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RESEARCH ARTICLE

Development of Lateral Flow Test for Serological Diagnosis of Tularemia [1]

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Abstract: Tularemia is a highly contagious zoonotic infection caused by *Francisella tularensis*. Bacterial culture, serology and molecular methods are used in the diagnosis of tularemia. The agent is a dangerous pathogen, and the importance of serological tests in diagnosis has increased because of the difficulty in culturing the organism. In this study, a practical, fast and reliable lateral flow-based immunochromatographic test was planned to detect *F. tularensis* specific antibodies in the field. Partially purified lipopolysaccharide antigen obtained from the live vaccine strain of *F. tularensis* was used as antigen. To determine the sensitivity of the test, 17 true positive tularemia serum samples with known Microagglutination test results, and to determine of specificity of the test, 30 true negative serum samples were used. In this study, *Brucella*-positive patient sera of various titers from our laboratory's serum bank to determine possible cross-reactivity with *Brucella* antibodies were also tested. The sensitivity and specificity of the Lateral Flow Test (LFT) rapid diagnostic kit were evaluated in comparison to the standard microagglutination test. The sensitivity and specificity of the newly developed Lateral Flow Test (LFT) were found at 100% and 93.5%, respectively. LFT for tularemia revealed 5% cross-reaction with positive sera for brucellosis. Cross-reactions were observed at antibody titers of 1:20 and below. In conclusion, it was concluded that the newly developed lateral flow test is a fast, reliable, and practical alternative test for the serological diagnosis of tularemia and cross-reaction in the serological tests conducted for brucellosis and tularemia should always be considered.

Keywords: *Francisella tularensis, Lateral Flow Test, Serology*

Tulareminin Serolojik Teşhisi için Lateral Flow Testinin Geliştirilmesi

Öz: Tularemi, *Francisella tularensis* tarafından oluşturulan son derece bulaşıcı, infeksiyöz zoonotik bir hastalıktır. Tulareminin teşhisinde bakteriyel kültür, seroloji ve moleküler metodlar kullanılmaktadır. Etken tehlikeli bir patojen olup, kültürünün yapılmasında karşılaşılan zorluklardan dolayı teşhiste serolojik testlerin önemi artmıştır. Bu çalışmada *F. tularensis* spesifik antikorları saptamak için sahada uygulaması kolay, güvenli hızlı sonuç veren lateral akış temelli bir immunokromatografik test geliştirilmesi amaçlandı. Antijen olarak *F. tularensis* canlı aşı suşundan elde edilen kısmen purifiye lipopolisakkarit antijeni kullanıldı. Testin duyarlılığının saptanmasında Mikroaglutinasyon testi sonuçları belli olan tularemi yönünden 17 gerçek pozitif ve özgüllüğünün saptanmasında 30 gerçek negatif serum örnekleri kullanıldı. Çalışmada ayrıca *Brucella* antikorları ile olası bir çapraz reaksiyonu değerlendirmek için laboratuvarımız serum bankasında bulunan çeşitli titrelerdeki Brusella pozitif hasta serumları da test edildi. Lateral Flow Testi (LFT) hızlı tanı kitinin sensitivite ve spesifitesi standart olarak kabul edilen mikroaglütinasyon testi ile karşılaştırmalı olarak değerlendirildi. Yeni geliştirilen testin duyarlılığı ve özgüllüğü sırasıyla %100 ve %93,5 olarak bulunmuştur. Tularemi için geliştirilen testin bruselloz yönünden olası bir çapraz reaksiyonunun değerlendirilmesi amacı ile yapılan testlerde, bruselloz ile %5 oranında çapraz reaksiyon saptanmıştır. Çapraz reaksiyonlar 1:20 ve altındaki antikor titrelerinde gözlemlenmiştir. Sonuçta geliştirilen lateral flow test prototipinin hastalığın tanısında güven ile kullanılabilecek, hızlı ve pratik bir serolojik test alternatifi olduğuna ve bruselloz ve tulareminin serolojik testlerinde bu hastalıklar için çapraz reaksiyonun olabileceğinin daima göz önünde bulundurulması gerektiği sonucuna varılmıştır.

Anahtar sözcükler: *Francisella tularensis, Lateral Flow Test, Seroloji*

INTRODUCTION

Although the route of transmission of tularemia caused by *Francisella tularensis* to humans is mai nly via rabbit-like animals and rodents, it can occur in many other direct or indirect ways besides insects such as ticks and flies.

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Effective treatment of tularemia cases depends on the actual diagnosis. This situation shows the importance of early diagnosis of the disease.

Culture of bacteria, serologic diagnosis (microagglutination test (MAT), Enzyme-Linked ImmunoSorbent Assay (ELISA) and molecular methods can be used successfully to diagnose tularemia [1]. However, culture requires a high-security laboratory (Biosafety Level 3) and experienced personnel [2]. Antibodies against *F. tularensis* can be detected by agglutination and the ELISA [3-6]. The tube agglutination test (TA) has been used to detect antibodies to the pathogen for many years [3,4,7]. However, since the 1970s, the microagglutination test (MAT) has been used because the TA test is time-consuming, is not suitable for testing many specimens, and requires an excessive amount of antigen [8-10]. The antigen used in MAT is usually produced in-house by laboratories and there is no national or international standardization. There is also no standard for the dyes used to detect the MAT test antigen and reaction better. It has been reported that the ELISA in which lipopolysaccharide derived from *F. tularensis* as antigen is ten times more sensitive than the tube agglutination test for the diagnosis of tularemia in humans [11].

Rapid diagnostic tests have successfully saved time in human and veterinary medicine diagnoses. The history of biosensors began in 1962 with the development of enzyme electrodes by scientist Clark and Lyons [12]. These rapid diagnostic tests are widely used and further developed in agricultural production, food processing, environmental monitoring, clinical diagnostics, drug testing, biotechnology, and the determination of biological and chemical warfare agents [13]. The researchers note that the Lateral Flow Test (LFT) method is superior to other serological tests because it provides results in a short time, is easy to perform, interpretable and sensitive [14].

Lateral Flow Test can be described by some procedures that include precise interaction of Abs and Ags. LFT system consists of four main components: the sample pad that carries the analyte to the absorbent pad, the conjugate pad on which the tagged analytes are attached to the biocomponents, the Nitrocellulose membrane that contains the control and test line, and the absorbent pad that stores the waste. The principle of an LFT is based on the movement of a liquid sample through a polymer strip with attached molecules that interact with the analyte to provide a signal that can be detected visually [15]. The most critical elements of the assay are the antibodies and the membrane, but all other materials used should also be considered to ensure compatibility and consistency of the product. An LFT is a rapid, inexpensive, portable, and user-friendly assay. However, the results are mostly qualitative (on/off) or semi-quantitative [16].

This study aims to develop a rapid diagnostic test based on immunochromatography using the LPS antigen for the detection of antibodies to tularemia.

Material and Methods

Ethical Statement

Approval was received from Harran University Animal Experiments Local Ethics Committee (HU/HADYEK: 2018/003/02).

Reference Bacterial Strain

The *F. tularensis* LVS vaccine strain (NCTC 10857) was obtained from Prof. Dr. Aynur Karadenizli at Kocaeli University. The supplied strain was grown on Cystine Heart Agar (Difco) containing 2% hemoglobin solution (Oxoid) in a 5% CO_2 environment. Homemade standardized MAT antigen from previous study was used.

Positive and Negative Control Sera

Standard sera from tularemia positive individuals with known (MAT) results were obtained from the Turkish Health Authority, the National Tularemia Reference Laboratory and the Kocaeli University Department of Medical Microbiology. The reference titers MAT of these sera were 1:20, 1:80, 1:160, 1:640 and 1:1280. These sera were used as positive control sera for MAT, ELISA and LFT during the project. To calculate the specificity and sensitivity of the LFT prototype produced in the study, 17 tularemia antibody positive and 30 negative human serum samples from the HÜBAP (Harran University Experimental Research Project No: 18072) project were used. This study tested *Brucella* positive patient sera of various titers from our laboratory's serum bank with LFT to evaluate possible cross-reactivity with *Brucella* antibodies.

In the serological tests used in the study, the reference and test sera were tested ten times. The means and standard deviations of the optical densities (OD) detected by ELISA were determined. The sera from patients diagnosed with brucellosis were also tested ten times by MAT, ELISA, and LFT to evaluate cross-reactivity with *Brucella*. The mean and standard deviation of the OD values obtained by ELISA were determined *(Table 1)*.

ELISA

Partially purified LPS layer by Trizol treatment was used as the solid-phase antigen in ELISA [17]. After checkerboard analysis of the isolated antigen with positive and negative sera, the most appropriate antigen dilution was prepared with carbonate bicarbonate buffer (pH 9.6) and 100 µL of the antigen dilution determined by checkerboard analysis was added to each well of the 96-well plate (NUNC, 269620, Denmark). After washing

Table 1. *Test results of MAT, LFT and, ELISA with reference MAT antigen*

Serum Titers Tested with the Reference MAT Antigen	In House MAT	In House ELISA Mean OD±Standard Deviation	LFT
F. tularensis MAT 1:20	1:20	Positive (0.546±0.111)	Negative
F. tularensis MAT 1:80	1:80	Positive (0.747±0.094)	Positive
F. tularensis MAT 1:160	1:160	Positive (1.346±0.089)	Positive
F. tularensis MAT 1:640	1:640	Positive (2.47±0.091)	Positive
F. tularensis MAT 1:1280	1:1280	Positive (3.09±0.102)	Positive
F. tularensis MAT Negative	Negative	Positive (0.145±0.061)	Positive
Brucella positive serum 1:80 Reference MAT	Negative	Positive (0.190±0.087)	Negative
Brucella positive SAT 1:320 Reference MAT	1:10	Negative (0.201±0.08)	Negative
Brucella positive SAT 1:1280 Reference MAT	1:20	Negative (0.225±0.072)	Negative

MAT: *Microagglutination test,* ***SAT:*** *Serum agglutination test*

and blocking, the positive and negative sera were diluted at 1:100 and added. After washing, recombinant A/G conjugate (Pierce 32490) labeled with HRPO was added. After washing, 100 µL of a chromogenic substrate (2 µg ortho-phenylenediamine and 0.03% H_2O_2 in 0.1 M citrate buffer (pH: 5.5) was added. After the plates were kept at room temperature for 10 to 15 min, 100 µL of 4 N H_2SO_4 was added to each well to stop the reaction and the absorbance values of the plates were read at 490 nm using an automated ELISA reader (VERSAmax 3.13/B2573). The mean of the negative serum OD's plus three standard deviations (SD) was determined as the ELISA cutoff value [18].

Lateral Flow Test Strips

In the preparing test strips for the diagnosis of tularemia, LPS antigen was obtained from the *F. tularensis* strain and prepared according to the method Eugene and Hackett [17] used for the test line. Strips prepared from all inactive bacterial solutions and LPS antigen were used for comparison.

Preparation of Colloidal Gold

Five mL of a 1% Hydrogen tetrachloroaurate trihydrate stock solution was mixed with 500 mL of distilled water and heated to boiling. Then 5 mL of a 1% sodium citrate solution was added to the gold solution and boiled until the color turned red. After boiling for another 5 min, the solution was ready and was stored in a black bottle at +4°C until use [19].

Preparation of Colloidal Gold Probes

After determining the optimal concentration, protein A/G diluted in the indicated ratio was added to the pH-adjusted colloidal gold solution, mixed and incubated at room temperature for 25 min. Then, a 10% (w/v) Bovine serum albumin (BSA)(Sigma) solution was added at a ratio of 1/10 and kept at room temperature for 10 to 15 min. The mixture was centrifuged at 15000 g and +4°C for 30 min. After centrifugation, the pellet was suspended in PBS and used as conjugate [20].

Preparation of Immunochromatographic Test Strips

Francisella tularensis LPS and purified polyclonal human IgG (Merck, Germany) were applied to a nitrocellulose membrane using a lateral flow dispenser as test and control, respectively and dried at 37°C for 2 h. The prepared colloidal gold probes were sprayed onto the glass fiber membrane using a lateral flow dispenser and dried entirely at 37°C. After the pads were dried, they were combined and cut using a cutter. The resulting strips were placed in plastic cassettes [20].

Measurement of Specificity and Sensitivity of Test Strips

Positive and negative reference serum panels were used to measure the sensitivity and specificity of the test. The sensitivity and specificity of the LFT rapid diagnostic kit were evaluated in comparison to the MAT test, which is the accepted standard. For this purpose, the following formula was used.

Sensitivity = *Francisella* Positive Sera/*Francisella* Positive Sera+ False Negative

Specificity = *Francisella* Negative Sera/*Francisella* Negative Sera+ False Positive

Results

Each reference and test sera were tested ten times to evaluate the reliability and reproducibility of the serological tests used in the study. The means and standard deviations of the optical densities determined by ELISA were calculated. Sera from patients positive for brucellosis were tested ten times by MAT, ELISA and LFT to evaluate possible cross-reactivity with *Brucella*. The mean and standard deviation of the OD values obtained by ELISA were determined. All the results in the present tables were prepared considering the values obtained from these

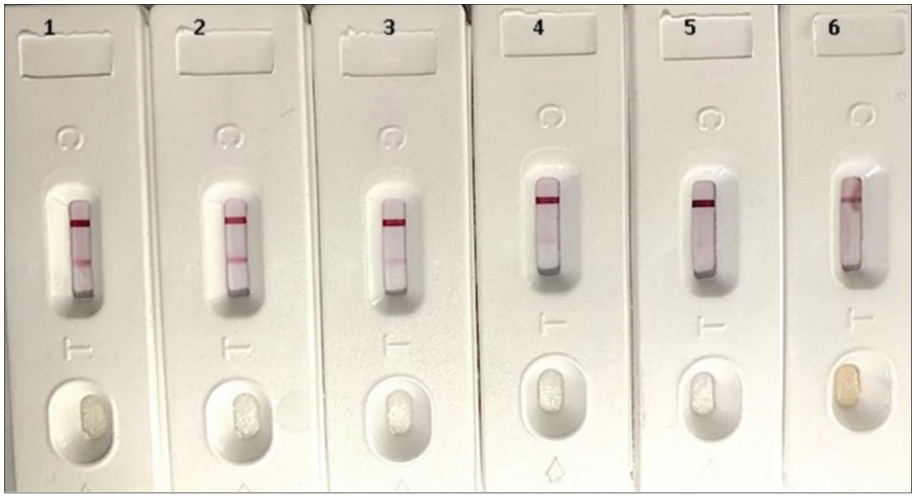
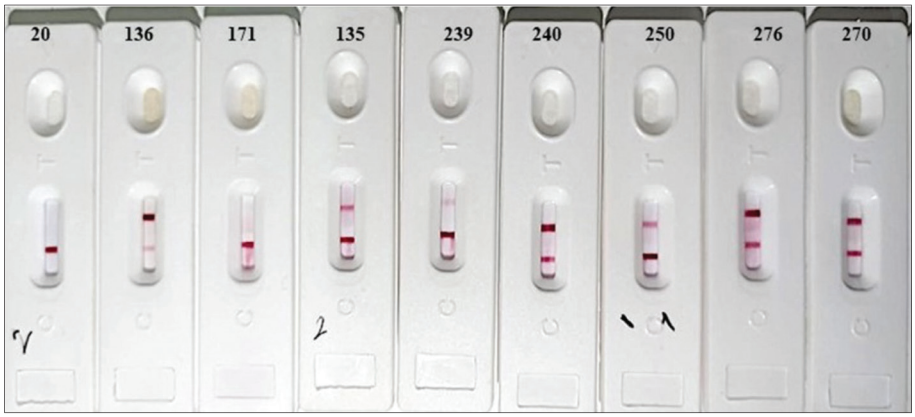


Fig 1. LFT results of sera tested with Reference *F. tularensis* MAT antigen and Reference *B. abortus* SAT antigen. 1. MAT 1:1280 2. MAT 1:640 3. MAT 1:160 4. MAT: 1:80 5. MAT 1:20 6. Negative serum

Fig 2. LFT results of *Francisella* antibody positive MAT sera



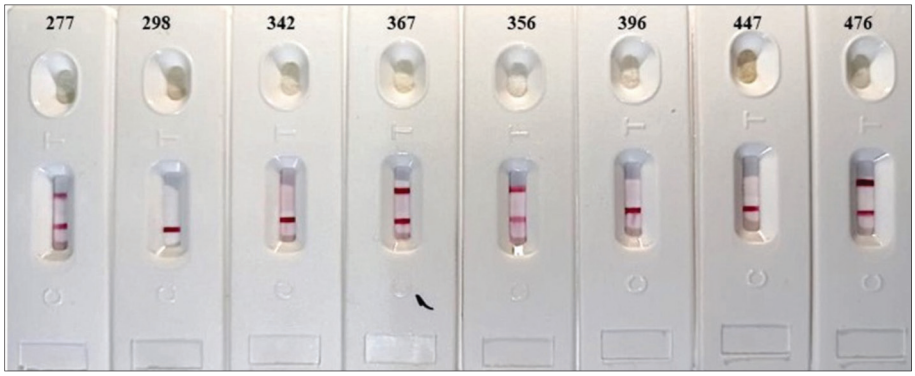


Fig 3. LFT results of *Francisella* antibody positive MAT sera

averages. When the positive reference sera MAT were tested with the LFT developed at the end of the study, all positive sera gave positive results with the LFT, except for the MAT 1:20 positive serum *(Fig 1)*.

Positive reference sera for *F. tularensis* and positive sera for brucellosis in various titers were tested with the LFT developed in the study MAT and ELISA. The results are shown in *Table 1*. It was found that all tests gave the same results when the positivity criterion for tularemia was assumed to be 1:20 and higher. However, while ELISA and MAT could detect a titer as low as 1:20, LFT could not detect this titer *(Table 1)*.

To calculate the specificity and sensitivity of the LFT prototype, 17 positive and 30 negative human serum samples, also used in the previous HÜBAP material (18072), were tested for tularemia with LFT. The results are shown in *Fig. 2* and *Fig. 3*.

A comparison of these sera with the results from MAT is shown in *(Table 2)*. Accordingly, the sensitivity of LFT was 100%, while the specificity was calculated to be 93.5%. In our study, the intensity of the LFT test line in sera 136 and 476 with a titer of 1:1280 was observed quite intensively with MAT and the results showed complete agreement with MAT *(Table 2)*.

Table 2. *LFT test results of F. tularensis positive MAT positive sera*

***F. tularensis* Positive Serum No**	**LFT Result**	**MAT Titer**
20	Negative	1:20
136	Positive	1:1280
171	Negative	1:20
135	Positive	1:320
239	Positive	1:80
240	Positive	1:640
250	Positive	1:160
276	Positive	1:640
270	Positive	1:640
277	Positive	1:320
298	Negative	1:20
342	Negative	1:20
367	Positive	1:640
356	Positive	1:320
396	Negative	1:20
447	Negative	1:20
476	Positive	1:1280
30 Tularemia negative serum (1:20 and above negative)	29 Negative	30 Negative
Total=17 positives (1:20 and above positive)	----- 11 Positive	17 Positive 11 Positive
F. tularensis LFT sensitivity	100%	100%
F. tularensis LFT specificity	93.5%	100%

Table 3. *Results of Brucella positive sera with different titers by Francisella LFT*

Number of *Brucella* Positive Sera	***Francisella* LFT Negative**	***Francisella* LFT Positive**
40	38 (1:80-1:1280 arası)	2 (1:1280 ve 1:2560)
Result	95%	5%

In addition, to determine a possible cross-reaction with brucellosis, various titers of *Brucella* antibodies of positive human sera from the serum bank of our laboratory were tested by LFT. The result was that 38 of 40 positive sera were negative (95%), while two serum samples were positive (5%) *(Table 3)*.

Discussion

Francisella tularensis is a pathogen that causes severe lethal infections in humans and some mammals. Studies have shown that successful antibiotic therapy for tularemia depends on timely diagnosis [21]. Because of this, an early and reliable diagnosis of the disease is essential. Diagnosis of the disease is based mainly on serological tests, as the pathogen is a dangerous and highly contagious microorganism [22]. However, there are no standardized commercial antigens and test kits that can be used for the serological diagnosis of the endemic diseases in Turkey. Therefore, the LFT prototype with LPS antigen was developed in the study.

It is known that ELISA and MAT are susceptible tests for diagnosing the disease [1,2,11,23]. A recent study conducted in our country showed that seropositivity rates for 72 human sera were found as 4.2% for ELISA and MAT. Seropositivity for 190 serum samples from sheep were found as 3.2% for MAT and 4.7% for ELISA. These figures show that tularemia cannot be considered as an insignificant disease in human and sheep. Therefore, rapid tests can be important in diagnosis and epidemiology of the disease [24].

This study, obtained positive results with the LFT from all positive sera except the MAT 1:20 positive serum. Because a single serum sample with a titer of 1:160 or greater is considered positive in the final diagnostic criteria for the disease [2], it is believed that failure to detect a titer of 1:20 in the LFT is not thought to reduce the sensitivity of the test. Titers of 1:10 to 1:80 are already widely associated with cross-reactivity [10].

In this study, the sensitivity of the LFT was found to be 100%, whereas the specificity was calculated to be 93.5%. Splettstoesser et al.[25] in their study on the development of the LFT, determined a sensitivity of the test of 98.3% and a specificity of 96.5% and reported that this test is a reliable test for detecting the disease in the field. Kilic et al.[6] have used a commercial LFT kit and found that the method had a sensitivity of 99.3% and a specificity of 94.6%. The values for sensitivity and specificity obtained in our study using the LFT method show significant similarity with the results obtained by all these researchers.

In the study, brucellosis positive human sera of different titers from the serum bank of our laboratory were also tested with LFT to evaluate possible cross-reactivity with brucellosis. The result was that 38 of 40 positive sera were negative (95%), whereas two serum samples were positive (5%) *(Table 3)*. Kilic et al.[6]. reported positive results in 5 of 50 brucellosis positive sera using the LFT kit. Cross-reactivity was observed in brucellosis and tularemia, albeit at low levels. It is known that the similarity of LPS in the cell wall structure of these two pathogens is responsible for these cross-reactions [22]. These findings are consistent with the results of our study and the possibility of cross-reactivity between these two diseases should always be considered in serological testing for brucellosis and tularemia.

It is concluded that the LFT is a valuable serologic test that can be used to diagnose of tularemia. It is safe, practical and provides rapid results. It is expected that using this test in the field will contribute to the control of disease and the timely measures to be taken.

Availability of Data and Materials

The authors declare that data supporting the study findings are also available to the corresponding author (O.Y. Tel).

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Conflict of Interest

The authors declared that there is no conflict of interest.

Author's Contributions

Experimental design and correction of the manuscript; OYT, data collection, lab work, first draft of the manuscript; SEG, AGY, OK, AK fieldwork; OK, SEG, AGY, OYT. All authors have read and agreed to the published version of the manuscript.

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