Improvement of Enzyme-Linked Immunoelectrotransfer Blot Assay by Quantitative Approach for Foot-and-Mouth Disease diagnosis

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Abstract: Foot-and-mouth disease (FMD) is a highly contagious animal disease that causes devastating economic losses. The trade of live animals and derived products is only possible if the exporting country is free from disease, according to the World Organization for Animal Health (WOAH) code for FMD. One of the most important ways to prove disease-free is to measure the levels of non-structural protein antibodies (NPS) of FMD virus in the target population sampled. For detection of the disease status of a herd, mass screening and assays such as Enzyme-linked immunosorbent assay (ELISA) and Enzyme-linked immunoelectrotransfer blot assay (EITB) were developed and described in the WOAH diagnostic manual. In this study, recombinant FMDV NS proteins were produced and tested with sera panels collected from uninfected and naturally infected animals using a quantitative Western blot assay as an improved EITB, which enables numerical documentation and statistical analysis. NSP band intensities were used to determine the cut-off values, differentiating infected from non-infected animals and revealing variable sensitivity among the different NSPs. The quantitative EITB results also showed a correlation with the NSP-ELISA results.

Keywords: *Enzyme-Linked Immunoelectrotransfer Blot Assay, Foot-and-Mouth Disease Virus, Non-Structural Proteins, NSP-ELISA, Quantitative Computerised Western blot (QCWB).*

Şap Hastalığı Teşhisi için Enzim Immunotransfer Blot Testinin kantitatif bir yaklaşım ile iyileştirilmesi

Özet: Şap hastalığı büyük ekonomik kayıplara neden olan çok bulaşıcı bir hayvan hastalığıdır. Dünya Hayvan Sağlığı Örgütü Şap Hastalığı koduna göre canlı hayvanların ve bunların ürünlerinin ticareti, ancak, ihraç eden ülkenin hastalıktan ari olması şartıyla mümkündür. Hastalıktan ariliğin gösterilmesi için en önemli yöntemlerden biri örneklenen hedef popülasyonun Şap virüsü yapısal olmayan proteinlerine (YOP) karşı oluşan antikor seviyelerinin ölçümü temeline dayanır. Bir sürünün hastalık durumunun belirlenmesinde yaygın tarama ve Enzim bağlı Immunosorbent test (ELISA) ve Enzim bağlı Immunotransfer testi (EITB) geliştirilmiş ve WOAH (Dünya Hayvan Sağlığı Örgütü) teşhis el kitabında tanımlanmıştır. Bu çalışmada Şap virüsünün yapısal olmayan proteinleri rekombinant olarak üretilmiş, enfekte olmayan, aşılı ve doğal olarak enfekte olan hayvanlardan toplanan serumlardan oluşan panellerle, sayısal belgelemeye ve istatistik analize olanak veren, iyileştirilmiş, kantitatif bir Western blot kullanılarak test edilmiştir. YOP bant yoğunlukları, hasta ve enfekte hayvanların birbirinden ayırt edecek eşik değerlerin ve farklı YOP'lerin bu teşhiste farklı hassasiyet gösterdiğinin belirlenmesinde kullanılmıştır. Kantitatif EITB sonuçları aynı zamanda YOP-ELISA sonuçları ile korelasyon göstermiştir.

Anahtar Kelimeler: *Enzim-Bağlı İmmunoelekrotransfer Blot Testi (EITB), Şap Virüsü, Yapısal Olmayan Proteinler (YOP), YOP-ELISA, Kantitatif Western blot.*

Introduction

Foot-and-mouth disease (FMD) is one of the most contagious and economically important diseases of cloven-hoofed animals. The causative agent is a virus that belongs to the family *Picornaviridae*. Like other picornaviruses, the virus has an approximately 8 kb RNA genome that encodes a single polyprotein split into 12 different proteins. Among these proteins, there are structural virus capsid proteins, VP1-4, and non-structural proteins (NSPs), L, 2A, 2B, 2C, 3A, 3B, 3C, and 3D. FMD virus infection elicits antibodies against both structural and non-structural proteins. Theoretically, purified FMD vaccines containing inactivated virions would be expected to elicit antibodies against structural proteins but not against NSPs (Kitching, 2002; Kweon et al., 2003).

FMD threatens the livestock industry of developed countries due to their non-vaccination policy (Orsel and Bouma, 2009). The disease is one of the most critical factors causing poverty in developing countries (Perry and Rich, 2007). Vaccination has been successfully utilised to control and eradicate the disease in many countries in Europe and South America. FMD was eradicated from large areas without any animal culling (Bergmann et al., 2005). Vaccination has become a more profitable strategy for future European outbreaks (Orsel and Bouma, 2009). However, vaccination can mask clinical symptoms, and infected animals may become carriers (defined as having the infectious virus in the pharynx more than 28 days post-infection). The role of FMD carriers is unclear in transmission, but they still pose a risk for FMD-free parts of the world (Davies, 2002).

An effective surveillance system has to be set up, and not only the clinical disease but also the risks must be controlled to gain FMD-free status in a formerly endemic country or region. In an FMD-free zone where vaccination is practised, serological assays must be able to discriminate between previously infected and vaccinated animals, as described in the WOAH code (Anonymous, 2011). Many ELISA-based tests have been developed for differentiating between infected and vaccinated animals (DIVA) by targeting antibodies to the NSPs. Among these tests, a 3ABC recombinant protein has been found to be the most successful protein thus far for this purpose (Clavijo et al., 2004).

Due to some NSPs within FMD vaccines (if not purified), some sera from vaccinated animals can give positive reactions in the absence of infection by NSP ELISA tests (Lee et al., 2006). Therefore, follow-up studies supported by confirmatory DIVA assays are critical in surveillance studies to reveal false positive animals having no FMD history.

A highly sensitive enzyme-linked immunoelectrotransfer blot (EITB) test that utilizes recombinant NS proteins (2C, 3A, 3B, 3ABC, and 3D) produced in *Escherichia coli* (*E. coli*) has been developed as a confirmatory assay (Bergmann et al., 1993; 1998; 2000; 2003; 2005). Although the assay has diagnostic potential as it is sensitive, reliable,

rapid, and economical for FMD (Bergmann et al, 1993), there are some drawbacks to limiting its use, such as it is not as simple as ELISA and it needs more laboratory equipment. Besides, *E. coli* infections of calves or vaccinations against *E. coli* generate anti-*E. coli* antibodies in the cattle sera may produce several non-specific bands with recombinant antigens produced in *E. coli* expression system in Western blot assays (Shen et al., 1999). Another complication of this assay is that the molecular weight and strength of the bands may change slightly depending on the test conditions. Qualitative and empirical solutions to overcome these problems increase Western blot assay subjectivity. Hence, standardization of the assay becomes difficult.

This study aimed to improve the known EITB by a quantitative approach. Improving the EITB will facilitate the screening of FMD infection in cattle populations and identify actual NSP antibody-positive animals.

Materials and Methods

The cattle sera used in this study are not subject to the ethical guidelines for using animals in research according to the current regulation in Türkiye.

Cattle Sera:
Group 1: Cattle sera from animals with a history of clinical disease were collected one month after an A Iran/2005 FMD outbreak at Samsun Karaköy State Farm in the Black Sea Region of Türkiye in 2008. The animals were vaccinated multiple times against FMD previously.

Group 2: Cattle imported from a disease-free country to a private dairy farm located at Aydın Germencik in the Aegean Region of Türkiye were vaccinated twice with oil-adjuvanted FMD bivalent vaccine (Turvac Oil, O_1 Manisa, and A_{22} Iraq) at one-month intervals, intramuscularly. The sera were collected one month after the second vaccination.

Group 3: Sera from non-infected and non-vaccinated cattle collected from the farm from which the animals of Group 2 were obtained. The sera were collected from imported animals before vaccination (Table 1).

Table 1. Number of animals, vaccination and disease status.

| Groups	Number	Country of origin	Vaccination status	Clinical Infection in herd
Group 1	119	Türkiye	Multiple	Yes
Group 2	79	New Zealand	Twice	No
Group 3	62	New Zealand	Unvaccinated	No

Production of recombinant proteins: Oligonucleotide primers were designed to target the FMDV O_1 Manisa strain (GenBank® Accession Number: AY593823.1) NSP coding regions; 2B, 2C, 3A, 3B, 3C, 3AB, and 3ABC. BamHI and HindIII restriction endonuclease recognition sites, initiation and termination codons were added near to the 5' and 3' ends of each primer, respectively. RT-PCR and purification of PCR products were performed as previously described (Clavijo et al., 2006). The pET30c vector (Novagen, Madison, WI, USA), which carries an N-terminal His-Tag sequence, was used for cloning the target sequences. The ligation reaction was performed according to the manufacturer's protocol following the restriction digest of both the PCR products and the vectors. *E. coli* JM109 cells (Promega, Madison, WI, USA) were used for cloning, and *E. coli* BL-21 (DE3) pLysS (Novagen, Madison, WI, USA) cells were used for the expression of these proteins.

All protein extractions and purifications were performed using nickel-nitrilotriacetic acid (Ni-NTA) metal-affinity chromatography matrices (QIAexpressionist kit, Qiagen, Germantown, MD, USA) under denaturing conditions. The eluted protein concentrations were measured using a BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions.

Design of multiplex assay system: The multiplex assay system used two gels containing 2C-3B or 3AB-3A recombinant protein mixtures. To provide equal spread, running, and transfer conditions and to prevent within-gel variation, the protein mixtures were loaded onto the gel without a comb. Each serum was tested in both the 2C-3B and 3AB-3A antigen systems. Antibody probing was performed in a 20-lane multiscreen apparatus (Miniprotean II multiscreen, Bio-Rad Laboratories, Hercules, CA, USA). One of the lanes was probed with anti-his MAb (monoclonal anti-polyhistidine, H1029, Sigma-Aldrich, St. Louis, MO, USA) to standardise the band densities and used as molecular weight markers. Other lanes were probed with the bovine test sera.

Quantitative computerised Western blot test (QCWB): According to the sizes of the recombinant proteins, two sets of protein mixtures have been designed. The serum samples have been tested in the assay using two different gels for Mixture 1 (2C and 3B) and Mixture 2 (3AB and 3A) (Figure 1).
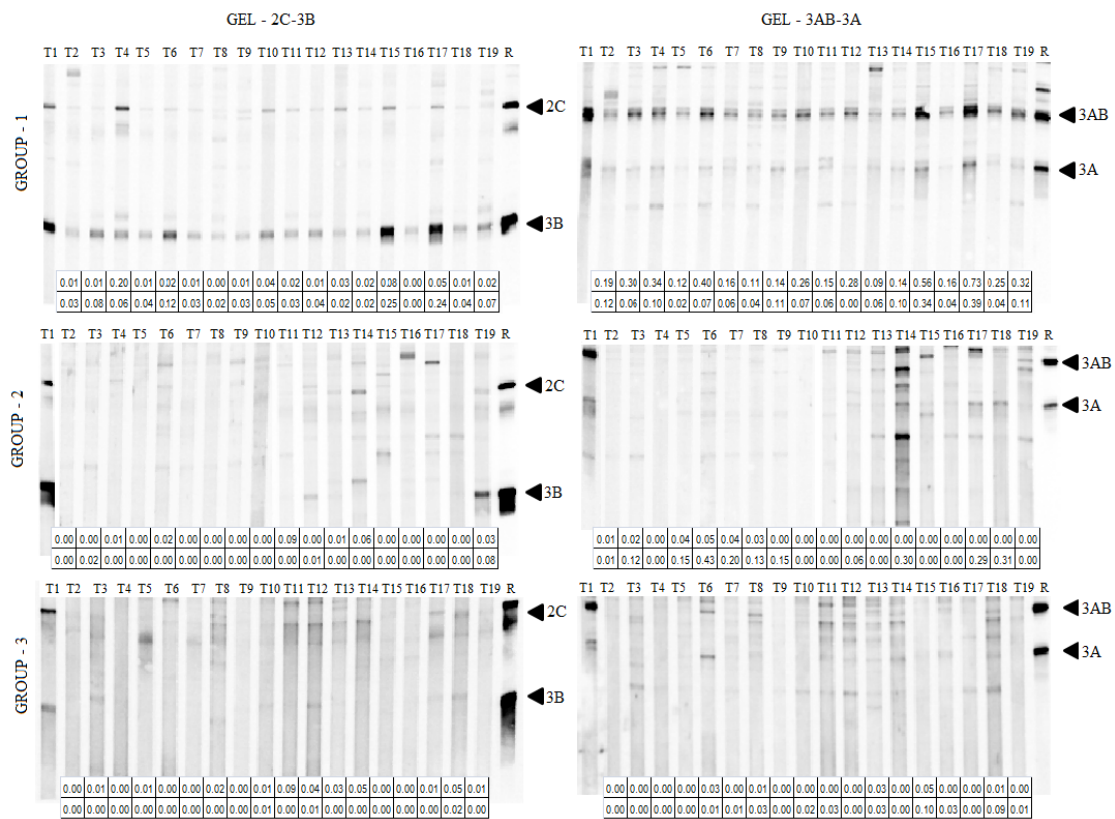
Figure 1. Multiplex assay format and calculating the relative intensity values (RTI). Lane R, probed with monoclonal anti-polyhistidine, is a reference lane. The other lanes, T1-T19, were probed with bovine sample sera. The RTI value of each band was shown under the lane of each serum.

Purified recombinant NS proteins were electrophoresed in 4-12% polyacrylamide gels (acrylamide/bis-acrylamide 40% solution) to evaluate their reactivities to MAb Anti-polyhistidine and bovine sera in QCWB. The separated proteins were transferred to nitrocellulose membranes by electrophoresis at 25 V for 25 minutes with a semi-dry blotter (Scie-Plas, Cambridge, UK). The blots were first blocked with TBS-T-M, which contains 5 mM Tris, 30 mM NaCl with pH 7.6, and 0.1% Tween-20 (0777-1L, Amresco, USA) and 5% dried skimmed milk (Marvel, UK) in a vertical motion shaker. After five minutes washing step with TBS-T (TBS-0.1% Tween-20), the membranes were probed with primary antibodies (anti-his MAb or bovine sera at a dilution of 1:750 in TBS-T-M, respectively) in the shaker. After three times five minutes shaking with TBS-T, the membranes were probed with secondary antibodies, which are secondary goat anti-mouse (31430, Thermo Fischer Scientific, Waltham MA, USA) or rabbit anti-bovine HRP conjugates (A5295, Sigma-Aldrich, St. Louis, MO, USA), at a dilution of 1:1000 in TBS-T-M. After three times five minutes shaking with TBS-T, the blots were incubated with the chemiluminescent substrate (SuperSignal West Pico Substrate Thermo Fisher Scientific, Waltham MA, USA). The blots were then imaged using an imaging station (Gel Logic 1500, Kodak, Rochester, NY, USA). Specific band densities were acquired as the net intensity and converted to the relative intensity values (RTI), which were processed using the software (KODAK Molecular Imaging Software v 4.0.5) provided with the image station. Bands of interest were identified by their exact weight by matching with the bands probed with anti-his MAb using the software tools. Therefore, both non-specific bands and the background colour were excluded to improve the interpretation of the test, which might be affected due to the impurity of the recombinant proteins.

Lane R, probed with monoclonal anti-polyhistidine, is a reference lane. The other lanes, T1-T19, were probed with bovine sample sera. Band images were digitised by Kodak Molecular Imaging Software as the net intensity (T_N or R_N). T_N for each NSP band in the bovine lanes was converted to RTI as a percentage of the R_N of the same NSP band in the reference lanes according to the formula (RTI=1xT_N/R_N). The RTI value of each band was shown under the lane of each serum.

Enzyme-linked immunosorbent assay (ELISA): NSP ELISA tests (PrioCHECK FMDV-NS, Prionics, Switzerland) were performed according to the manufacturer's instructions for all sera samples. The percentage inhibition (PI) values were categorised into two groups, 50–69 and 70–100, based on validation criteria for PI values of weak positive and

positive controls indicated in the manufacturer's instruction manual. The weak positives were repeated twice and tested with another 3ABC ELISA kit (AniGen FMD NSP Ab ELISA, Bionote Inc. Gyeonggi-do, Republic of Korea) according to the manufacturer's instructions as well.

Statistical Analysis: Statistical analysis was performed using two different software suites (SPSS Statistics 20, IBM, New York, NY, USA, and Medcalc V.13.2.2.0 Ostend, Belgium). Comparisons between the groups were performed using receiver operating characteristic curve (ROC) and Student's t-test analyses. The ROC analysis is the most common method to evaluate the performance of a diagnostic test. This analysis allows the comparison of different test and operator efficiencies, determining appropriate cut-off values, and monitoring the laboratory results quality (Greiner et al., 2000). ROC curves that display the diagnostic performances of the proteins were drawn. The optimal cut-off values of band intensities for each protein were determined using the ROC curves. The diagnostic sensitivity and specificity of the test were also established with the help of these cut-off values. A comparison of the areas under the ROC curves for sensitivity and specificity calculations has been made by using Medcalc software for the simultaneous use of protein results for the overall performance of the test. Correlations between EITB and ELISA results were computed using the SPSS software mentioned above using Pearson's correlation coefficient. P values of less than 0.01 were considered significant. Significant differences between the groups were examined by Student's t-test for independent samples, and P values < 0.05 were considered significant.

Results

Quantitative computerised Western blot test (QCWB): FMD Virus O_1 Manisa reference strain NSPs 2B, 2C, 3A, 3AB, 3ABC, 3B, and 3C recombinant proteins were expressed in *E. coli*. The 2B, 3ABC, and 3C proteins could not be used for further work and were excluded from the tests due to the degradation or low yield of these proteins.

The 2C, 3B, and 3AB proteins did not react with sera from the non-infected groups (Groups 2 and 3). From the infected group, detection of all of the FMDV proteins tested was achieved. The band densities, read by the software, were transformed into numerical values, and the RTI values were given under each lane (Figure 1).

ELISA: NSP ELISA (PrioCHECK FMDV NS) results from the groups are shown in Figures 2a, b, and c. All 119 animals in the infected group (Group 1) tested positive. Four of these 119 animals gave values
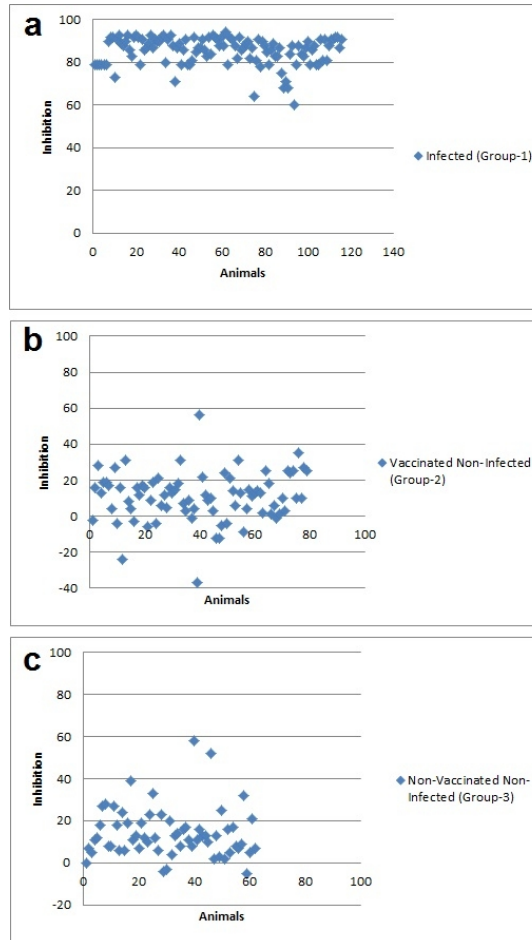
Figure 2. NSP ELISA percentage inhibition values of Group 1 (a), Group 2 (b) and Group 3 (c) Inhibition value < 50% is defined as negative, while an inhibition value of ≥ 50% is positive for the infection.

between 50 and 70% inhibition; in other words, these four were weak positives while the other 115 were strongly positive. One animal was determined to be a weak positive (56% inhibition) in the vaccinated but non-infected group (Group 2). In contrast, 2 out of 62 animals in the non-vaccinated and non-infected group (Group 3) were determined to be weak positives (52% and 58% inhibition). The

weak positives obtained by NSP-ELISA were retested, and the results showed that sequential tests detected all infected animals (Group 1). Only one animal also tested positive in PrioCHECK had false reactivity in the vaccinated non-infected group (Group 2). The repeated seven weak positive sera results are given in Table 2.

Table 2. Percentage Inhibition and Relative Intensity values of the weak positive sera.

Ear Tag	Category	NSP-ELISA				Quantitative EITB			
		PrioCHECK	PrioCHECK 2nd test	PrioCHECK 3rd test	AniGen	2C	3B	3AB	3A
4276	Group 3	**58**	39	31	19	0	0	0	**0.12**
4268	Group 3	**52**	34	22	28	0	0	0	0
6421	Group 2	**56**	**55**	48	48	**0.02**	**0.08**	0	0
6672	Group 1	**64**	**67**	**67**	**83**	**0.03**	**0.02**	**0.08**	**0.06**
6538	Group 1	**68**	**81**	**78**	**85**	0	0	**0.10**	**0.38**
6531	Group 1	**68**	**86**	**82**	**74**	**0.02**	0	**0.29**	**0.45**
6557	Group 1	**60**	**65**	**67**	**81**	0	0	**0.07**	**0.79**

-The percentage inhibition and relative intensity values above the cut-off levels marked as bold characters. The AniGen test interpretation method was similar to the PrioCHECK ELISA.

Statistical Analysis: The derived minimum, maximum, mean, standard error, and standard deviation values are evaluated by statistical analysis. Statistical analysis of the data in Table 3 showed that

Table 3. The Pearson correlation of the Relative Intensity (RTI) values of the bands.

	ELISA	RTI 2C	RTI 3B	RTI 3AB	RTI 3A
ELISA	1	0.233*	0.366*	0.552*	0.265*
RTI 2C	0.233*	1	0.577*	0.615*	0.430*
RTI 3B	0.366*	0.577*	1	0.742*	0.205*
RTI 3AB	0.552*	0.615*	0.742*	1	0.571*
RTI 3A	0.265*	0.430*	0.205*	0.571*	1

* Correlation is significant at the 0.01 level (2-tailed).

the reaction against the proteins correlated well with each other and ELISA results. The highest correlation was detected between 3AB and 3B (r: 0.742), followed by that between 3AB and 2C (r: 0.615). Briefly, the highest correlation with the ELISA results was detected with the 3AB protein (r: 0.552), followed by 3B, 3A, and 2C. The area under the curve was calculated by ROC curve analysis; the largest area under the curve was for 3AB, followed by 3B, 3A, and 2C (Figure 3).
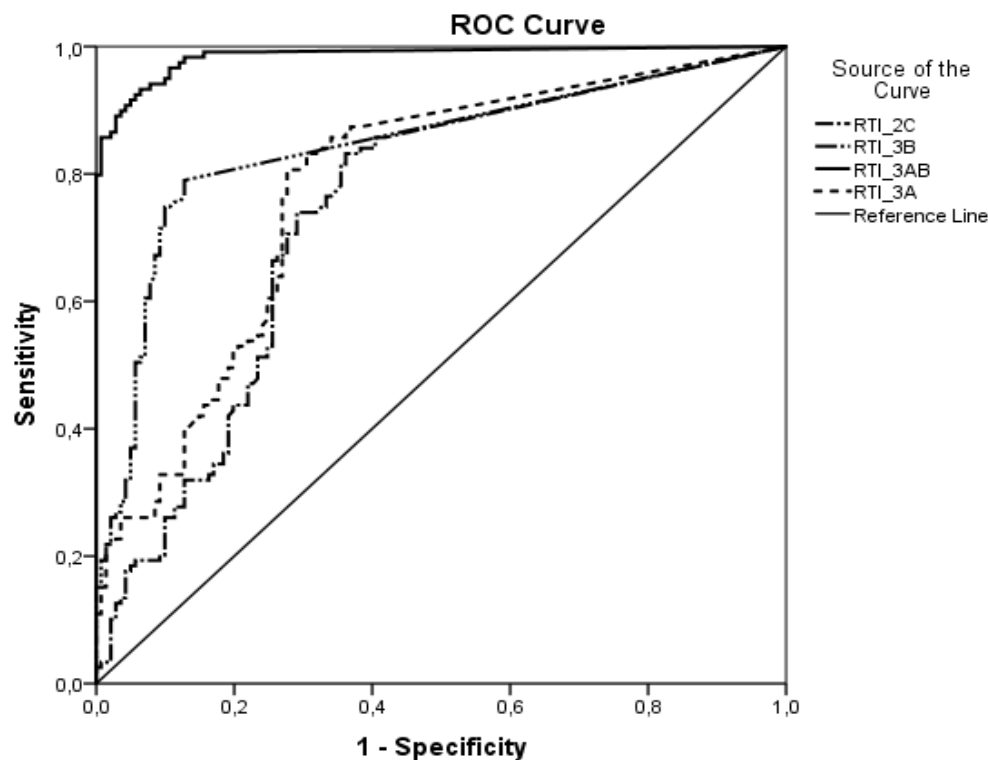
Figure 3. The Receiver Operating Characteristic (ROC) curve analysis of Relative Intensity (RTI) values of individual recombinant proteins in quantitative EITB assay.

The optimum cut-off values for the RTI of the bands were determined by using the ROC curve to obtain the maximum sensitivity and specificity values for the test. (Table 4). A statistically significant difference was found among the RTI values of the 3A protein between the vaccinated (Group-2) and the non-vaccinated, non-infected groups (Group-3), as assessed by independent Student's t-tests ($p < 0.05$).

Table 4. Cut-off, sensitivity and specificity values of the proteins and protein combinations.

Proteins	Cut-Off (RTI)	Sensitivity	Specificity
2C	0.010	0.739	0.709
3B	0.002	0.790	0.872
3AB	0.044	0.924	0.936
3A	0.035	0.807	0.723
3AB and 3B	*	0.756	0.993
3AB and 3A	*	0.781	0.964
3B and 2C	*	0.655	0.929
3AB, 3B and 2C	*	0.621	0.993
3AB, 3B, 2C and 3A	*	0.546	0.993

*Cut-off values for single use of the proteins are also valid for multiple use of the proteins.

Discussion

The detection of infection is essential for the control and eradication of FMD. In some countries where widespread vaccination is used, when animals have antibodies to the structural proteins, then clinical disease may not be apparent, and detection of previously infected animals can only be possible using NSP ELISA tests. According to the WOAH code (Anonymous, 2011), NSP-seropositivity of animals in which NSP-free FMD vaccine is applied should be confirmed by retesting with a confirmatory test and follow-up investigation for the presence of ongoing infection where the clinical disease is absent. To evaluate the NSP ELISA test results, some alternative tests such as Dot blot, Luminex-based multiplex assay, or another ELISA have been developed (Clavijo et al., 2004; Fu et al., 2011; Paton et al., 2006).

This study was designed to improve the well-known EITB assay, which is generally used to confirm of NSP-ELISA results. Recombinant proteins are used in both tests. Production of recombinant NSPs of FMDV can be difficult because of their toxic effect on the bacterial expression system (Lewis et al., 1991). Moreover, purification of the proteins can also be troublesome because some proteins may be lost during the extraction or purification steps (Bergmann, 2000). According to our experience, the production of some FMDV NS proteins, such as 2B and 3C, was difficult, probably, the 2B was a too small, and the 3C was a protease. Despite choosing a special expression system, BL-21 (DE3) pLysS, which is suitable for expressing toxic proteins, the 2B, 3C, and 3ABC proteins could not be produced in

sufficient amounts for the assay. In addition, the 3C protein was degraded during its storage.

The number of sera used in a single test was increased using of a multichannel Western blot apparatus so that 38 samples could be tested simultaneously compared to 24 stripes of conventional EITB assay (Anonymous, 2011). The subjectivity of the test was also reduced by using Kodak Molecular Imaging software, which converts band densities into numerical RTI values.

In a similar study (Mackay 1998), it was found that there was a high correlation between indirect ELISA and EITB for reactive and non-reactive sera results. According to their indirect ELISA results, 3A, 3B, and 3ABC had the best results. Another study (Bronsvoort et al., 2006) showed that infected animals could be differentiated from vaccinated animals by detecting of antibodies against the 2C, 3A, and 3ABC proteins.

In this study, the RTI values correlated well with each other and with the ELISA results. The most concordant result with ELISA was obtained for the 3AB protein (Table 3). Furthermore, it was found that the 3AB, 3B, 3A and 2C proteins could be used for differential diagnosis by ROC analysis (Figure 3). According to the curve, the highest diagnostic sensitivity was obtained for 3AB, followed by 3B, 3A, and 2C. These findings agree with the first study's results (Mackay, 1998), except for 2C. Our findings showed that the 2C proteins could also be used as another indicator of infection in the EITB assay.

It was reported that using several different NS proteins increases the test's specificity (Bergmann, 2005). In this study, when the 3AB and 3B proteins were evaluated together, and both results were

positive, the sensitivity and the specificity were measured at 75.63% and 99.29%, respectively. Similarly, when the reactions to all three or four proteins were simultaneously positive, the specificity values were above 99%. Consequently, combining two or more proteins improved the test's specificity compared to using single proteins. Conversely, depending on the combinations chosen, the sensitivities were reduced compared to the use of single proteins. Specificity should take the highest priority here because the quantitative EITB is designed as a confirmatory test following ELISA and it is desired to have a low false positivity rate. The NSP ELISA tests have specificity problems and should be complemented by an alternative assay such as EITB (Espinoza et al., 2004). In our study, a few weak positives detected in the first NSP test were evaluated together with the sequential tests and quantitative EITB. The results are consistent with the animal groups when the 3AB-3B pair is considered (Table 4). The results of this study also revealed that not all of the proteins have to be used for evaluation. The best combination was the 3AB-3B pair for optimum specificity and sensitivity.

There is a continuing debate about the presence of trace amounts of NSPs in the FMDV vaccine formulations (Espinoza et al., 2004). An early study (Lubroth, 1996) showed that 2C was removed entirely during the vaccine preparation process, and therefore repeated vaccination could not elicit antibodies against 2C. On the other hand, another study (Dekker and Gijsen, 1998) found 3A in the supernatant of virus cultures. Our data support these findings, as there were significantly more animals reactive to 3A protein in Group 2 than in Group 3 ($p < 0.05$).

In conclusion, quantitative EITB can contribute to evaluating the results obtained by NSP-ELISA tests as an essential complementary tool. False positives and negatives can be detected more precisely with the help of this computerized assay. Using this test may lead to better estimations of the true prevalence and incidence of the disease. However, this quantitative EITB should be validated with standard sera panels against various strains provided by international laboratories and with some controls for linearity, gel-to-gel variation, and reproducibility. In addition, because the assay utilises anti-bovine conjugates, sheep sera and anti-sheep conjugates should also be studied to validate of the assay.

Conflict of Interest

We did not have any real, potential or perceived conflict of interest.

Ethical Approval

This study is not subject to HADYEK permission in accordance with Article 8 (k) of the "Regulation on Working Procedures and Principles of Animal Experiments Ethics Committees".

Similarity Rate

We declare that the similarity rate of the article is 13% as stated in the report uploaded to the system.

Author Contributions

Motivation / Concept: FÖ
Design: CÇ, FÖ
Control/Supervision: FÖ
Data Collection and / or Processing: ÜP, BS
Analysis and / or Interpretation: AB, AB
Literature Review: CÇ
Writing the Article: CÇ
Critical Review: FÖ

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