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Research Article

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Development of Recombinant Nucleocapsid Protein-based Enzyme-linked Immunosorbent Assay for Serological Detection of Winter Dysentery Disease

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Abstract

Winter Dysentery Disease is an acute and contagious viral disease that adversely affects dairy cattle on farms in Thailand. An indirect enzyme-linked immunosorbent assay (ELISA) based on the recombinant nucleocapsid (rN) protein which has been developed for antibodies detection against Winter Dysentery Disease was produced by an *Escherichia coli* protein expression system. The epitope antigen N protein was designed using almost total N gene fragments (8-430 aa, N gene). It was identified to be approximately 48 kDa as the rN protein and can bind bovine coronavirus dairy cattle positive serum by western blot analysis. The conditions of the ELISA method were optimized. The rN protein was standardized with a coating antigen concentration of 5 µg/well. The rN protein was tested with sera in both infected and uninfected dairy cattle. The dilution of the primary antibodies was identified as 1:50 using a checkerboard titration. The intra- and inter-assays were repeatable. The cut-off of the corrected OD450 value from mean ± 2SD (standard deviations) was established at 0.049. The percentage of specificity, sensitivity and accuracy between the developed rN-ELISA and a SVANOVIR® BCV-Ab ELISA kit was 96.3, 84.8 and 86.1%, respectively. Cohen's kappa value of the developed ELISA compared with the commercial test kit was 0.71. The correlation coefficient of absorbance values from these two tests was 0.68. The recombinant nucleocapsid protein ELISA method might be helpful for bovine coronavirus diagnosis and surveillance.

Keywords

Bovine coronavirus; Dairy cattle; ELISA; Winter dysentery disease

Introduction

Winter Dysentery Disease (WD) is an acute and contagious viral disease of ruminants [1]. The main clinical signs involve the gastrointestinal tract and respiratory tract [2] and include bloody diarrhea, haematochezia, fever, weakness and nasal discharge. This disease occurs in the winter months, most commonly from November through March [3,4]. Furthermore, WD is a main economic problem in the dairy industry globally resulting in decreased the milk production and weight loss [5,6]. Diagnosis of WD can be detected based on history, clinical signs, lesions, virus isolation, serological test and molecular techniques [7]. Bovine coronavirus (BCoV) is the cause of WD resulting in diarrhea in adult cattle and calves [8]. BCoV can be detected by using both electron microscopy and an antigen capture ELISA test from the feces of affected cattle. Moreover, serological detection of BCoV can be detected using virus neutralization which is the "gold standard" method, Hemagglutination-inhibition (HI) and Enzymed-Linked Immunosorbent Assay (ELISA) [9-11]. Treatment for WD should be the focus for providing supportive treatment for affected cattle; furthermore, if an animal becomes dehydrated, it may be effectively treated with oral fluids.

BCoV is a single-stranded, non-segmented, positive-sense RNA genome of approximately 30 kb [12]. BCoV virion is enveloped and pleomorphic-to-spherical in shape and about 80-200 nm in diameter. It is classified in the order Nidovirales, family Coronaviridae, subfamily Coronavirinae, genus betacoronavirus (Group 2 Coronavirus) subgroup 2A [13]. Its genome provides 13 open reading frames (ORFs) which are flanked by 5' and 3' untranslated regions and contains five structural proteins and non-structural proteins. Furthermore, the five major structural proteins are encoded within the genomic RNA ,namely hemagglutinin-esterase (HE) protein (ORF3), spike (S) glycoprotein (ORF4), small membrane (E) protein (ORF8), transmembrane (M) protein (ORF9), and nucleocapsid (N) protein (ORF10) [12,14]. BCoV can be mainly transmitted via the fecal-oral and aerosol routes [15]. The incubation period is 3-8 days. Adult cattle with low serum antibody levels to BCoV are more susceptible to developing the disease than those with high antibody levels. It has been shown that acquired antibodies remain detectable for years, even without reinfection [16]. Nowadays, BCoV infection seems to be endemically present in cattle on all continents [17]. BCoV was first reported by Mebus in the USA [18,19]. In Thailand, the first reports of seroprevalence of BCoV were in bulk tank milk samples at 93% in the Muaklek area, Saraburi province [20] and more recently from BCoV molecular diagnosis in dairy cattle [21].

The N protein of BCoV is a 50-60 KD phosphoprotein that is bound to viral genomic RNA to form a helical nucleocapsid with 1,347 bp in length. N protein plays a role in the replication of viral RNA since the antibody directed against the N protein inhibits the in vitro RNA polymerase chain reaction [22]. It is the highly immunogenic and conserved amino acid sequences of the nucleocapsid gene among various strains which induce antibodies of serological diagnosis [23]. Moreover, the N protein plays important roles in viral transcription and translation [24].

Enzyme-linked immunosorbent assay (ELISA) is a sensitive and specific method for detection of BCoV in infected herds. ELISA based on purified recombinant protein may have a high immunodominant epitopes [25]. Nucleocapsid protein-based ELISA for detection of antibodies against other species of coronavirus has been used in some reports with IBV, PED and SARS [26-28]. N protein expressed in *E. coli* provided better immunogenicity than that expressed in insect cells. N protein expressed in *E. coli* revealed a good correlation with the commercial ELISA kit for IBV [26,29]. In the present study, the

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N gene was cloned and expressed from a Thai field isolate in the *E. coli* system. The expressed and purified recombinant nucleocapsid protein was used as the coating antigen for an indirect ELISA, and the developed rN-ELISA was tested with specific antibodies from infected dairy cattle and then compared with the available commercial ELISA kit (SVANOVIR® BCV-Ab; Svanova, Sweden).

Materials and Methods

Sample collection and RT-PCR technique

Serum samples were collected from 231 dairy cows in 33 dairy herds on 27 farms in the western region and 6 farms in the central region of Thailand. RNA was extracted from diarrheic cattle feces using a FavorPrep™ Viral Nucleic Acid Extraction Kit I (Favorgen®; Biotech Corporation; Taiwan) according to the manufacturer's instructions. The cDNA samples were synthesized from the extracted RNA. Eleven microlitres of extracted RNA were added into a PCR tube containing 4 µl of 5X Reaction Buffer, 2 µl of Random hexamer primer, 2 µl of dNTP, 0.5 µl of RNase Inhibitor and 1 µl of RevertAid Reverse Transcriptase (Thermo Scientific™; Thermo Fisher Scientific Inc.; U.S.A.). The mixture was incubated for 10 min at 25°C followed by 60 min at 42°C, and then the reaction was terminated by heating at 70°C for 10 min in a thermal cycler (Biorad T100™; BIORAD®; U.S.A.).

Primer design and N gene cloning

Pairs of primers were designed for almost the full-length of the N gene (GenBank accession No. M31053). A predicted 1,269 base pairs (nt 22 to 1290 from the N gene) of amplicon were amplified by forward primers PNF 5'- CAATCCAGTAGTAG AGCGTC -3' and reverse primers PNR 5'- GTCCTCTGCAGTCAACTCTC -3'. PCR reactions were set up in 20 µl volume containing 18 µl PCR master mix (2.5 units Taq DNA polymerase (Invitrogen™; Thermo Fisher Scientific Inc.; U.S.A.) in 1x PCR-$MgCl_2$ buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP), 0.5 µl of each primer and 2 µl of the DNA template. The total volume was made up to 20 µl. The reaction conditions were performed with one cycle of pre-denaturation at 94°C for 5 min followed by 35 cycles at 94°C for 30 sec, 54.5°C for 1.5 min and 72°C for 1.5 min and then one cycle of post-elongation at 72°C for 5 min. The expected PCR products were stained with GelRed™ nucleic acid staining (Biotium Inc.; U.S.A.), visualized using a UV illuminator after 1.5% agarose gel electrophoresis in 1x Tris-acetate-EDTA (TAE) buffer pH 8.3 (40 mM Tris, 20mM acetic acid, and 1mM EDTA), and analyzed using the Gel Doc™ EZ System (BIORAD®; U.S.A.). The 1,269 bp of PCR product from the N gene was purified using a FavorPrep™ GEL/PCR Purification Kit (Favorgen®; Biotech Corporation; Taiwan) according to the manufacturer's instructions. The purified PCR product was ligated into RBC TA cloning vector (RBC Bioscience Corp.; Taiwan) and transformed into a competent *E. coli* strain JM109 using the heat shock technique. The bacterial colonies that carried the N-inserted plasmids were confirmed using a PCR technique and nucleotide sequencing. Nucleotide sequence analysis was performed using the BioEdit program version 7.0.5 (Ibis Therapeutics; U.S.A.) Finally, the nucleotide sequence was submitted to the nucleotide basic local alignment search tool (BLASTn) software (http://blast.ncbi. nlm.nih.gov/) to identify BCoV sequences with related similarities.

Subcloning of N gene into expression vector

The *E. coli* strain JM109 carrying the N-inserted plasmids and pQE80L expression vectors were individually cultured overnight in lysogeny broth (LB) containing 100 µg/ml of ampicillin. Both of them were extracted using a FavorPrep™ Plasmid Extraction Mini Kit (Favorgen®; Biotech Corporation; Taiwan) according to the manufacturer's protocols. The N-inserted plasmids and pQE80L vectors were digested with *Bam*HI and *Hind*III restriction enzymes (FastDigest™; Thermo Scientific™; Thermo Fisher Scientific Inc.; U.S.A.) then ligated together. The ligation mixture was transformed into a competent cell of the *E. coli* strain M15 using the heat shock technique. The N-inserted gene in each bacterial colony was examined using the conventional PCR technique with PNF and PNR primers.

Protein expression in *E. coli*

A single colony of each bacterium containing either the N-inserted plasmid or no inserted plasmid (pQE80L vector) was grown overnight for a starter culture in LB broth containing 100 µg/ml of ampicillin and 25 µg/ml of kanamycin in an incubator shaker at 180 rpm and 37°C. The starter cultures were subcultured at a concentration of 1:50 in LB broth containing 100 µg/ml of ampicillin and 25 µg/ml of kanamycin. They were shaken until the optical density (OD_{600}) reached 0.6. The recombinant N protein was induced by adding isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 0.25 mM for 6 hr. The cell pellet and supernatant were separated by using centrifugation at 10,000 rpm and 4°C for 5 min. The recombinant N protein added to a 2X SDS-PAGE loading buffer was detected using 12% SDS-PAGE and Coomassie brilliant blue staining.

Protein purification

The pellet fraction containing the recombinant N protein was lyzed using enzymolysis (lysozyme 1 mg/ml) and sonication. Histidine-tagged N fusion proteins were dissolved using a serial urea concentration and sonication, with each urea concentration being 1 M urea, repeated three times and 2 M urea, repeated three times, respectively. Purification was achieved using affinity column chromatography containing Ni-NTA (ÄKTA start™; GE-Healthcare; U.K.) according to the manufacturer's protocols. Purified recombinant N proteins were detected using 12% SDS-PAGE. Each purified recombinant N protein concentration was determined using a spectrophotometer (Nanodrop 2000; Thermo Scientific™; U.S.A.), according to the manufacturer's instructions. Purified recombinant N proteins were stored at -20°C until analysis.

Western blot analysis and biological function evaluation

The proteins from the N-purified supernatant, N pellet fraction and pQE80L pellet fraction were run in SDS-PAGE and transferred to a nitrocellulose membrane using a Trans-Blot® SD Semi-Dry Electrophoretic Transfer Cell (BIORAD®; U.S.A.) according to the manufacturer's instructions. The nitrocellulose membrane was blocked with 5% skim milk in 1XPBS-T (phosphate buffered saline containing 0.05% Tween 20) at 37°C for 1 hr. A 1:3,000 dilution of Anti-His antibody (GE-Healthcare; U.K.) in 1X-PBS-T contained 1% skim milk (which was used for detection of the 6X histidine-tag protein on a recombinant protein) and incubated at 37°C for 1 hr. The membrane was washed with 1X PBS-T for 5 min, repeated three times. A secondary antibody was added of goat anti-mouse IgG-horseradish peroxidase (HRP) (KPL; U.S.A.) and was diluted 1:1,000 with 1% skim milk in 1X-PBS-T and incubated at 37°C for 1 hr. The membranes were washed with 1X-PBS-T for 5 min, repeated three times followed by development of the color of the 3,3',5,5'-Tetramethylbenzidine (TMB) membrane substrate (KPL; U.S.A.) within 15 min. The reaction

was stopped by soaking the membrane with 1X-PBS-T. Positive and negative dairy cattle sera to BCoV were tested using a commercial ELISA kit (SVANOVIR® BCV-Ab; Svanova, Sweden) and were used to evaluate for biological functions of the recombinant proteins. The nitrocellulose membrane-bound proteins from SDS-PAGE were blocked with 5% skim milk and 10% horse serum in 1X-PBS at 37°C for 1 hr. Each positive and negative dairy cattle serum was diluted 1:50 with 1% skim milk, 10% horse serum and 10% lysozyme pQE80L in 1X-PBS and incubated at 37°C for 1 hr. The membrane was washed with 1X-PBS-T for 5 min, repeated three times. Then, it was added to a dilution of 1:5,000 goat anti-bovine IgG-HRP (KPL; U.S.A.) in 1X-PBS with 1% skim milk and 10% horse serum and incubated at 37°C for 1 hr. The membranes were washed with 1X- PBS-T for 5 min, repeated three times and developed for the color of the TMB membrane substrate (KPL; U.S.A.) within 15 min. The reaction was stopped by soaking the membrane with 1X-PBS-T.

Enzyme-linked immunosorbent assay establishment

Recombinant protein coating: The 96-well flat-bottomed microtiter plates (MaxiSorp, Nunc; Roskilde, Denmark) were initially coated with 10 µg/well (100 µl/well) recombinant nucleocapsid (rN) protein diluted in coating buffer (carbonate-bicarbonate sodium, 0.1 mmol/L pH 9.6) and incubated at 37°C for 1 hr. By checkerboard titration, the optimal protein concentration 5 µg/well (dilution 1:50) was determined. The plates were then incubated with blocking solution (5% skim milk with 10% horse serum in 1X-PBS) at 37°C for 1 hr.

Primary antibody: The anti-BCoV antibody titer was also determined using the commercial antibody ELISA kit for BCoV (SVANOVIR® BCV-Ab; Svanova, Sweden) according to the manufacturer's instructions. Serum samples with a positive percentage (PP) of less than or equal to 10 were considered negative, while values more than 10 were considered positive. PP was calculated from the corrected OD value of the sample divided by the negative control and then divided by the corrected OD value of the positive control and multiplied by one hundred. By checkerboard titration, 100 µL of serum samples in primary buffer (1% skim milk, 10% horse serum and 10% lysozyme pQE80L in 1X-PBS) were diluted two-fold serially (1:25, 1:50, 1:100). Following incubation at 37°C for 1 hr, the wells were washed with 1X-PBS-T, repeated three times. The optimal serum sample dilution at 1:50 was determined.

Secondary antibody: A volume of 100 µl/well of goat anti-bovine IgG-HRP (KPL; U.S.A.) diluted 1:5,000 (0.1 µg/ml) in 1X-PBS with 1% skim milk and 10% horse serum. It was added to every well, followed by incubation at 37°C for 1 hr. The wells were washed with 1X-PBS-T, repeated three times.

Substrate TMB substrate (KPL; U.S.A.) 100 µl/well was dispensed immediately into every well and incubated for 15 min. The reaction was stopped by stop solution (0.5 M HCl) at 100 µl/well. The plates were read at 450 nm in an ELISA reader (Infinite® F50; Tecan; Männedorf, Switzerland).

Assay validation: Cut-off value, sensitivity and specificity. The cut-off value for the rN-ELISA was determined using the corrected OD_{450} values obtained from 27 negative dairy cattle sera and calculated from a mean of the OD_{450} from the N protein well minus the OD_{450} from the pQE80L protein (*E. coli* control antigen) well and two standard deviations (2SD) of negative sera. The sensitivity of the rN-ELISA was evaluated with positive dairy cattle sera against foot and mouth disease virus, bovine leukemia virus, bovine ephemeral fever virus and bovine viral diarrhea virus. The specificity of the rN-ELISA was evaluated with negative dairy cattle sera. The sensitivity specificity and accuracy of the rN-ELISA were evaluated by comparison with the commercial ELISA kit (SVANOVIR® BCV-Ab; Svanova, Sweden) by testing 231 dairy cattle sera from 33 dairy cow herds.

Coefficient of variation: Precision was estimated using the coefficient of variation (CV) from three positive dairy cattle sera, three negative dairy cattle sera and PBS. The intra-assay variation was obtained from quadruplicates of these in the same plate. The inter-assay variation was obtained from quadruplicate of these run on four different days. The percentage of CV was calculated from the SD divided by the average and multiplied by one hundred.

Cohen's kappa value: Agreement between the rN-ELISA and the commercial ELISA in classification of sera as positive or negative antibody was identified using Cohen's kappa test [30], considering values of more than 0.7 as substantial agreement.

Correlation coefficient: The data were used to analyze the relationship between the rN-ELISA and the commercial ELISA. The correlation coefficient (r) of the rN-ELISA was evaluated by comparison with the commercial ELISA (SVANOVIR® BCV-Ab; Svanova, Sweden) by testing 231 dairy cattle sera from a dairy cow farm.

Results

The partial N gene was amplified using the PCR technique from diarrheic dairy cattle fecal samples and was produced from the *E. coli* protein expression system. Agarose gel electrophoresis of diarrheic fecal samples yielded desired amplicons that were 1,269 base pairs (Figure 1). In addition, after purification of samples agarose gel, and sequencing analysis, using the nucleotide basic local alignment search tool (BLASTN), they possessed 99% nucleotide identities to the nucleocapsid protein of bovine coronavirus. GenBank accession number MF737175 was assigned to TWD4.

The pellet and supernatant of each *E. coli* containing the N gene-inserted plasmid were collected and analyzed using 12% SDS-PAGE. A protein band that increased after being induced by 0.25 mM IPTG was found to be approximately 48 kDa in the pellet fraction. In SDS-PAGE, it was shown that the N protein did not secrete in the supernatant (Figure 2). The expressed proteins along with the N-terminus 6× histidine-tag from the pellet fraction were detected using western blotting with the Anti-His antibody. The protein band was visualized

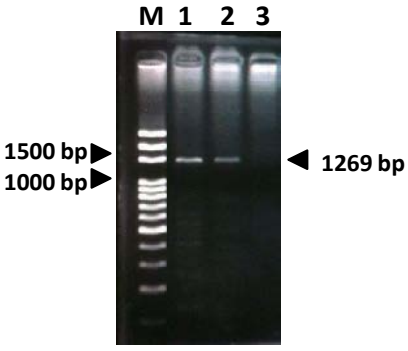

Figure 1: PCR amplification Lane M: 100 bp DNA ladder (Solis BioDyne; Tartu, Estonia), lane 1: positive control, lane 2: diarrheic dairy cattle fecal sample, lane 3: negative control.

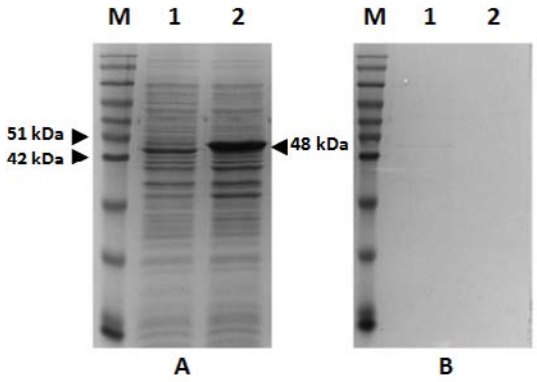

Figure 2: SDS-PAGE analysis showing the expressed N from *E. coli*. The samples show pellet fraction (A) and supernatant fraction (B) which were collected and evaluated using SDS-PAGE. M is represented by a Chromatein prestained protein ladder (Vivantis; Suite V., Chino. CA, U.S.A.) Lane 1 is pQE80L control plasmid., Lane 2 is N-inserted plasmid.

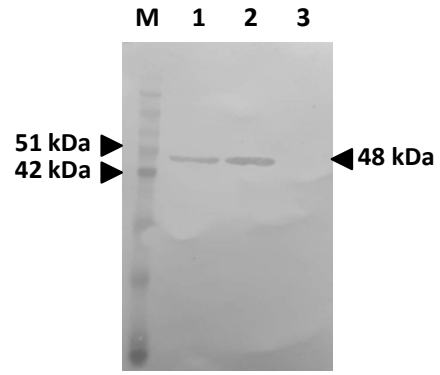

Figure 3: Western blotting with Anti-His antibody. Lane 1 is purified N protein, Lane 2 is supernatant fraction from *E. coli* containing N-inserted plasmid, and Lane 3 is supernatant fraction from *E. coli* containing pQE80L control plasmid. M is represented by a Chromatein prestained protein ladder (Vivantis; Suite V., Chino. CA, U.S.A.).

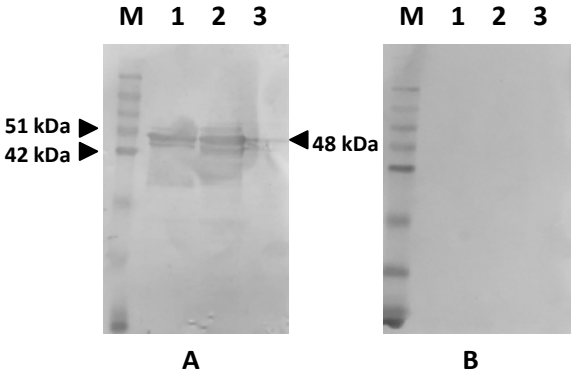

Figure 4: Evaluation of biological rN function by western blotting using dairy cattle antibodies. The proteins from supernatant fraction reacted with positive (A) and negative (B) dairy cattle serum. M is represented by a Chromatein prestained protein ladder (Vivantis; Suite V., Chino. CA, U.S.A.) Lane 1 is purified N protein, Lane 2 is N-inserted plasmid, and Lane 3 is pQE80L control plasmid.

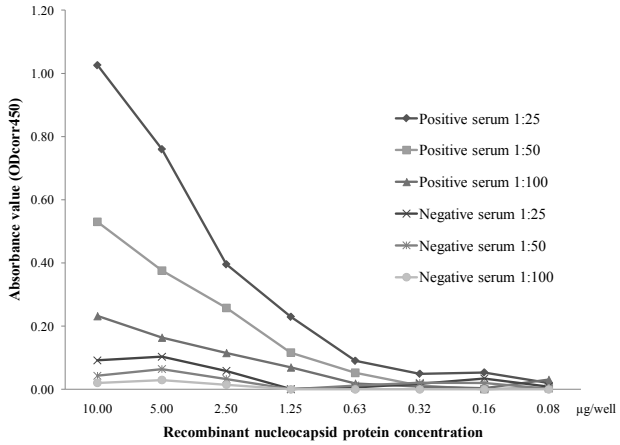

Figure 5: Checkerboard titration between recombinant nucleocapsid protein concentration and dairy cattle antibodies in ELISA. Two-fold serum dilution started with a dilution of 1:25 and recombinant nucleocapsid protein dilution started with 10 µg/well.

on a nitrocellulose membrane with both purified-N protein and the supernatant fraction from *E. coli* containing N-inserted plasmid at a molecular weight of about 48 kDa that was expected. Indistinct *E. coli* protein bands appeared from both *E. coli*-contained plasmids with the N gene and the purified-N protein supernatant (Figure 3). The biological function test of the rN protein showed that rN protein only reacted with positive serum against bovine coronavirus of dairy cattle (Figure 4A) while no bands appeared from the negative dairy cattle serum using western blotting (Figure 4B).

The optimal rN protein antigen and *E. coli* control antigen concentration in the developed ELISA (5 µg/well) were selected using checkerboard titration (Figure 5). Moreover, the optimal rN protein was selected by dissolving with 1 M urea, repeated three times and 2 M urea, repeated three times, respectively, but *E. coli* control antigen was selected from pQE80L dissolved with 1mg/ml lysozyme .The primary sera are all of the dairy cattle sera. They were diluted at 1:50, because the non-specific background of the *E. coli* control antigen OD_{450} values were minimal. The secondary antibody dilution was selected as 1:5,000, according to the manufacturer's recommendation.

The mean OD_{450} and SD of 27 negative sera of the rN-ELISA was 0.013 and 0.0183, respectively. The cut-off value of the rN-ELISA from the mean OD_{450} and two standard deviations of the negative sera were 0.049. The sensitivity, specificity and accuracy was 84.8% 96.3% and 86.1%, respectively, of the developed rN-ELISA relative to the commercial ELISA (Table 1). Out of the 204 commercial ELISA-positive serum samples, 173 of the samples were identified to be positive by the rN-ELISA. Out of the 27 commercial ELISA-negative serum samples, 26 of the samples were identified to be negative by the rN-ELISA. In addition, 173 were considered to be true positive because they were revealed in the commercial ELISA as well as having rN-ELISA readings above the optimized cut-off, while 18 rN-ELISA-positive serum samples were diagnosed as false negative because the readings were below the cut-off. Furthermore, 26 samples had corrected OD_{450} values of the rN-ELISA below the optimized cut-off (true negative) and one serum had a corrected OD_{450} value of the rN-ELISA above the optimized cut-off and hence was considered to be false positive. By using three positive dairy cattle sera, three negative dairy cattle sera and PBS in quadruplicate, the intra-assay CV was indicated in the range 2.33-13.33% and the inter-assay CV was in the range 4.48-18.75% (Table 2). Cohen's kappa value

Table 1: Cut-off value, number of positive and negative, percentage of sensitivity, specificity and accuracy results.

Cut-off		Commercial ELISA kit		Sensitivity (%)	Specificity (%)	Accuracy (%)
		Positive	Negative			
Mean + 1SD	Positive	186	5	91.2	81.5	90
	Negative	18	22			
Mean + 2SD	Positive	173	1	84.8	96.3	86.1
	Negative	31	26			
Mean + 3SD	Positive	154	0	75.5	100	78.4
	Negative	50	27			

Table 2: Repeatability of the ELISA test kit for intra- and inter-assays with four replications using positive and negative dairy cattle sera. The results are shown as the mean of OD + SD and CV%.

Sample	Mean OD_{450} + SD		Intra-assay CV%	Inter-assay CV%
	Intra-assay	Inter-assay		
Negative sera	0.015 ± 0.002	0.016 ± 0.003	13.33	18.75
	0.043 ± 0.001	0.043 ± 0.007	2.33	16.28
	0.019 ± 0.001	0.021 ± 0.003	5.26	14.29
Positive sera	0.205 ± 0.006	0.189 ± 0.018	2.93	9.52
	0.264 ± 0.019	0.268 ± 0.012	7.20	4.48
	0.428 ± 0.024	0.363 ± 0.019	5.61	5.23
PBS	0.017 ± 0.001	0.018 ± 0.002	5.88	11.11

OD_{450}: Optical $density_{450}$, SD: Standard deviation, CV: Coefficient of variation

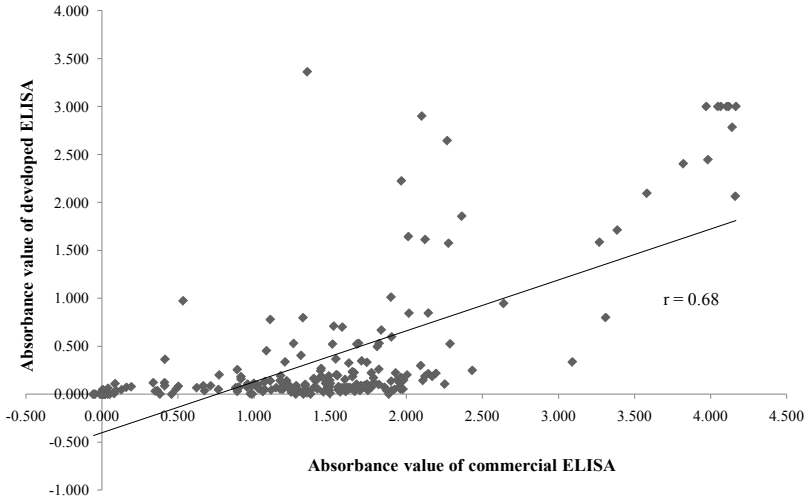

Figure 6: Correlation coefficient between the developed ELISA and commercial ELISA from 231 positive and negative dairy cattle sera.

was 0.71. The correlation coefficient between the rN-ELISA and the commercial ELISA was 0.68 (Figure 6). The corrected OD_{450} values of the rN-ELISA were in the range 0.000-3.362 and correlated with the corrected OD_{450} values of the commercial ELISA that were in the range 0.000-4.166. Positive dairy cattle sera against foot and mouth disease virus, bovine leukemia virus, bovine ephemeral fever virus and bovine viral diarrhea virus (data not shown) provided negative results in the rN-ELISA.

Discussion

The purpose of this study was to develop a simple and dependable recombinant protein ELISA method for the detection of BCoV infection. Although the commercial ELISA kit for serological detection of BCoV is available now, but the cost of this kit is quite expensive in Thailand. Moreover, the commercial ELISA kit has the disadvantage of requiring the propagation of large quantities of virus and the purification of virus particles before use as ELISA antigens. The current study designed primers for almost the full-length of the nucleocapsid gene, expected to cover the immudominant epitope and conserved all BCoV strains because there has to date been no reported strain of BCoV in Thailand. These primers were able to detect the N gene of BCoV which was confirmed by percent identity using the BLASTn software. The N gene of BCoV was highly conserved due to strong selective structural constraints that limit its revolution [31].

The suspected size of the rN protein was 48 kDa from the SDS-PAGE analysis while a predictable size of the rN protein was 46 kDa, because the suspected protein was included with 6X His tagged protein (2 kDa). Furthermore, the suspected protein with the 6X histidine tag can help to confirm the suspected protein by using affinity column chromatography. Heterologous protein production in *E. coli* is preferred to develop diagnostic methods due to it having a fast growth rate and being an inexpensive media with well-understood genetics [32]. The rN protein produced from *E. coli* can be discriminated between positive and negative dairy cattle sera using western blotting. This result revealed that the rN protein can

maintain biological function (Figure 4A). The recombinant protein has an advantage over the whole virus as it forms immunodominant epitopes and is lacking non-specific moieties [33]. The rN protein was found to be appropriate for immunological assays and that expressed in *E. coli* was more immunogenic than the protein expressed in insect cells [29]. The N protein of human coronavirus (HCoV) is attracting attention as a crucial target of antibody test methods because it has high antibody reactivity [34]. Moreover, N protein provided an excellent diagnostic reagent in combination with ELISA. Comparison using *E. coli* expressed between SARS-CoV S glycoprotein and N protein proved that N protein was a better choice of antigen for use in diagnostic ELISA [35]. The use of a recombinant ELISA has never been developed for the detection of antibodies against BCoV, so the rN protein antigen and *E. coli* control antigen had to be selected using checkerboard titration. The dairy cattle serum samples were a 1:50 dilution for the rN-ELISA developed in this study, while a 1:25 dilution was recommended by the commercial ELISA kit (SVANOVIR® BCV-Ab; Svanova, Sweden), for which the rN protein antigen achieved a good sensitivity which may be attributed to its high epitope density. In addition, the high background reaction of rN-ELISA could be decreased by absorbing the tested sera with horse serum. The cut-off value selection is one of the most important factors in developing ELISA [36]. A sample of 27 negative dairy cattle sera collected from dairy herds was used to confirm the cut-off value of the rN-ELISA, which was valid for application to dairy farms. The specificity, sensitivity and accuracy of the assays were determined by evaluating sera from the field samples in Thailand (n=231) and showed the percentage of sensitivity, specificity and accuracy were sufficient to detect BCoV infection. The low-to-medium of coefficient of variation for the intra- and inter-assay values warranted the repeatability and reproducibility of the ELISA [37]. The Cohen's kappa value of 0.71 revealed substantial agreement between the rN-ELISA and the commercial ELISA. The correlation coefficient (r) between the rN-ELISA and the commercial ELISA was 0.68 (Figure 6) and indicated that the strength of the two tests was good. This rN-ELISA showed no cross-reactivity against FMD, BLV, BEF and BVD. Finally, these results suggested that the rN protein induces a good immune response. Thus, the detection of antibodies against the rN protein in dairy cattle using the rN-ELISA may be considered an indicator of the infection or immune status of a dairy herd infected with BCoV.

In conclusion, this is the first report of the development and validation of a recombinant nucleocapsid protein BCoV enzymed-linked immunosorbent assay for antibodies detection in Thailand. The rN protein had biological function and an indirect ELISA development based on recombinant nucleocapsid protein to detect anti-BCoV antibodies in this study was found to be sensitive, specific and accurate when compared to the commercial ELISA kit (SVANOVIR® BCV-Ab; Svanova, Sweden). This indicated that the recombinant nucleocapsid protein-based indirect ELISA was a simple, safer, rapid and inexpensive method capable of widely screening and monitoring large numbers of samples to evaluate serological diagnosis as well as epidemiological surveillance [26].

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