Current status of Foot and Mouth Disease in goats in Brunei Darussalam and the efficacy of the ELISA-based detection method in detecting the presence of the disease

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Abstract

Brunei Darussalam has been recognized as a Foot and Mouth Disease (FMD) free country. Nonetheless, control efforts must be exercised to maintain this status. A reliable and precise diagnostic test is needed for rapid detection, which is essential for fast decision-making by the national authorities. This study aims to update the current status of FMD in the country, and to prove that the ELISA method used by the Veterinary Laboratory Services (VLS) of Brunei Darussalam is reliable and sufficient for detecting the presence of FMD. According to data collection over the past 6 years, no FMD was detected in the blood serum from goats, which confirms that the country is free from FMD. The ELISA test that the VLS conducts has been validated and accredited to ISO / IEC 17025:2017 since 2019 and has also participated in Proficiency Testing with satisfactory results, confirming that the test is sufficient for detecting the presence of this disease. The method achieved 98% and 100% for Diagnostic Sensitivity and Diagnostic Specificity, with null false positives and only 1 false negative. The test was also found to be satisfactory in terms of repeatability, reproducibility and selectivity. With these satisfactory validation procedures, continuing use of the existing ELISA-based screening method can be recommended, to maintain the FMD-free status of the country. However, other detection methods, such as improved PCR-based techniques, need to be employed from time to time to exclude the possibility of experimental error that may occur during the ELISA-based assay.

Index Terms: Foot Mouth Disease, ELISA, goats, Brunei Darussalam, diagnostics

1. Introduction

Foot and Mouth Disease (FMD), which is unrelated to hand-foot and mouth disease, is a highly infectious viral disease in cloven-hoofed ruminants such as cattle, sheep and goats. It is caused by a FMD virus of genus *Aphthovirus* in family of *Picornaviridae*.[1] Affected ruminants with FMD will suffer from fever and show blister-like sores on their tongue, in the mouth, on their lips, between their hooves and on the teats.[2]

Any occurrence of FMD in any country must be reported to the World Organization for Animal Health (WOAH) as it is an OIE-listed disease.[3] This disease seriously affects ruminant production and may cause economic losses, as the affected countries can be excluded from international animal trade.[4] Domestically, the price of meat at the farm level can only be recovered after export bans are lifted by importing countries.[5,6]

Due to its importance, control measures and efforts are very much needed to ensure this disease will not be present in Brunei Darussalam. Successful control efforts will ensure that the Sultanate continues to be recognized as an FMD-free country.[7] One condition necessary to ensure this is to have a precise and reliable diagnostic laboratory test for the early detection of FMD. This test will help to ensure a fast response by initiating rapid control measures for controlling and preventing the spread of the disease. In our laboratory, Enzyme-Linked Immunosorbent Assay (ELISA) is used for detection of this disease. The objective of this study is to update the current status of FMD in the Sultanate of Brunei Darussalam, and to analyze the efficacy of ELISA as a powerful tool for detecting the presence of FMD.

2. Experimental Approach

2.1 Data collection and analysis

A total of 2, 083 goat blood samples were collected and submitted by VLS clients (goat farm owners in Brunei Darussalam) from 2016 to September 2022. These samples were collected from all four districts in Brunei Darussalam. One blood sample was collected per goat, which was selected randomly from the farm.

All samples were tested using the PrioCHECK FMDV Antibody ELISA Kit (Thermo Fisher Scientific, Netherlands) following the procedure in the supplier manual. For every test conducted, two different types of negative and positive control were also analyzed to assess the validity of the test. These controls comprised previous test samples that were known to be positive or negative, as well as controls provided with the testing kit itself. The ELISA plate was read using an ELISA plate reader (Biorad Laboratories, USA) at 450 nm.

The percentage inhibition (PI) of each control and test serum was calculated according to the following formula:

$$PI = 100 - \left\{\left(\frac{OD_{450}test\ sample}{OD_{450}max}\right)\ x\ 100\right\} \qquad (1)$$

where OD_{450} max denotes the mean OD_{450} value of the negative controls.

For test validity, the OD_{450} of the negative controls must be >1.000. The mean percentage inhibition of the positive controls must be >70%. Not meeting any of these criteria is reason enough to discard the results of that specific test plate. If the OD_{450} of a test sample is higher than OD_{450} max, the Percent Inhibition can be interpreted as 0%. A value of PI < 50% indicates that antibodies against the NS protein of FMDV are absent in the test sample, while a value of PI ≥ 50% indicates that antibodies against the NS protein of FMDV are present in the test sample.

2.2 Validation of the FMD ELISA test

Validation is a procedure which ensures the fitness of an analysis that has been developed and optimized for an intended purpose.[8] Four major criteria were used to validate the FMD ELISA testing. These included Selectivity, Repeatability and Reproducibility.

Selectivity was checked by testing 7 blood serum samples which were known to be positive for NDV (Newcastle Disease Virus) and IBD (Infectious Bursal Disease) antibodies using the Priocheck FMD Kit. These samples were tested by two operators. For the test to be selective for FMDV antibody detection only, the presence of the NDV and IBDV antibodies in the samples must not produce percentage inhibition (PI) values greater than 50%.

Repeatability was demonstrated by having one operator test 9 positive FMD samples on the same day. Reproducibility was checked by having two different operators analyze 9 positive FMD samples using the Priocheck FMD Test kit.

For every test, the quality control procedure depended on the presence and testing of positive and negative FMD controls. These controls were laboratory reference controls provided by the Australian Animal Health Laboratory (AAHL).

2.3 Statistical analysis

To test for the accuracy, precision, sensitivity and specificity of the test, a statistical analysis was conducted following OIE standards using the data collected during the analysis.[9] The statistical measures used included the standard deviation, coefficient of variation, RSD, diagnostic specificity and selectivity.

Small values of the standard deviation showed that the analysis was precise and demonstrated that the readings had no significant variations.[10] For comparing the precision of two analyses, the F-test was employed. If the F-value exceeds a critical value provided by a statistical table, a significant difference exists between the results of the analyses.[10]

The Diagnostic Specificity and Sensitivity were calculated using formulas provided by the OIE.[8] These were used to assess how accurately the FMD ELISA test classifies truly negative (TN) or truly positive (TP) samples, respectively. DSe measures how accurately a test is able to detect truly positive results. A highly sensitive test would show few false negative (FN) results. According to Reid and Allen, a test with high DSe values should also show few false positive (FP) results.[11]

3. Results and Discussion

3.1 Data Collection

To the best knowledge of the authors, a total of 4, 012 goats are currently present in Brunei Darussalam, located in various places in all four districts. All the goat blood samples collected and tested from 2016 to September 2022 tested negative for FMD (see ***Table 1***), which further strengthens the country's status as being FMD-free. However, the laboratory has yet to test epithelial samples from affected ruminants, due to their unavailability. Epithelial samples are much preferred over blood samples[3]. But it is a difficult task to obtain infected epithelial samples because of sample instability and the possibility that the import of such samples might be prohibited. Hence, this study was conducted using certified reference blood samples known to be positive or negative, and provided by the AAHL.

3.2 Validation: Selectivity

The results for all the serum samples known to be positive for ND and IBD showed that their PI values were less than 50% (see ***Table 2***). This suggests that the presence of the NDV and IBDV antibodies in the samples does not have any cross-reactivity with the 3ABC specific monoclonal antibody (mAb) for FMD coated in the test plate. This confirms that the test effectively quantifies only the targeted FMDV antibodies, even in the presence of other disease reference controls.[8] Therefore, the tests are selective only for FMDV antibodies.

3.3 Validation: Repeatability

The results for all the serum samples known to be positive for FMD showed that their PI values were more than 50%. This suggests that the presence of FMD antibodies in the samples does have cross-reactivity with the 3ABC specific monoclonal antibody (mAb) for FMD coated in the test plate. OIE guidelines recommend a minimum of three replicates and a working dilution.[8] However, due to the limited availability of kits, this testing protocol was not adopted. Instead, the laboratory decided to have two different operators analyze 9 positive samples at different times. One of these runs was used to test repeatability. The standard deviation was found to have a low value of 1.01 (see ***Table 3***), which indicates that the test is precise.

3.4 Validation: Reproducibility

The results obtained by both analysts for all the serum samples known to be positive for FMD showed that their PI values were more than 50%. This suggests again that the presence of FMD antibodies in the samples had cross-reactivity with the 3ABC specific monoclonal antibody (mAb) for FMD coated in the test plate. The standard deviation was found to have low values of 0.88 and 1.01, which again indicates that the test is precise.[10] The F value of the two analyses was 0.748, and is lower than the F critical value F(0.05, 8, 8), which is 3.44 at 95% confidence level (see ***Table 4***). Hence the means of the two

analyses were not significantly different. This confirms that the results of the test are repoducible.[10]

3.5 Diagnostic sensitivity (DSe) and specificity (DSp)

To be considered reliable, a test is expected to have a high DSe (close to 100%), to be rapid (results often available in less than 24 hours), and to generate highly repeatable and reproducible results. A high DSp is expected for a confirmatory diagnosis. As part of the validation method for this test, the DSe and DSp were found to be 98% and 100%, respectively (see ***Table 5***). This confirms that the test is sensitive to FMD and specific only to FMD. These results are comparable to the requirements for the validation certificate issued by Priocheck FMD, with 183 cattle sera for DSe and 160 sera for DSp.[12]

3.6 Validation: Proficiency Testing

To ensure that the diagnostic test is reliable, the laboratory has participated in proficiency testings (PT), which involve blind samples comprising samples that are both positive and negative for FMD. Since 2019, the laboratory has participated in 2 PT conducted by Pak Chong (Thailand) and Vetqas (United Kingdom), and both showed satisfactory results. This indicates that the diagnostic test is reliable and can be used as part of country's effort to control and detect the presence of FMD.

3.7 Control Efforts

Other control efforts carried out by Brunei Darussalam include international collaboration with OIE and ASEAN laboratories, and importation only from FMD-free countries. Brunei Darussalam has participated in the South-East Asia and China Foot and Mouth Disease (SEACFMD) Campaign since 2010. SEACFMD aims to enhance the productivity and economic yield of the livestock sector by controlling and eradicating FMD in South-East Asia, China and Mongolia.

Though it is difficult to implement in Brunei Darussalam and other places, the movement of animals should be restricted. The movement of animals has been proven to be the main source of FMD outbreaks.[13,14] It has been suggested that animal movement should be controlled by first confirming that all animals are negative for FMD before their transfer to other cities.[14] This is crucial, as some animals may not develop fever and may not show obvious lesions. But they may still be infected and infect susceptible animals. Under this proposal, no animals could be moved without permits and health certificates from the respective authorities.

4. Conclusion

This study showed no detection of FMD in the Sultanate in the period from 2016 to September 2022. The ELISA test was also found to be sufficient for use as one of the control strategies. However, a few discrepancies must still be tackled. These include the need for further diagnostic tests, such as the PCR method, and for the testing of more reliable samples, such as epithelial samples, in large quantities. With these improvements, the testing regime will allow the national authorities to make informed decisions when combating this disease, if it ever appears. Further and more specific tests may also need to be developed, as there are 7 different serotypes of FMD found worldwide, and vaccination against one serotype will not provide immunity to the other 6 serotypes.

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Table 1. Total number of goat blood samples collected for FMD testing from 2016 to September 2022.

Year	No. of FMD Samples	Positive Results
2016	138	0
2017	314	0
2018	222	0
2019	621	0
2020	451	0
2021	240	0
Sept 2022	97	0

Table 2. Test results for the positive ND and IBD samples, used to check the selectivity of the FMD ELISA test.

Sample	Mean	PI Value (%)	Positive / Negative
ND1	1.510	8.21	Negative
ND2	1.455	11.55	Negative
ND3	1.994	-21.22	Negative
ND4	1.799	-9.36	Negative
ND5	1.577	4.16	Negative
ND6	1.841	-11.91	Negative
ND7	1.481	10.00	Negative
IBD1	1.519	7.66	Negative
IBD2	1.888	-14.74	Negative
IBD3	1.607	2.34	Negative
IBD4	2.029	-23.31	Negative
IBD5	1.831	-11.31	Negative
IBD6	1.629	1.00	Negative
IBD7	1.752	-6.50	Negative

Note: A PI value (%) $\geq$ 50% indicates that antibodies against the NS protein of FMDV are present in the test sample.

Table 3. Results and analysis for the known positive FMD samples, used to check the repeatability and precision of the FMD ELISA test. The critical number is the standard deviation.

No.	Mean Absorbance	PI value (%)
1	0.247	83.93
2	0.238	84.52
3	0.250	83.77
4	0.252	83.60
5	0.270	82.43
6	0.226	85.30
7	0.228	85.17
8	0.223	85.49
9	0.229	85.10
Mean		84.3678
Standard Deviation, s		1.013949922

Table 4. Results of the F-test for the known positive FMD samples, used to check the reproducibility and precision of the FMD ELISA test.

Sample	Analyst A	Analyst B
	PI value (%)	PI value (%)
1	84.68	83.93
2	86.55	84.52
3	85.95	83.77
4	85.75	83.60
5	84.21	82.43
6	86.85	85.30
7	86.62	85.17
8	85.81	85.49
9	85.71	85.10
Mean	85.7922	84.3678
Standard Deviation, s	0.87696605	1.013949922
Variance	0.769069444	1.028094444
Observations (n)	9	9
Degree of Freedom, df (n-1)	8	8
F value	0.748053302	
Critical Value F (0.05, 8, 8)	3.44 (from F-distribution table)	

Table 5. Diagnostic sensitivity and specificity estimates, calculated from a hypothetical set of results for samples tested from known infected and non-infected control samples.

		Number of reference samples required			
		Known Positive (57)		**Known negative (53)**	
Test Results	**Positive**	56	TP	FP	0
	Negative	1	FN	TN	53
		Diagnostic Sensitivity (Dse) TP/ (TP + FN) =56 / (56+1) = 98%		Diagnostic specificity (Dsp) TN / (TN + FP) =53 / (53+0) =100%	

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