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Development and Evaluation of Recombinant NS3 Protein-based ELISA for the Detection of Bovine Viral Diarrhea Virus (BVDV) Antibodies

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ABSTRACT

Background: Bovine Viral Diarrhea (BVD) is an important infectious disease that has a significant economic impact on dairy farms. Early detection of antibodies against BVD virus within herds can help reduce economic losses resulting from impaired animal health and reproductive performance. However, commercial ELISA tests tend to be expensive and unaffordable for smallholder farmers in Thailand. This study aims to develop an in- house indirect ELISA for the detection of antibodies to BVD.

Methods: Gene synthesis and codon optimization were performed prior to protein expression. The prepared recombinant NS3 protein was confirmed through western blot analysis. Assay validation involved a comparison with an indirect commercial ELISA using a set of field serum samples (n=497) as references to evaluate its diagnostic accuracy. Measurements for test agreement and assay repeatability were calculated to assess performance.

Result: The area under the receiver operating characteristic (ROC) curve was 0.86, indicating good discrimination between BVDV-positive and negative animals. The kappa coefficient, with an identified optimal cut-off value, was 0.577, indicated moderate agreement between the two tests. Additionally, the assay exhibited acceptable repeatability, with coefficients of variations (CVs) of 8.8% and 15.1% for intra-assay and inter-assay variations, respectively. This developed ELISA demonstrates both discriminatory ability and repeatability, making it a valuable alternative tool for the serodiagnosis of BVDV infection, especially in low-resource settings.

Key words: Bovine Viral Diarrhea Virus, Cows, ELISA, NS3, Recombinant protein.

INTRODUCTION

Bovine Viral Diarrhea (BVD) is a significant infectious disease with a global impact, primarily affecting cattle (Scharnbock *et al*., 2018). The disease is caused by the BVD virus (BVDV), which belongs to the *Pestivirus* Genus within the *Flaviviridae* family and is characterized as an enveloped, single linear positive-stranded RNA virus (Tautz *et al*., 2015). BVDV infections often result in subclinical or non-specific clinical signs, including high fever, depression, inappetence, lethargy, respiratory issues, diarrhea and reproductive disorders (Khodakaram-Tafti and Farjanikish, 2017). Furthermore, BVDV can give rise to persistently-infected (PI) animals, which continually shed the virus in their secretions and excretions, thus facilitating ongoing virus circulation within herds (Lanyon *et al*., 2014).

BVDV infection in Thailand leads to economic losses on dairy farms due to reduce reproductive performance (Kampa, *et al*., 2011) and its direct adverse effects on animal health, resulting in decrease milk production (Yue *et al*., 2021). Amonongart *et al*. (2020) found a seroprevalence of 28.44% in two provinces located in the western region of Thailand. Furthermore, a recent 2022 study reported a significant individual prevalence rate of 36.89% among dairy farms in western part of Thailand (Thongtem *et al*., 2023).

Enzyme-Linked Immunosorbent Assay (ELISA) has become a widely adopted and routine diagnostic method due to its reliability, simplicity, rapidity and cost-effectiveness and accessibility in many laboratories, making it suitable for large-scale investigations (Hosseini *et al*., 2018). Currently, in Thailand, a commercial whole-virus indirect

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ELISA kit (IDEXX BVDV Total Ab Test, USA) is employed for the detection of BVDV antibodies, offering high sensitivity (96.3%) and specificity (99.5%), as claimed by the manufacturer (Lanyon *et al*., 2013). However, this commercial ELISA comes with a notable drawback- it is relatively expensive and its cost escalates when dealing with a low number of samples. Furthermore, whole-virus-based assays have a potential downside of generating false-positive results due to non-specific reactions with co-purified cellular proteins from the antigen preparation process (El-Duah *et al*., 2019; Rosati *et al*., 1994). In contrast, the in-house developed ELISA offers flexibility as the antigen can be produced from properly-stored *E. coli* with the target gene in varying quantities, catering to specific testing needs.

In this study, the authors describe the development of an in-house ELISA using the non-structural 3 (NS3) recombinant protein as an economical alternative for BVDV serodiagnosis in Thailand. NS3 is recognized as one of the most immunogenic proteins of BVDV (Mahmoodi *et al*., 2014). Notably, the NS3 protein demonstrates a high degree of conservation among pestiviruses, with over 94% identity within Pestivirus A (BVDV-1) and more than 89% identity between Pestivirus A (BVDV-1), B (BVDV-2) and H (HoBi-like pestivirus) (Riitho *et al*., 2020). Therefore, the primary objective of this study is to develop a reliable indirect ELISA based on the NS3 recombinant protein for the detection of BVDV antibodies.

MATERIALS AND METHODS

Ethical approval

This research was approved by the Animal Care and Use for Scientific Research Committee of Kasetsart University, Bangkok, Thailand (ACKU64-VET-003).

Study population

The sample collection was carried out in the western region of Thailand from 2019 to 2021. Participating farms were selected based on the availability of comprehensive individual-levels dairy cow databases, recorded by Kasetsart University Veterinary Teaching Hospital. A total of 497 blood samples were collected from dairy cows aged over 1 year in Nakhon Pathom, Kanchanaburi, Ratchaburi, Phetchaburi and Prachuap Khiri Khan Provinces (Fig 1).

Serological test

All laboratory processes were conducted at Kamphaeng Saen Veterinary Diagnostic Center, Kasetsart University. The disease status of individual animals was classified using an indirect commercial ELISA (IDEXX BVDV Total Ab Test, USA) as a standard reference test according to the manufacturer's instructions. The interpretation of the results, whether the sample was identified as positive or negative, was based on the S/P (Sample-to-Positive) ratio. A negative classification was given to sample with an S/P ratio of less than 0.20, while a positive designation was assigned to samples with a ratio greater than or equal to 0.30 S/P.

Preparation of recombinant NS3 protein

In summary, the NS3 gene from the BVDV strain Singer Arg (GenBank: MH133206) was identified, focusing on previously reported amino acid sequences (Li *et al*., 2013). A specific NS3 region was selected based on its conservation among 60 BVDV strains, hydrophilicity profile and six epitopes identified by Li *et al*. (2013). This chosen NS3 region comprised 381 amino acids and had a predicted protein molecular weight of 42 kDa. To optimize its expression in *Escherichia coli* (*E. coli*), the NS3 gene was synthesized into 6xhistidine-tagged pET-28b (+) plasmid by

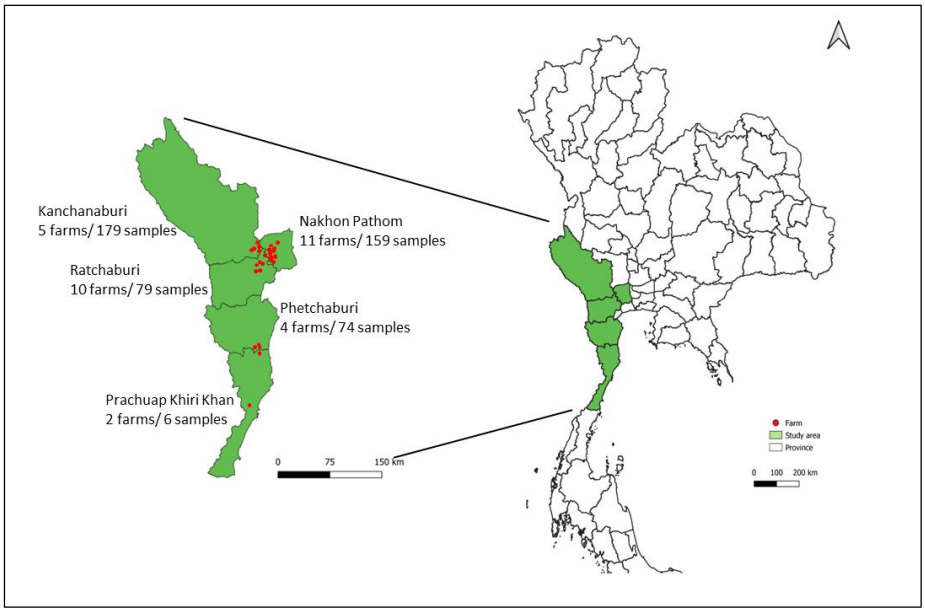


Fig 1: Geographical locations and distribution of blood samples collected from dairy cows aged over 1 year in Western Thailand.

U2Bio (Thailand) Co., Ltd. The NS3-inserted plasmids were introduced into *E. coli* strain BL21 (U2Bio, Korea). Protein expression was achieved by 1 mM of Isopropyl β-D-thiogalactopyranoside (IPTG) for four hours. Analysis using 12% SDS-PAGE revealed a band of recombinant protein with the expected molecular weight, visualized by Coomassie Brilliant Blue staining. Additionally, the present of recombinant NS3 protein was confirmed through western blot analysis using anti-histidine antibodies. Purification was carried out by dissolving the pellet in progressive increasing concentrations of urea (1 M, 2 M, 4 M, 6 M and 8 M) and attained using affinity column chromatography equipped with Ni-NTA (AKTA startTM, GE-Healthcare, UK) following the manufacturer's instructions. The purified recombinant NS3 protein's antigenicity was assessed via western blot analysis, utilizing positive and negative bovine sera.

Development of recombinant NS3 protein-based ELISA

The ELISA working conditions were fine-tune through a checkerboard titration. The purified recombinant NS3 protein and *E. coli* control protein was coated on the 96-well ELISA plate. The protein dilutions range from 5 μg/well to 0.04 μg/well in a carbonate-bicarbonate buffer pH 9.6 at 37°C for 1 hour. Following the washes with phosphate-buffered saline with Tween 20 (PBS-T), a blocking buffer consisting of 300 μl of phosphate-buffered saline (PBS) with 3% Bovine Serum Albumin, 20% rabbit serum and 0.02% NaN3 was incubated at 37°C for 30 minutes. Afterward, the blocking buffer was removed and 100 μl of serum samples, diluted to 1:12.5, 1:25 and 1:50 in a primary antibody buffer (comprising 5% skim milk, 10% horse serum, 20% of 10 mg/ml *E. coli* protein lysate and 0.02% NaN3 in 1XPBS buffer), were added and incubated at 37°C for 30 minutes.

Following a series of washes, 100 μl of 1:7500 dilution of Horseradish peroxidase (HRP)-conjugated goat anti-bovine Immunoglobulin G in 1XPBS containing 1% skim milk and 10% horse serum was introduced to the wells as a secondary antibody for another 30-minute incubation at 37°C. The plates were washed again before the addition of the 3,3',5,5'-Tetramethylbenzine (TMB) substrate solution (KPL, USA) and incubated at room temperature for 15 minutes. The reaction was halted using 0.25M HCl as the stop solution, facilitating the measurement of OD. The results were interpreted by subtracting the OD450 value from the control well using the OD value of the NS3-protein well.

The cut-off was selected based on test results of reference positive and negative serum samples obtained from the commercial kit. Non-parametric receiver operating characteristic (ROC) curve and parametric ROC with a 95% confidence interval were generated to illustrate the test performance (Greiner *et al*., 2000). Additionally, the optimal cut-off was determined using the two-way sensitivity-specificity plot depending on the particular application proposed in the assay. A sample was classified as positive when the OD450 value was higher than the cut-off value. A McNemar's Chi-square test was employed to compare the proportion of positive animals between the results obtained from the commercial and the developed ELISA. Subsequently, the level of agreement between the two tests was assessed using a Cohen's kappa statistic.

Repeatability assessment

The repeatability of the test was assessed for both the intra-assay and inter-assay variability. For this assessment, a single sample was tested 144 times, spread across three separate 96-well plates, all using the same developed ELISA protocol. Within-plate, the coefficient of variation (CV) was computed for each plate and the average of these CVs across the three plates provided an estimate of intra-assay variation. The CV for inter-assay variability was also calculated among these three plates. Furthermore, between-run repeatability was evaluated using serum controls. Two replicates of both negative and positive control samples were tested in ten runs. The mean values of replicates were considered acceptable if they fell within the range of the mean ± 2 standard deviation (SD), calculated from all runs of the assay (Jacobson, 1998). Mean±1, ±2 and ±3 SD were calculated to establish initial upper and lower control limits for each control sample.

All statistical analyses including ROC curve, two-way sensitivity-specificity plot, Cohen's kappa statistic and CV calculations, were carried out using Microsoft Excel and Stata statistical software (Release 16, StataCorp LLC, College Station, TX, USA).

RESULTS AND DISCUSSION

Western blot analysis confirmed the successful expression of the target protein, revealing a molecular weight of approximately 42 kDa (Fig 2). The BVDV-positive serum displayed recognition of the protein, indicating its antigenicity. In contrast, no reactivity was observed with the negative serum. In Fig 3, the checkerboard titration displayed that using 1.25 μg/well of protein and serum diluted to 1:12.5 yielded the greatest discrepancy in OD values between positive and negative samples. However, for the ELISA working dilution, a protein concentration of 0.63 ug/well and a serum dilution of 1:25 were selected, as lower background readings in the control well were achieved, effectively minimizing non-specific binding.

Out of the 311 collected serum samples, a commercial ELISA identified 59 as positive and 252 as negative. All of these samples, along with the inclusion of 186 positive stock samples, were used as reference samples to assess the performance of the newly-developed ELISA. In total, 245 positive samples and 252 negative samples were subjected to testing using the developed ELISA.

The discriminatory power of the developed assay is illustrated in Fig 4. The resulting Area Under the Curve (AUC) was 0.86, indicating a good discriminatory test ($0.8 \leq AUC < 0.9$), in accordance with the interpretation guidelines by Nahm (2022). A two-way sensitivity-specificity plot, shown in Fig 5, revealed that equal sensitivity and specificity (approximately 79%) were achieved at a cut-off value of 0.364.

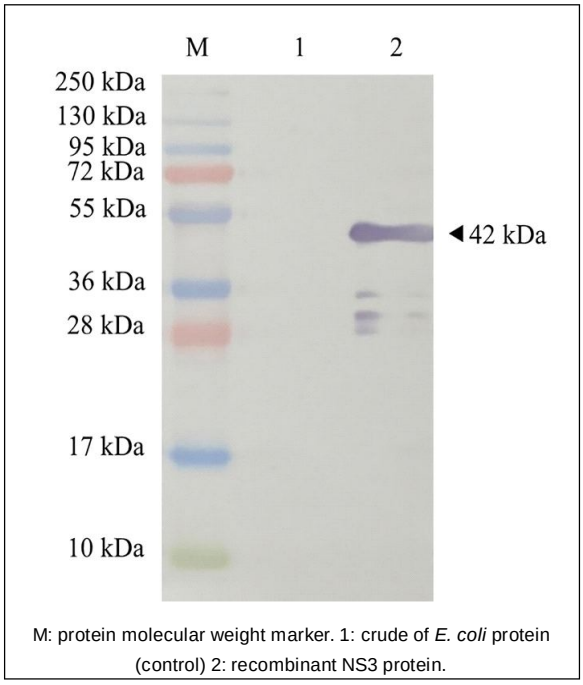
Measurement of test agreement

The test results obtained from both the commercial and the developed ELISA were categorized based on the determined optimal cut-off value of 0.364, as presented in Table 1. The McNemar's chi-square test yielded a non-significant result (*P*=.922), suggesting a lack of substantial evidence that the proportion positives of the two tests were dissimilar. Furthermore, the calculated kappa value was 0.577, with a 95% confidence interval ranging from 0.506-0.649. This value fall within the category of moderate strength of agreement strength, as per the classification by Landis and Koch, (1977).

Repeatability assessment

The results of the precision assessment for the developed assay are presented in Table 2, indicating its strong precision. Both the intra- and inter-assay CVs for raw OD450 value were under 20%. The intra-assay CV ranged between 7.7% and 9.7% with an average of 8.8%. The inter-assay CV stood at 15.1% across the three plates. Additionally, Levey-Jennings charts were plotted using the mean OD450 values of positive (Fig 6A) and negative control duplicates (Fig 6B) over ten runs of the assay. The results demonstrated acceptable between-run repeatability, as all mean values fell within the range of ±2 SD of the mean of all runs. The means of positive and negative control OD450 results were 0.565 and 0.098, respectively. The upper and lower control limits for each control sample were shown in Table 3.

As the NS3 protein became a candidate antigen for ELISA (Chi *et al*., 2022; Yitagesu, *et al*., 2021) and was prepared in various immunogenic regions, vector choices and expression systems, it is worth noting that the study of the whole NS3 protein of BVDV by Mahmoodi *et al*. (2015) revealed high sensitity and specificity. However, the full-length NS3 (683 amino acids with about 80-kDa molecular weight) when combined with maltose binding protein resulted in a larger protein size, which may lead to a lower yield of target protein expression (Bao *et al*., 2006). Subsequently, shorter sequences were shown to be more favorable for plasmid construction and protein expression (Qiu *et al*., 2022). Furthermore, the use of smaller fragments



M: protein molecular weight marker. 1: crude of *E. coli* protein (control) 2: recombinant NS3 protein.

Fig 2: Western blot analysis of recombinant NS3 protein with anti-histidine antibodies.

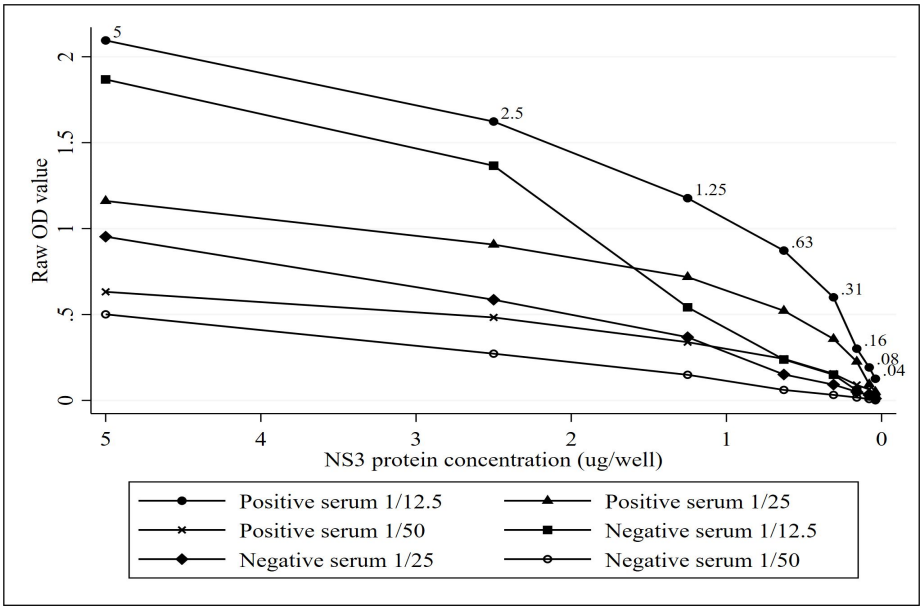
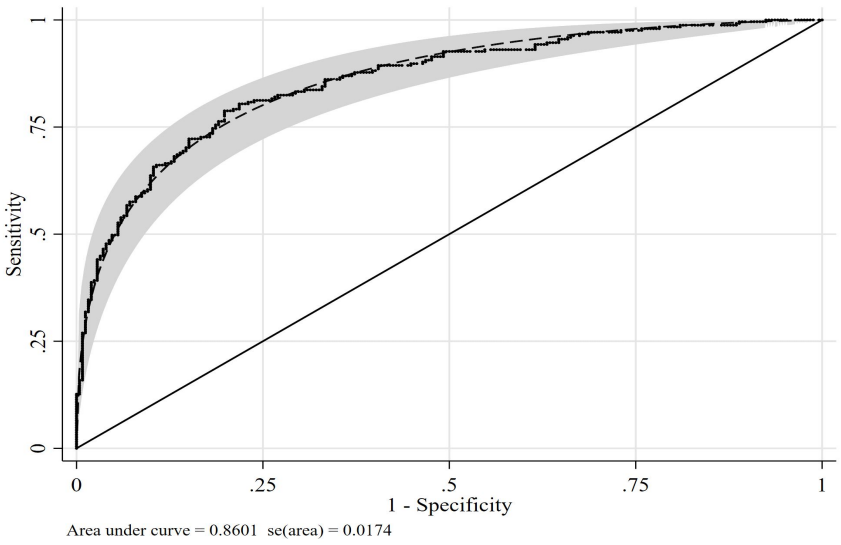


Fig 3: Checkerboard titration for optimization of ELISA working dilutions including 1) recombinant NS3 protein solution two-fold serial diluted as target antigens and 2) serum samples diluted at 1/12.5, 1/25 and 1/50 as primary antibodies.

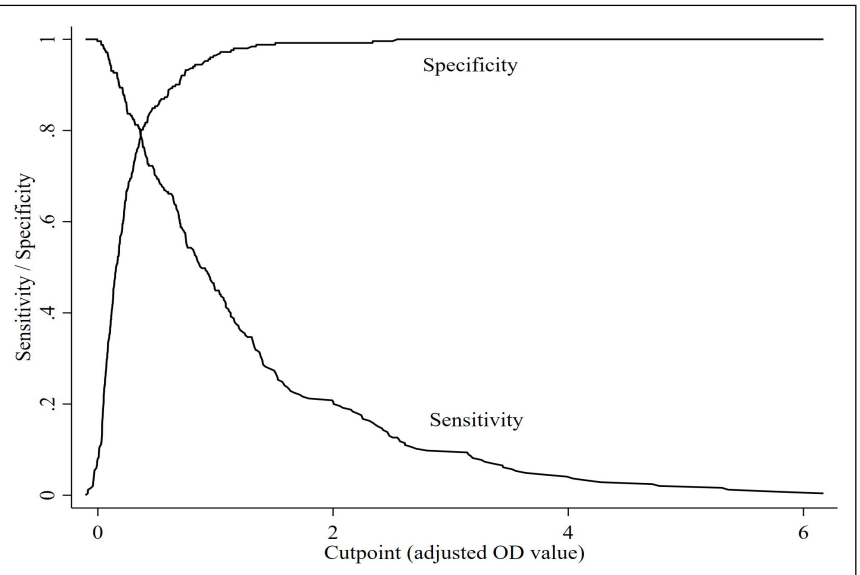
concentrates more specific epitopes and avoids the non-significant part of NS3 (Bhatia *et al.*, 2008). In this current study, we specifically selected the region of interest within the NS3 protein based on the displayed epitopes, hydrophilicity profile and conserved region. This resulted in a protein with a length of only 381 amino acids and a molecular weight of 42 kDa.

For protein expression, this study chose the *E. coli* expression system due to its simplicity, rapidity, high yield and cost-effectiveness (Francis and Page, 2010). However, codon usage bias in native sequences can limit expression levels. To address this, the present study designed and synthesized a codon-optimized gene sequence to enhance heterologous protein expression in *E. coli* (Gustafsson *et al.*, 2004). The NS3 gene region was successfully incorporated into the pET-28(+) plasmid and codon adaptation indices (CAI) were maximized (increasing from 0.39 to 0.98 in fragment 1 and from 0.4 to 0.99 in fragment 2),



The sensitivity of the test was plotted against the false positive rate (1-specificity) with possible cut points. The dashed line represents a parametric ROC curve with a 95% confidence interval displayed in the grey area. Connected points show a non-parametric ROC curve. The 45º straight line represents a test that lack the ability to discriminate between diseased and non-diseased animals.

Fig 4: ROC curves with a 95% confidence interval.



The intersection is at an S/P ratio of 0.364 where sensitivity and specificity are equal at approximately 79%.

Fig 5: Selection of optimal cut points using a two-way sensitivity-specificity plot.

indicating the potential for high-level expression in the target host.

The AUC value of 0.86 indicates strong test discrimination (Nahm, 2022), suggesting an 86% probability that serum from a randomly selected BVDV-positive cow will yield a higher OD450 reading than that from a negative cow. Instead of standard ROC curve, this study employed a two-way sensitivity-specificity plot, also known as a two-graph ROC plot, to facilitate the selection of optimal cut-off values. This plot highlights various cut points for maximizing sensitivity or specificity, taking into account the costs of false positive and false negative results. The study determined the optimal cut point, set at 0.364, to achieve equal sensitivity and specificity, as reflected by a kappa coefficient of 0.577, indicating moderate agreement (0.41 to 0.6). Kappa's sensitivity to the prevalence of the condition of interest was considered, with our study group having a prevalence of approximately 0.5, expected to yield a robust kappa value (Vach, 2005).

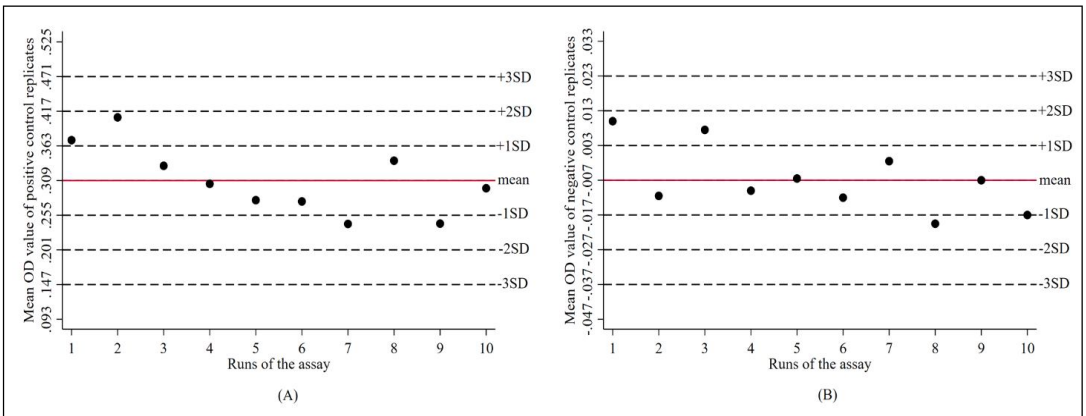
The developed ELISA successfully identifies 193 negative serum samples out of 251 negative references, indicating the potential for false positives that can diminish the assay's specificity. Generally, the use of recombinant protein as coated antigens for ELISA is expected to provide higher specificity compared to the whole viral antigens employed in commercial ELISA kits. This is because cross-reactions with mammalian cell proteins in cell culture can lead to false-positive results in the whole-virus-based assay (Reddy *et al*., 1997). However, the conflicting results observed may be attributed to false negatives generated by the commercial ELISA. In whole-virus-based assay, there may be an insufficient concentration of protein with immunogenic significance (Reddy *et al*., 1997). Another potential factor contributing to the significant occurrence of

Table 1: Test results categorized according to the cut-off value of 0.364 for the agreement study comparing the commercial ELISA and the recombinant NS3 protein-based ELISA.

Commercial ELISA	Recombinant NS3 protein-based ELISA		Total
	Positive	Negative	
Positive	193	52	245
Negative	53	199	252
Total	246	251	497

Table 2: Intra-assay and inter-assay variability: Mean, SD, range and %CV of raw OD value from three plates running 144 repetitions on the same serum sample.

Variability	Plate number	Number of replicates	Mean	SD	Range	%CV
Intra-assay variability	1	48	0.435	0.039	0.343-0.517	9.0
	2	48	0.456	0.035	0.397-0.533	7.7
	3	48	0.340	0.033	0.271-0.409	9.7
Inter-assay variability		144	0.410	0.062	0.271-0.533	15.1



Data points on each chart represent the mean value of controls in duplicates. Mean OD values for 10 runs are represented by the red solid line, while ±1, ±2 and ±3 SD are indicated by the dashed lines.

Fig 6: Levey-Jennings charts for positive (A) and negative controls (B) of the individual assays.

Table 3: Upper and lower control limits of OD results for positive and negative serum controls.

Control limits	Positive control		Negative control	
	Upper limit	Lower limit	Upper limit	Lower limit
1SD	0.643	0.487	0.105	0.091
2SD	0.721	0.410	0.112	0.084
3SD	0.799	0.332	0.119	0.078

false-positive reactions could be the hydrophobic binding of serum immunoglobulin components to plastic surfaces in individual samples. This phenomenon, often referred to as background noise reaction, is a common issue encountered during ELISA development, regardless of the type of antigen used (Terato *et al*., 2014).

This developed assay also displayed strong repeatability, as indicated by the CV values for both inter and intra-assay variation of the raw OD value readings, which remained well below 20% (Jacobson, 1998). To properly assess the repeatability of the assay, subsequent tests using field serum samples must be carried out following the same optimized procedures and conditions mentioned earlier. This study has established stringent validity criteria for the developed ELISA to ensure consistency across multiple runs. Specifically, each ELISA plate run should include duplicates of one positive and one negative control. An ELISA assay is deemed valid if the averaged OD value of positive control falls within the range of 0.40-0.70 (calculated as the mean ± 2SD from the between-run repeatability results, as previously mentioned), with a difference of more than 0.30 OD units between the average positive control and the negative control. In cases where these conditions are not met, the results should be considered suspect and invalid.

To ensure the practical application of our developed assay, the cut-off value was carefully selected by considering the costs associated with false positives and false negatives. It is worth noting that the cost of false negative can have a continuous, detrimental impact on production within Thai dairy herds due to potential subclinical BVDV infections. These hidden infections can lead to significant economic losses. In Thailand, previous research has primarily focused on BVDV seroprevalence, while the economic consequences of BVDV infections remain poorly documented. Nonetheless, there is compelling evidence indicating a substantial negative impact on the reproductive system, as observed through prolonged first-service intervals, calving- to-conception intervals, calving intervals and reduced overall pregnancy rate (Kampa *et al*., 2011), all of which contribute to economic losses. On the other hand, in Thailand, where control policies or eradication measures like test-and-cull are not widely implemented, the consequences of false positive results appear to be less significant. Additionally, if a BVDV-seropositive cow is identified, further sample collection and confirmatory tests are typically carried out to validate the suspected animal's current infection status. This use of sequential testing ultimately results in a higher level of specificity in the screening process (Dohoo *et al*., 2009). Overall, it is evident that the cost associated with false positives in BVDV testing is more substantial than that of false negatives. As a result, an ELISA based on recombinant NS3 protein has been developed for the screening purpose to be used as an alternative to the routine assay.

For a screening assay, a suggested cut-off value at the S/P ratio of 0.18 offers a high sensitivity of 90% without significantly compromising specificity (54%). If the S/P ratio obtained from a serum sample is below 0.18, the sample is categorized as negative. Conversely, if the S/P ratio is greater or equal to 0.18, the sample is deemed positive. However, the sensitivity and specificity of the developed ELISA were maximized concurrently but only at a moderate level of 78%. Out of 246 positive sera, only 193 samples tested positive using the developed assay. This occurrence might be attributed to the relatively low antigenicity of the prepared NS3 protein, as indicated by western blot analysis of positive serum, which exhibited a weaker signal compared to the amount of protein loaded in SDS-PAGE run concurrently under the same conditions. Another potential reason for the occurrence of false negative results is the stage of infection, as antibodies against the non-structural protein are produced later in the infection and decreased earlier compared to other structural proteins (Mohanty *et al*., 2021). Commercial kits based on whole BVDV are considered superior in terms of detecting antibodies during various infectious periods, as they contain a more diverse array of antigenic determinants from the virus. This diversity sometimes leads to false negative results when using the newly-developed ELISA. Using different regions of NS3 and a combination of multiple immunogenic proteins from BVDV such as E0, E2 and NS3, in the development of ELISA could potentially enhance accuracy of diagnostic parameters. This idea is supported by a previous study conducted by (Chimeno Zoth and Taboga, 2006), which achieved high sensitivity and specificity relative to the viral neutralization test through the use of three recombinant antigens.

CONCLUSION

The recombinant NS3 protein of BVDV was successfully produced and employed as an antigen in an indirect ELISA. This newly developed test is characterized by its simplicity, speed and cost-effectiveness. In performance evaluations against a commercial ELISA, it demonstrated good

discrimination ability and satisfactory repeatability. Consequently, the recombinant NS3 protein-based ELISA serves as a practical and accessible option for BVDV antibody detection, offering improved serodiagnosis accessibility for dairy farms in Thailand.

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Conflict of interest statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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