Kafkas Universitesi Veterinar Fakultesi Dergisi
ISSN: 1300-6045 e-ISSN: 1309-2251
Journal Home-Page: http://vetdergikafkas.org

Kafkas Univ Vet Fak Derg
28 (4): 431-436, 2022
DOI: 10.9775/kvfd.2021.26927

RESEARCH ARTICLE

Milk Non-Enzymatic Immunofiltration Assay "mNERIFA": An Alternative Rapid Bovine Milk Test for Anti-*Brucella* Antibody Detection [1]

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[1] *This study was presented as a poster presentation at the 4th International Eurasian Conference on Biological and Chemical Sciences held on 24-26 November, 2021 in Ankara, Türkiye*

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Article ID: KVFD-2021-26927 Received: 13.12.2021 Accepted: 09.06.2022 Published Online: 09.06.2022

Abstract: Rapid milk tests for the indirect diagnosis of brucellosis are limited and generally not preferred due to insufficient diagnostic performances and inappropriate handling process. In this study, a rapid immunofiltration assay known as Non-Enzymatic Immunofiltration Assay (NERIFA) was evaluated based on diagnostic performances and applicability for the detection of anti-*Brucella* antibodies in bovine milk samples as milk NERIFA (mNERIFA). For this purpose, wheys, that were obtained from rennin precipitated milk constituted with reference sera, were used as test material. Besides, an enzyme-linked immunosorbent assay (ELISA) was also developed for the evaluation of mNERIFA. After approval, the ELISA was evaluated as important (P<0.001) by Receiver Operating Procedures (ROC) analysis based on the diagnostic index (J) score specified as the Youden index (J: 0.963) and the Area Under the Curve (AUC) value (0.996), its results compared with mNERIFA by kappa statistical analysis. ELISA's comparison with mNERIFA based on diagnostic performances indicated that ELISA's performance was in perfect agreement with mNERIFA (κ=0.97) and the agreement was 0.97% (0.92-0.99%) with CI 95%. In the optimization process of mNERIFA, it was found that there was no significant difference between the individual and pooled whey samples (P>0.05), and a pool of 5 samples can be used instead of individual testing. In this study, it was concluded that mNERIFA may be recommended as a rapid test for anti-*Brucella* antibody detection in bovine milk samples based on the diagnostic performances, applicability, and pooling capacity.

Keywords: *Bovine brucellosis, Milk NERIFA, Milk test, Rapid test, Serology*

Süt İmmünofiltrasyon Testi "mNERIFA": Anti-Brusella Antikor Tespiti İçin Alternatif Hızlı Süt Testi

Öz: Hızlı süt testleri brusellozun indirekt teşhisi amacıyla sınırlıdır ve yetersiz teşhis özellikleri ve uygun olmayan işleme süreci nedeniyle genellikle tercih edilmez. Bu çalışmada, Non-Enzimatik İmmünofiltrasyon Testi (NERIFA) olarak bilinen hızlı bir immünfiltrasyon testi, süt NERIFA olarak tanısal performanslarına ve anti-*Brucella* antikor tespiti yönünden sığır süt örnekleriyle değerlendirildi. Bu amaçla referans serumlardan oluşturulan rennin ile çökeltilmiş sütten elde edilen süt serumu test materyali olarak kullanıldı. Ayrıca, mNERIFA'nın değerlendirilmesi için bir enzim immünosorbent testi (ELISA) geliştirildi. ELISA'nın, Receiver Operating Procedures (ROC) analizi ile Youden endeksi olarak bildirilen tanı indeksi (J: 0.963) ile Eğri Altındaki Alan (AUC) (0.996) değerine dayalı olarak uygunluğu (P<0.001) onaylandıktan sonra, mNERIFA ile kappa istatistiksel analizi ile karşılaştırıldı. ELISA'nın tanısal performanslara dayalı olarak mNERIFA ile karşılaştırması, ELISA'nın performansının mNERIFA ile mükemmel uyum içinde olduğunu (κ=0.97) ve bu uyumun %95 güven aralığında %0.97 (%0.92-%0.99) olduğu belirlendi. mNERIFA'nın optimizasyon sürecinde, bireysel ve havuzlanmış süt serumu örnekleri arasında anlamlı bir fark olmadığı (P>0.05) ve bireysel testler yerine 5 örneklik bir havuzun kullanılabileceği belirlendi. Bu çalışmada, mNERIFA'nın, tanısal performanslarına, uygulanabilirliğine ve havuzlama kapasitesine bağlı olarak sığır sütü örneklerinde anti-Brusella antikorlarının tespiti için hızlı bir test olarak önerilebileceği sonucuna varılmıştır.

Anahtar sözcükler: *Hızlı test, Seroloji, Sığır brusellozisi, Süt NERIFA, Süt testi*

How to cite this article?

Genç E, Serdar G, Kılıçoğlu Y, Genç O: Milk non-enzymatic immunofiltration assay "mNERIFA": An alternative rapid bovine milk test for anti-*Brucella* antibody detection. *Kafkas Univ Vet Fak Derg,* 28 (4): 431-436, 2022.
DOI: 10.9775/kvfd.2021.27927

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Introduction

Brucellosis is one of the seven neglected prevalent zoonotic diseases that affect animals and human beings worldwide [1]. It is a costly, highly contagious disease that affects many farm animals [2,3]. The disease is usually manifested by abortion, retention of the placenta, stillbirth, infertility with the scattering of the organism in uterine discharges and milk. Diagnosis is based on isolation and detection of *Brucella* spp. from abortion material, mammary secretions, or postmortem tissues [4-6]. *Brucella* culture is accepted as a reference as the gold standard and it may give rise to false negative results in serological evaluation. Although serological tests are used for the screening and monitoring in surveillance and control and eradication phase of the disease [7], but no single valid serological test is available to detect every stage of infection with *Brucella* spp. [8] with higher accuracy. Because of this, bovine brucellosis diagnosis is performed with serial or parallel tests in blood sera and generally, Rose bengal test (RBT) and Complement fixation test (CFT) are preferred in combination to confirm the diagnosis in many countries [7,9]. ELISAs being more sensitive and specific, are recommended in place of both CFT and RBT and other conventional tests [5] by OIE as a suitable screening test [7,10]. However, a limited number of tests mostly Milk Ring Test (MRT) and ELISA [11-13] and less frequently chromatographic tests [14] are being used for brucellosis screening based on testing anti-*Brucella* antibodies in milk.

Rapid tests are preferable in field conditions due to higher sensitivity, specificity, rapidity, and applicability [15,16]. Among these tests, immunochromatographic [14,16] and immunofiltration [17-19] based formats have been suggested for serological detection of bovine brucellosis, but their applicability in milk is very limited. Immunochromatographic rapid tests are more common [20-22] compared with immunofiltration based formats. However, there is no information with immunofiltration tests to detect anti-*Brucella* antibodies in milk.

In rapid tests, either in immunochromatographic or immunofiltration, nanoparticles as detector molecules are conjugated to antibodies that recognize the antigens and antibodies [23], and in some cases, proteins such as proteins A, and G are preferred in place of antibodies due to their higher affinities [13]. Recently, the gold nanoparticles conjugated with proteins have been used commonly in different species for the detection of different antibody isotypes. The affinity of protein G is better for the detection of bovine IgG isotypes and the strong optical absorption of gold nanoparticles with protein G enables its colorimetric detection by the naked eye [23,24]. These properties of protein G conjugated with gold nanoparticles make it special for rapid testing for both antigen and antibody detection. This study aimed to determine the diagnostic performance of mNERIFA based on detector Gold/Protein G (G/PG) nanoparticles for brucellosis detection as an alternative test in bovine milk samples.

Material and Methods

Ethical Statement

Ondokuz Mayıs University Animal Experiments Local Ethics Committee provided an ethics report for this study (E-68489742-604.02.03-255675).

Blood Sera

A total of 87 reference sera was included in the study. Of these, 60 were obtained from certified *Brucella*-negative herds and 27 were from aborted dairy cows. The sera were also confirmed serologically by iELISA. All positive references were from aborted cows and in which *Brucella abortus* biotype 3 isolates were isolated from vaginal swabs and milk samples.

Milk Samples

Milk samples obtained from a certified *Brucella*-negative herd was tested for the presence of anti-*Brucella* antibody by Milk Ring Test (MRT) (MRT Antigen Institute Pourquier, France), and then it was used for preparation of whey for ELISA and mNERIFA testing.

Milk Whey Preparation

Since milk samples were not suitable for the evaluation of mNERIFA, wheys were prepared from the milk samples. For this purpose, all control reference sera were transferred into the milk samples at a ratio of 1: 2 considering the probability of binding of the antibodies to milk fat globules in place of transferring directly the reference sera to wheys. Briefly, milk samples were prepared by the reference sera. The mixture was then incubated with microbial coagulating solution (Turkish rennet, Yayla) at room temperature for 30 min. After centrifugation at 3.000xg for 10 min at 4°C, the wheys were collected [25] and used to determine the diagnostic performance of the ELISA.

Reference Sera

An OIE serum containing 1000 Complement Fixation Test Unit (CFTU) anti-*Brucella* antibody was used for the optimization of CFT, ELISA, and mNERIFA. Monoclonal anti-*Brucella* LPS antibody (LPS-mAb, clone 4B5A) was used as a reference antibody for checking the LPS consistency of the crude LPS antigen.

Antigens, Reagents and Bacteria

Antigens and other reagents such as complement and amboceptor were obtained commercially and used in CFT (Virion Serion, Germany). Crude lipopolysaccharide

(LPS) antigen used in ELISA and mNERIFA was prepared from *B. abortus* S19 vaccine strain by the hot phenol method described by OIE [10]. Ring test antigen was used for the selection of the milk samples to be tested with mNERIFA.

Pooling Samples

The pooling process was performed with 2 to 5 combinations of positive and negative wheys to determine how the pooling process visually affected the test results. For this purpose, milk wheys prepared from negative and positive references were pooled with the combination of 2, 3, 4, and 5 samples, and compared for background development and visual efficiency.

Complement Fixation Test (CFT)

Sera from brucellosis-free dairy cows used in the study as reference standard was confirmed by CFT and it was performed according to the method described by OIE [7].

Indirect Enzyme-Linked Immunosorbent Assay (iELISA)

The iELISA procedure was carried out following the method [26] for testing the wheys with some modifications. Briefly, microplates were coated with *B. abortus* crude LPS antigen prepared in carbonate buffer (0.1 M, pH 9.6) and kept overnight at +4°C. The microwells blocked with 1% fish gelatine in phosphate-buffered saline (PBS) containing 0.2% Tween 20 (FG-PBST) were incubated at 37°C for 1 h. After washing, wheys and reference sera were diluted 1:50 and 1:100, respectively, in FG-PBST and transferred to microwells. Then, conjugate (alkaline phosphatase conjugated sheep anti-bovine IgG, Novus Biologicals, NB776) diluted 1: 4 000 were added to wells and incubated for 1 h at 37°C. After pNPP (p-Nitrophenyl Phosphate, Amresco), the plate was left 1 h at 37°C for the reaction development. The absorbance was read at 405 nm in ELISA reader (Multiskan EX, Fisher Scientific, Shanghai) after stopping with 1 N NaOH. The assay was carried out in duplicate and results were assessed by ROC analysis.

Milk Non-Enzymatic Rapid Immunofiltration Assay (mNERIFA)

The NERIFA [18] was developed for the detection of anti-*Brucella* antibody was performed with milk wheys. Briefly, the test cassettes shown in *Fig. 2* was prepared to contain the control and test dots. Two kinds of material known as absorbant pad and laminated nitrocellulose membrane (Schleicher & Schuell BioScience GmbH, Dassel, Germany) were inserted into casette respectively and two sides of the membrane were dotted with control and the respective antigen. Each test contained bovine IgG (1 mg/mL, Sigma-Aldrich) as control and crude LPS (1:200) as the test antigen. In this test procedure, laminated nitrocellulose membrane was firstly regenerated with a blocking solution (1% PBST/FG). Then, wheys generated from milk were added to the cassette after 1:2 dilution in blocking solution. Following flow-through it was washed 3 times with the washing solution. Then, the progression was maintained by CG- protein G conjugate (gifted, Abcam ab270696). One minute later, the stopping solution was added and the results were interpreted by comparing to the control side and checking with respective control sera of 2 and 20 CFTU/mL by their color intensity. In negative sample, the interpretation of the result was carried out as only control dot visible, while in positive sample two dots were visible. The cassettes presenting the test results were shown in *Fig. 2* with representative samples. A total of 27 positive and 60 negative wheys both individually and pooled were tested by mNERIFA and the results were evaluated by eye inspection.

Determining the Limit of Detection

The detection limit of mNERIFA was defined by OIE reference serum containing 1000 CFTU/mL. Two-fold dilutions of the reference serum from 8 U to 0.125 U was prepared for determining the limit of detection in mNERIFA.

Statistical Analysis

The accuracy of the ELISA was determined by ROC analysis and optimal cut-off selection was performed with Youden Index by MedCalc statistic [27]. The diagnostic performance of mNERIFA and the degree of agreement between ELISA and mNERIFA was estimated by kappa statistic with 95% CI using Medcalc. Statistical differences between individual and pooled whey samples in mNERIFA were determined by P-value and a P-value less than 0.05 was considered statistically significant. The significance level of ROC analysis (AUC values) was evaluated ($P<0.001$) as important.

Results

The ELISA results obtained from control standards and reference serum were optimized and evaluated for diagnostic performance and accuracy. An optimal cut-off value was selected based on the Youden index (J) score (0.963, cut-off>0.45) and evaluated by ROC analysis *(Fig. 1)*. Based on the cut-off value, sensitivity and specificity of the test were determined as 96.3% (CI 95%, 81-99.9%), 100% (CI 95%, 94-100%), respectively. As the accuracy of the test being 0.996, the ELISA was accepted to be accurate ($P<0.001$) for testing of the wheys for mNERIFA *(Table 1)*.

mNERIFA was performed with reference wheys and compared to ELISA by Medcalc statistics based on

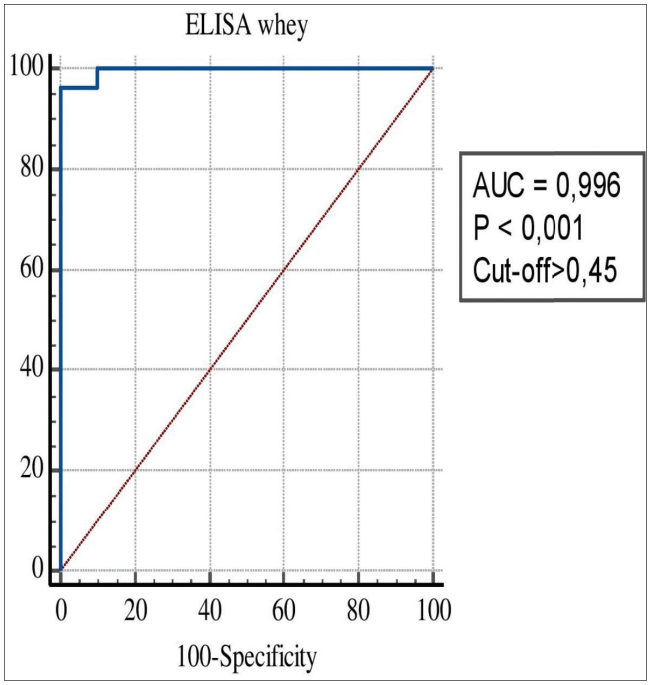

Fig 1. ROC analysis results of ELISA. Cut-off>0.45, Youden Index (J): 0.963, sensitivity: 96.3%, specificity: 100%

Table 1. *Diagnostic performances of ELISA and mNERIFA relative to control reference sera*

Diagnostic Criteria	ELISA Positive (n=26) Negative (n=59)	mNERIFA Positive (n=26) Negative (n=58)
Sensitivity	96.3% (81.03-99.91%)	96.3% (81.03-99.91%)
Specificity	98.3% (91.06-99.96%)	96.7% (88.47-99.59%)
Accuracy	97.7% (91.94-99.72%)	96.5% (90.25-99.28%)

diagnostic criteria. These results indicated that ELISA's performance was in perfect agreement with mNERIFA (κ=0.97) and the overall proportion agreement was 0.97% (CI 95%, 0.92-0.99%). Sensitivity, specificity and accuracy results were 96.3% (CI 95%, 81.3- 99.91%), 96.7% (CI 95%, 88.47-99.59%) and 96.5% (CI 95%, 90.25-99.28%) respectively *(Table 1)*.

mNERIFA results were also investigated by pooling up to 5 samples and some representative results are shown in *Fig. 2*. No difference was observed in terms of both individual and pooled samples with mNERIFA (P>0.05).

The detection limit of the mNERIFA was determined as 2 CFTU/mL and it was accepted as the cut-off level in the test. In addition, different reference sera were evaluated based on OD values of the ELISA compared with OIE reference serum quantitatively, and accordingly 20 CFTU was selected as the optimal low OD sera unit. Therefore, 2 CFTU/mL and 20 CFTU/mL sera as cut-off and low concentration control sera were included respectively in the panel of mNERIFA testing.

Fig 2. mNERIFA results with representable whey samples. mNERIFA results are visualized representatively. The first line numbered 1,2 and 3 show negative, 2, and 20 CFTU antibody results, respectively. The second line illustrates pooling negative results (no 4, 5 and 6) and 3rd and the bottom line show pooling (7,8,9) and individual positive sera (10,11,12) of different OD values selected respectively

Discussion

Various rapid serological tests have been developed to accurately determine the test performance based on sensitivity, specificity, sensitivity + specificity (performance index), accuracy in milk [8,9,28,29]. Most of these rapid tests are antibody detection tests such as lateral flow assays (LFAs) and agglutination-based MRT [13,20,21,30]. Different Lateral Flow tests and ELISAs have been developed and improved considerably for the detection of anti-*B. abortus* antibodies in milk [5,11,30,31]. In ELISAs, anti-immunoglobulin isotype enzyme conjugates are generally used for the detection of anti-species immunoglobulins against reactive antigens [5,9,23,29]. However, Protein G's affinity for IgG subclasses instead of detecting only one isotype antibody has replaced anti-immunoglobulin antibodies in these tests. Therefore, peroxidase and alkaline phosphatase-labeled Protein Gs substituted for enzyme-labeled secondary antibodies in ELISA, LFA, and ERIFA [17,31,32] and different LF tests and ELISAs for the detection of anti-*B. abortus* antibodies in milk have been developed and improved considerably [18]. Although the same mechanism works with NERIFA, only LFA is used for bovine brucellosis detection in milk. In this study, we hypothesized that NERIFA, which is also compatible with bovine, ovine, and

human brucellosis detection [18,19], may be an alternative test for milk screening. Specific antibodies are detected by the binding of the protein G/gold conjugate (protein G/GC) to the reacting *Brucella* LPS in lieu of secondary antibodies on laminated nitrocellulose membrane in mNERIFA. Due to the clogging of the membrane by milk and fat globules, an ELISA was optimized in which a whey-dependent assessment could be performed. The ELISA results were determined by its performance by ROC analysis. ELISA was found to be significant (P<0.001) depending on the results of the diagnostic index (J) score specified as the Youden index (J: 0.963) and the Area Under the Curve (AUC) value (0.996) for testing whey samples and comparing mNERIFA *(Fig. 1)* in the study.

mNERIFA was performed with the reference wheys and compared to ELISA based on diagnostic performances by Medcalc statistics. Wheys were prepared from control and OIE reference sera after being treated with rennin and a total of 87 including 27 positive and 60 negative wheys were tested for mNERIFA evaluation. Based on diagnostic properties; sensitivity, specificity and accuracy were found 96.3% (81.3-99.91%), 96.7% (88.47-99.59%) and 96.5% (90.25-99.28%), by mNERIFA respectively. These results are concordant with the ELISA and lateral flow assays but higher than MRT [21,33]. Positive sera used in the study were from the aborted cattle and *B. abortus* biotype 3 was the etiological agent in all events. Therefore, it was evaluated as gold standard sera with the negative sera which were from *Brucella*-negative herd. Although all positive sera were from gold standards, only one in 27 was found to be negative in both ELISA and CFT, it was evaluated as positive and accepted as false negative in ELISA and mNERIFA. Since the sensitivity of serological tests based on bacterial detection is low [4,14,29], that resulted in a decrease from 100% to 96.6% in sensitivity in the study. Although the decrease in the sensitivity of the test, these results indicated that diagnostic performances of the mNERIFA were compatible with those studies [29-32] for the milk testing. We may suggest this prototype alternative testing of brucellosis in milk samples considering the diagnostic performances. Protein G has a strong binding activity to bovine IgG and its isotypes, but its affinity to IgM and IgA in milk are not cited [15,32]. Therefore, detection of antibodies other than IgG in milk may be a disadvantage for milk testing with mNERIA.

The detection limit and weak positive antibody unit detected by quantitative ELISA (data not shown) have been accepted as the criteria for evaluating mNERIFA. In the study, we have shown that 2 and 20 CFTU/mL sera would be necessary for the evaluation of the test results *(Fig. 2)*. We, therefore, suggest these control sera should be included in each test panel.

In order to investigate pooling effect into test performance, wheys were pooled with 2 through 5 positives and negatives. There was no difference (P>0.05) between the individual and pooled whey results.

According to the data obtained in the study, our results show that mNERIFA may be an alternative test for anti-*Brucella* antibody detection in milk samples. Although the whey preparation process is a disadvantage in mNERIFA, its diagnostic performances, rapidity, evaluation in 5 min, pooling capacity with 5 samples make mNERIFA an alternative for rapid milk test. Although this study was simulated in the lab from wheys generated from the control sera, large-scale field studies are required where mNERIFA can be standardized before being adapted for field use.

In conclusion, based on the diagnostic performances and applicability presented here mNERIFA may be recommended as an alternative serological test for bovine brucellosis milk testing.

Availability of Data and Materials

Data sets analyzed during the current study are available from the corresponding author (O. Genç) on reasonable request.

Acknowledgements

The authors sincerely thank F. Ferrajoli, S. Iyisan, N. Yurdusev, S. Arslan, Ö. Çelebi and Samsun Veterinary Control Institute for kindly providing monoclonal anti-LPS antibody (Clone 4B5A), *B. abortus* S19 vaccine strain, protein G gold conjugate, statistical analysis and providing of positive and negative gold sera, respectively.

Funding Support

This study was partly funded by the Scientific Research Council of Ondokuz Mayıs University in Samsun, Türkiye (Project number PYO.VET.1905.13.001).

Ethical Statement

Ondokuz Mayıs University Animal Experiments Local Ethics Committee provided an ethics report for this study (E-68489742-604.02.03-255675).

Competing Interests

The authors declared that there is no conflict of interest.

Authors' Contributions

EG, GS and YK were ranked based on the contribution rates for performing lab studies and literature search and the corrections. EG and GS contributed equally to this work. The determination of the subject and the writing stages were planned and carried out by the corresponding author OG. All authors have contributed to the revision and final proof-reading of the manuscript.

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