQ389635
KCD5	2004	South Korea	Enteric	DQ389636
KCD6	2004	South Korea	Enteric	DQ389637
KCD7	2004	South Korea	Enteric	DQ389638
KCD8	2004	South Korea	Enteric	DQ389639
KWD1	2002	South Korea	Winter dysentery	AY935637
KWD2	2002	South Korea	Winter dysentery	AY935638
KWD3	2002	South Korea	Winter dysentery	AY935639
KWD4	2002	South Korea	Winter dysentery	AY935640
KWD5	2002	South Korea	Winter dysentery	AY935641
KWD7	2002	South Korea	Winter dysentery	AY935643
KWD9	2002	South Korea	Winter dysentery	AY935645
KWD11	2002	South Korea	Winter dysentery	DQ389652
KWD13	2002	South Korea	Winter dysentery	DQ389654
KWD14	2002	South Korea	Winter dysentery	DQ389655
KWD15	2002	South Korea	Winter dysentery	DQ389656
KWD16	2002	South Korea	Winter dysentery	DQ389657
BC94Korean vaccine	1994	South Korea	Vaccine strain	EU401989
0501	2005	South Korea	Vaccine strain	EU686689
0502	2005	South Korea	Vaccine strain	EU401986
A3	1994	South Korea	Vaccine strain	EU401987
SUN5	1994	South Korea	Vaccine strain	EU401988
HCoV-OC43	1967	UK	Respiratory	NC005147

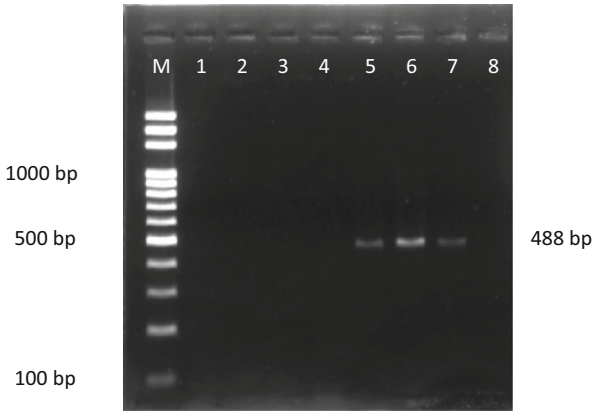

Fig. 1 Nested PCR amplification *lane M*: 100 bp DNA ladder(Solis BioDyne); *lanes 1–3*: three known positive samples using outer primers; *lane 4*: negative control; *lanes 5–7*: known positive samples using inner primers; *lane 8*: negative control

were analyzed by 1.5% agarose gel electrophoresis in 1× Tris-acetate-EDTA (TAE) buffer pH 8.3 (40 mM Tris, 20 mM acetic acid, and 1 mM EDTA),visualized with GelRed™ nucleic acid staining (Biotium) and analyzed by Gel Doc™ EZ System (Bio-Rad®).

Sequencing analysis

The PCR products from the nested PCR were purified by Thermo Scientific gene JET gel extraction kit; DNA fragments were sent to First Base Co., Ltd. (Selangor, Malaysia) for sequencing. Sequence quality analysis as performed by using BioEdit sequence alignment editor and the consensus sequences were assembled using the CAP contig assembly program of BioEdit. Finally, the sequences were submitted to the nucleotide basic local alignment search tool (BLASTn) software (http://blast.ncbi. nlm.nih.gov/) to find the most similar sequences and possible non-BCoV related similarities.

Table 3 Corrected OD values (OD_{corr}) and percent positivity values (PP) of TWD1–3 from SVANOVIR® BCV-Ab test

Sample name	OD_{corr} [a]	PP[b]	Interpretation
TWD1	1.277	84.570	Positive
TWD2	1.820	128.124	Positive
TWD3	1.771	124.674	Positive

[a] The optical density (OD) values in well coated with BCV viral antigen are corrected by subtracting the OD values of the corresponding wells containing the control antigen. $OD_{Corr} = OD_{BCV} - OD_{Control}$

[b] All corrected OD values for the test samples as well as the negative control are related to the corrected OD value of the positive control as follows: $PP = [OD_{Corr(Sample/NegativeControl)}/OD_{Corr\ (Positive\ Control)}] \times 100$ and interpretation of serum samples; PP interpretation <10 = negative and ≥10 = positive

Phylogenetic analysis

The three positive sample sequences, 50 BCoV, and 1 human coronavirus OC43 (HCoV-OC43) sequences obtained from the National Center for Biotechnology Information, USA (GenBank) (http://www.ncbi.nlm.nih.gov/genbank/), were aligned and constructed a phylogenetic tree by using the Molecular Evolutionary Genetics Analysis (MEGA, version 7). The GenBank accession numbers of 50 BCoV and HCoV-OC43 strains are indicated in Table 2. HCoV-OC43 strain was used as an outgroup. The phylogenetic tree was created by using the neighbor-joining method with bootstrap test (1000 replicates) based on the nucleotide sequence of the hypervariable region in BCoV. The evolutionary distances were computed by using the Tamura 3-parameter method.

GenBank accession number

GenBank accession number KX373886, KX373887, and KX373888 were assigned to TWD1, TWD2, and TWD3, respectively. These are the sequenced 448-bp fragment.

Results

Clinical inspection of affected dairy cattle showed watery diarrhea, bloody diarrhea, dehydration, melena or occult blood in feces, decrease milk production, and death in some cows.

Agarose gel electrophoresis of three diarrheic fecal samples yielded that out of the 25 samples the desired amplicons that were 488 base pairs (Fig. 1). In addition, after having been purified, the three samples yielded agarose gel, and they were sent sequencing analysis, by using the BLASTn. The result showed that they possessed 97–99% nucleotide identities to spike glycoprotein of bovine coronavirus.

The three cows were positive for fecal testing and also positive for the blood testing (Table 3). All blood samples analyzed the presence of the IgG antibodies to BCoV by commercially available indirect ELISA (SVANOVA, Biotech).

The sequence alignment by ClustalW multiple alignment of the BioEdit program indicated that nucleotide and amino acid sequences of the three TWD isolated in Thailand were high similar to each other (amino acid at position 39 of TWD1, TWD3 was proline, but TWD2 was serine) and were closely related to OK-0514-3 strain (virulent respiratory strain; RBCoV).The amino acid sequence identities among TWD1, TWD2, TWD3, and OK-0514-3 strain were 96.0 to 96.6%, which of those at T3I, H65N, D87G, H127Y, and Q136R were changed (data not shown).

The phylogenetic tree of partial spike glycoprotein genes of bovine coronavirus was composed of three major clades by using the 54 sequences generated in this study from field samples, GenBank reference strains, and vaccine strains.

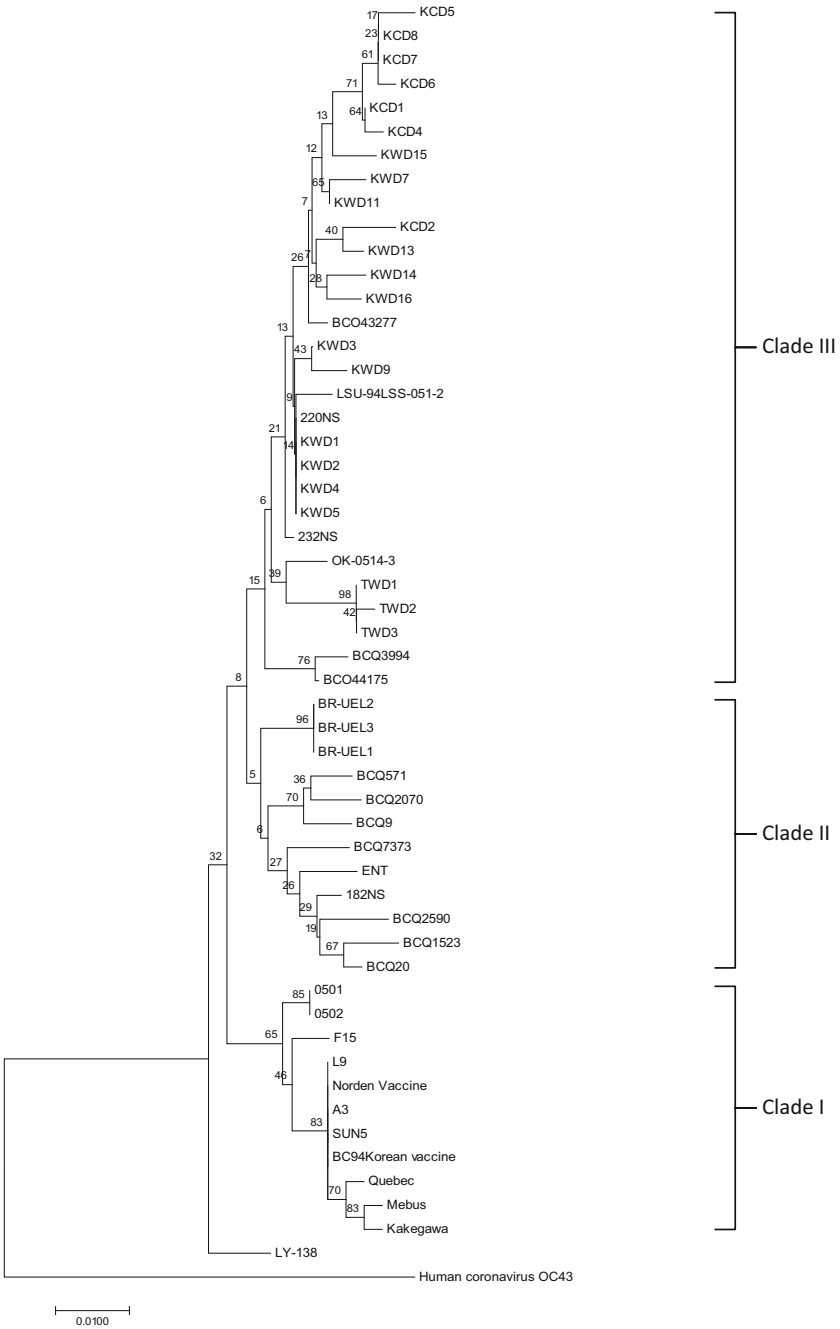

Fig. 2 The phylogenetic tree of the partial S gene sequences of BCoV. This tree was generated using the neighbor-joining method. Bootstrap values were obtained from 1000 replications. The *scale bar* represents the number of substitutions per site. TWD1–TWD3 were received from the three positive samples in Thailand. Other sequences were acquired from GenBank (Table 2), and HCoV-OC43 was used as an outgroup

TWD1, TWD2, and TWD3 were closely related to the isolated from three positive samples (99.8 to 100% similarity), and they also had 98.2 to 98.5% similarity with OK-0514-3 strain. In Asia, they had 95.9 to 98.5% similarity with 24 Korean strains and Kakegawa strain. Furthermore, the most distance between those of TWD and other published strains were the Mebus and Kakegawa strain (95.9 to 96.2% similarity) for the partial S1 gene by nested PCR in Thailand. TWD1, TWD2, TWD3, all of Korean calf diarrhea (KCD) and Korean winter dysentery strains (KWD), some American respiratory strains (OK-0514-3, LSU-94LSS-051-2, 232NS, 220NS), and Canadian respiratory strains (BCO43277, BCQ3994, BCO44175) were clustered on clade 3. All of the Brazilian strains (BR-UEL1, BR-UEL2, BR-UEL3),Canadian enteric strains (BCQ571, BCQ2070, BCQ9, BCQ1523, BCQ20), Canadian winter dysentery strain (BCQ7373, BCQ2590), American enteric strains (ENT), and American respiratory strains (182NS) were clustered on clade 2. The Korean vaccine strains(0501, 0502, A3, SUN5, BC94 Korean vaccine), F15, L9, Norden vaccine, Quebec, Mebus, and Kakegawa strain were clustered on clade 1, but LY-138 (American enteric strain) was clustered on a separate branch (Fig. 2).

Discussion

In this study, we aimed to identify bovine coronavirus in Thailand and compare our partial S1 sequences with some field and vaccine strains around the world in GenBank. During clinical inspection, the amplicons from nested PCR amplification and percent identity from the BLASTn software of three positive field samples were revealed. This is the first detection of bovine coronavirus in Thailand. Inner primers (Brandao et al. 2003) were able to be detected BCoV. However, the band of PCR with outer primers could not be detected in agarose gel because all of the three positive samples had probably low concentrations of BCoV or/and PCR inhibitors in feces that may yield false negative. The nested PCR technique will increase more sensitivity and specificity of DNA amplification than conventional PCR technique (Rustempasic et al. 2016).

The phylogenetic tree for the hypervariable region of the S1 subunit spike glycoprotein BCoV gene showed that the evolutionally distant, TWD1, TWD2, and TWD3 in our study were the isolated group together and most similar to OK-0514-3 strain that is a respiratory strain (RBCoV) (Fig. 2). Respiratory strain was closely related with enteric strain (EBCoV); Hasoksuz et al. (2002) said the BCoV strains may be diverging from an enteric tropism to a dual (respiratory and enteric) tropism over time via intermediates. Moreover, Cho et al. (2000) have observed that gnotobiotic and colostrum-deprived calves inoculated with respiratory isolates from BCoV in that all strains were pneumoenteric and were shed both nasally and rectally and induced diarrhea. It may explain that there were no variations between the respiratory and the enteric isolates (Hasoksuz et al. 2002). The amino acid substitutions of TWD1, TWD2, and TWD3 from OK-0514-3 strain have been related immunological escape mutation through changes in protein secondary structure (Hasoksuz et al. 2002; Yoo and Deregt 2001). All virulent TWD tended to be distant from the prototype strains, because the allelic variation resulted in genetic mutation over time. Similarly, Kanno et al. (2007) and Fulton et al. (2013) concluded that these isolates had distinctive genetic divergent from the prototype BCoV strains such as Mebus, Quebec, F15, and LY-138 strains.

In conclusion, this is the first report of molecular and phylogenetic diagnosis of bovine coronavirus in the dairy cattle from Thailand. It is important, as the data provides that there has been BCoV infection in Central Region, Thailand. Moreover, phylogenetic tree revealed closely relation between the three isolates, enteric, and respiratory BCoV strains. Further study will develop ELISA assay for serologic detection of winter dysentery disease.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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