Iran J Public Health, Vol. 44, No.8, Aug 2015, pp.1114-1120

Original Article

Rocket and Two Dimensional Immunoelectrophoresis in Diagnosis of Caprine Brucellosis

Davood MEHRABANI [1], *Zahra GHOLAMI [2], Jamshid KOHANTEB [2], Masood SEPEHRIMANESH [3], Seyed Mohammad Hossein HOSSEINI [4]

1. *Stem Cell and Transgenic Technology Research Center, Dept. of Pathology, Shiraz University of Medical Sciences, Shiraz, Iran*
2. *Dept. of Bacteriology and Virology, School of Medicine, Shiraz University of Medical Sciences, Shiraz, Iran*
3. *Gasteroenterohepatology Research Center, Shiraz University of Medical Sciences, Shiraz, Iran*
4. *Razi Vaccine and Serum Research Institute, Shiraz, Iran*

***Corresponding Author:** Email: medvetbiochem93@gmail.com

(Received 14 Nov 2014; accepted 20 May 2015)

Abstract

Background: Brucellosis is a major bacterial zoonosis of global importance with the causative organisms of Gram-negative facultative intracellular pathogens. The aims of this study were to standardize two immunoelectrophoretic techniques, rocket and cross immunoelectrophoresis, and compare their results with other conventional serodiagnostic tests.

Methods: Sera from 15 sheep, without any history of brucellosis vaccination, infected with *Brucella melitensis* M16 subcutaneously, were employed in a comparison of culture, precipitating, and immunoelectrophoretic tests. A 125 days serologic follow-up was performed after the infection was started. As a reference, these tests also done in the five healthy sheep.

Results: The results obtained with the rocket immunoelectrophoresis test correlated very well with those of the cross immunoelectrophoresis, whereas results of other tests such as culture, Rose Bengal, standard tube agglutination and 2-mercaptoethanol seruagglutination tests were inferior.

Conclusion: As agglutination test shows cross reaction and a prozone phenomenon, and in blood culture, the bacteria is not always detectable, so they are time consuming rocket and cross immunoelectrophoresis are recommended because their results can be obtained in a shorter time.

Keywords: Brucellosis, Immunological diagnosis, Immunoelectrophoresis, Zoonosis, Agglutination

Introduction

Brucellosis is one of the most common zoonotic diseases that is endemic in rural areas of Mediterranean, Middle East and Latin American countries. This disease is caused by *Brucella* genus and many species of them are human pathogens (1). Brucellosis can be transmitted from animals to humans in many ways: ingestion of infected meat or unpasteurized dairy products, direct contact of broken skin or mucous membrane with infected animal tissues, and inhalation of infectious aero (2). Although, genitourinary complications such as epididymo-orchitis and prostatitis are seen in cases of human brucellosis, but, person to person transmission is still considered uncertain (3). *Brucella* organisms are Gram-negative facultative intracellular pathogens that may affect a range of different mammals, including man, cattle, sheep, goats, swine, rodents and marine mammals (4). Species infecting domestic livestock are *B. melitensis* (goats and sheep), *B. suis* (pigs), *B. abortus* (cattle and bison), *B. ovis* (sheep), and *B. canis* (dogs). In most host species, the disease primarily affects the repro-

ductive system with a concomitant loss in productivity of animals affected.
Ovine brucellosis is induced by *B. melitensis* along with *B. ovis* which can infect sheep, cattle, and sometimes humans (5). Infectious food-borne brucellosis usually result in humans when contaminated or unpasteurized milk and cheese products are consumed (6). Therefore, control of brucellosis in animals, and thus prevention of human disease, depends on utilizing efficient diagnostic procedures. The diagnosis of brucellosis is mainly based on the detection of antibodies directed to the O-chain component of the lipopolysaccharide (LPS) antigen, expressed at the surface in *Brucella* species with smooth phenotype (4). Although the LPS antigens play the most important role in agglutination tests, other antigenic fractions of *Brucella* may be involved in other tests (7). Different methods are used for diagnosis and screening of animal and human infected populations. The diagnosis of brucellosis made by the isolation of *Brucella* species in blood cultures (BC) is successful in only 6-40 to 70-92% of cases (1, 8). Following *Brucella* infection, IgM appear within one week, reach a peak within 3 months and remain elevated for weeks to months. Moreover, IgG antibodies appear within 3 weeks of infection, they reach a peak within 6 to 8 weeks and they remain present, albeit at low levels, for months to years after the recovery of patients (9). Thus, laboratory diagnosis of brucellosis very often relies on detecting specific serum antibodies. Specific IgG antibodies can be detected by using of Coombs test, 2-mercaptoethanol (2-ME) slide agglutination test (SAT) and enzyme linked immunosorbent assay (ELISA) (10).
In this study, we tried to standardize two immunoelectrophoretic techniques, rocket and cross immunoelectrophoresis, and compare the results obtained with those of BC, Rose Bengal, SAT, and 2-ME.

Materials and Methods

Serum samples

We used 20 sheep in two groups: Sera examined in this study were obtained, over a period of 125 days, from 15 female Mehraban sheep without any previous history of brucellosis vaccination which were infected with 1.5×10^8 CFU per sheep *B. melitensis* M16 (Razi Ins, Hesarak, Iran) subcutaneously. Control sera were selected from other five healthy sheep without brucellosis who were not suffering from other bacteriological or parasitic diseases.

Cytosolic antigen preparation

Lyophilized *B. melitensis* M16 was cultured in *Brucella* broth (Sigma-Aldrich, Germany) at 37 °C for 24 h. After recultivation of bacteria in blood agar and *Brucella* agar, the surface of agar was washed with normal saline, pH 7.2. The resulting suspension was centrifuged at 3500 rpm for 30 min and then washed three times with normal saline pH 7.2. The washed bacterial cells and deionized water were mixed at a 1:1 ratio and disintegrated by sequential cold and hot extraction for 10 cycles of five min in liquid nitrogen of -196°C and 10 min in a water bath of 60 °C. After centrifugation at 3500 rpm for 30 min, cytosolic antigens were obtained in the supernatant and cell wall antigens were in the precipitate. Total protein of supernatant was assayed according to the Lowry method with some modification using crystalline bovine serum albumin as a standard (11, 12).

Ouchterlony double immunodiffusion (ODI)

The test was performed in mesh acetate-agarose (1% agarose, 0.6% sodium acetate, 1.03% sodium barbiturate). In the central well apply 10 μL of soluble antigen of *B. melitensis* M16, and the two side wells 10 μL of serum from infected sheep. Slides were incubated in a humid chamber at room temperature (RT) for 12 h. After that, the slides were washed with normal saline, dried at 60°C for 36 h, stained with Amido Black in 7% acetic acid for 5-10 min and destained in 5% acetic acid for 12 h (13).

Rose Bengal test (RBT)

The test was performed according to the method described previously (14). Briefly, 30 μL of serum was dispensed on a glass plate with white ceramic background and mixed with an equal volume of RBT antigen (Razi Ins, Hesarak, Iran), previously equilibrated at RT and shaken to resuspend any

bacterial sediment, using a toothpick. The tile was then rocked at RT for 4 min, instead of the 8 minutes recommended for human brucellosis, and any visible agglutination was taken as a positive result.

Standard tube agglutination test (SAT)

The antigenic suspension was prepared from *B. abortus* strain and test was done according to the method reported previously (7). The SAT titers equal or above 1:40 are considered positive.

2-ME test

To determine the sensitivity of sera to 2-ME, 0.2 ml of serum was diluted in 0.3 ml normal saline and mixed with 0.5 ml of 0.2 M 2-ME, incubated at 37 °C for 1 h, and the usual tube agglutination test performed (7). The 2-ME titers equal or above 1:20 are considered positive.

Rocket immunoelectrophoresis (RIE)

In RIE, negatively charged antigen samples are electrophoresed in an agarose gel-containing antibody, which is specific to that antigen. Acetate-agarose (1% agarose, 0.6% sodium acetate, 1.03% sodium barbiturate) was prepared and boiled to dissolve properly. Once the agarose solution cooled to 52 °C, 450 μL of sheep sera was added to the gel. The agarose solution was poured onto grease free glass plate (25 by 75 mm) placed on a horizontal surface, immediately. After incubation in a humid chamber at 4 °C for 20 min, wells were created with the help of gel puncher and 12 μL of prepared cytosolic antigen were added to the each well. The glass plate with the gel was placed on a horizontal electrophoresis system contain 1X TBE buffer and electrophoresis was done at 15 volts for 4 h.

Dimensional (crossed) immunoelectrophoresis (2DIEP)

2-DIEP, also known as crossed immunoelectrophoresis (CIE), consists of two sequential electrophoretic steps and is a useful technique for the quantitation of one or more proteins in a complex mixture. We used 2-DIEP, based on the protocol described previously (15).

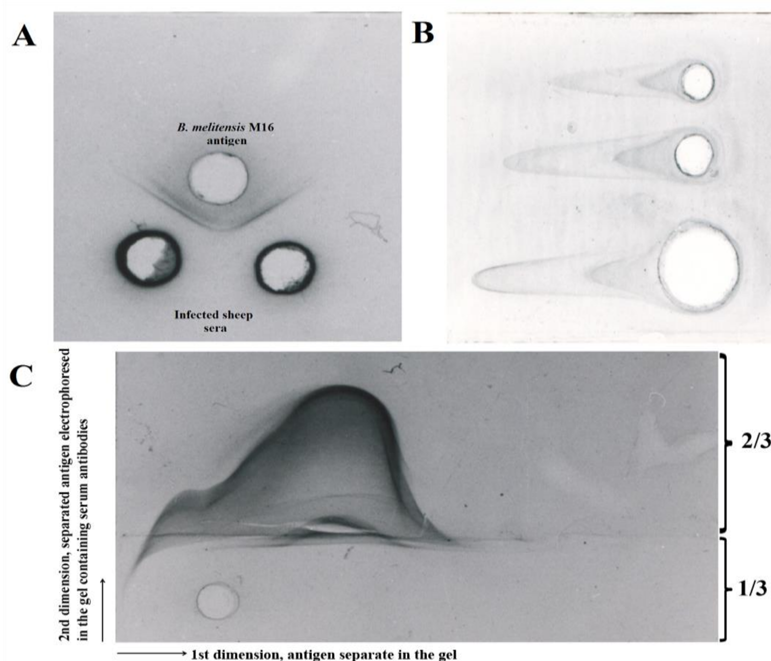

Fig.1: Precipitation line created in different immunological methods. A: The precipitation line in Ouchterlony double immunodiffusion (ODI) demonstrate the specificity of Ig(s) and antigens; The conical line in Rocket immunoelectrophoresis (B) and 2-dimensional immunoelectrophoresis (C) indicated the positive response

Results

The protein content of prepared cytosolic antigen was 2.3 $mg.ml^{-1}$. This supernatant was used as antigen source for other immunological methods. The shape of the precipitation line in ODI, RIE, and 2-DIEP is shown in Fig. 1 A, 1B, and 1C, respectively.

The precipitation line in ODI demonstrates the specificity of Ig (s) and antigens. On the other two tests, the precipitation lines demonstrate the positive response that getting started from days 6 and are finished at days 62. The percentage of positive samples detected in each method during the sampling days was compared by culture method and presented in Fig. 2. RBT could detect samples that are more positive in a long duration of time, until 111days post infection (DPI), and 2-ME, SAT, RIE, and 2-DIEP are in the next steps with at least positive samples detection in 83, 69, 62 and 62 DPI, respectively. On the other hand, RIE, 2-DIEP, SAT and 2ME had the highest positive sample detection between all derivate samples. The poorest technique in detecting brucellosis was culture with at least positive samples detection in 17 DPI. Furthermore, the results obtained with the RIE completely correlated with those of the 2-DIEP (Fig. 2).

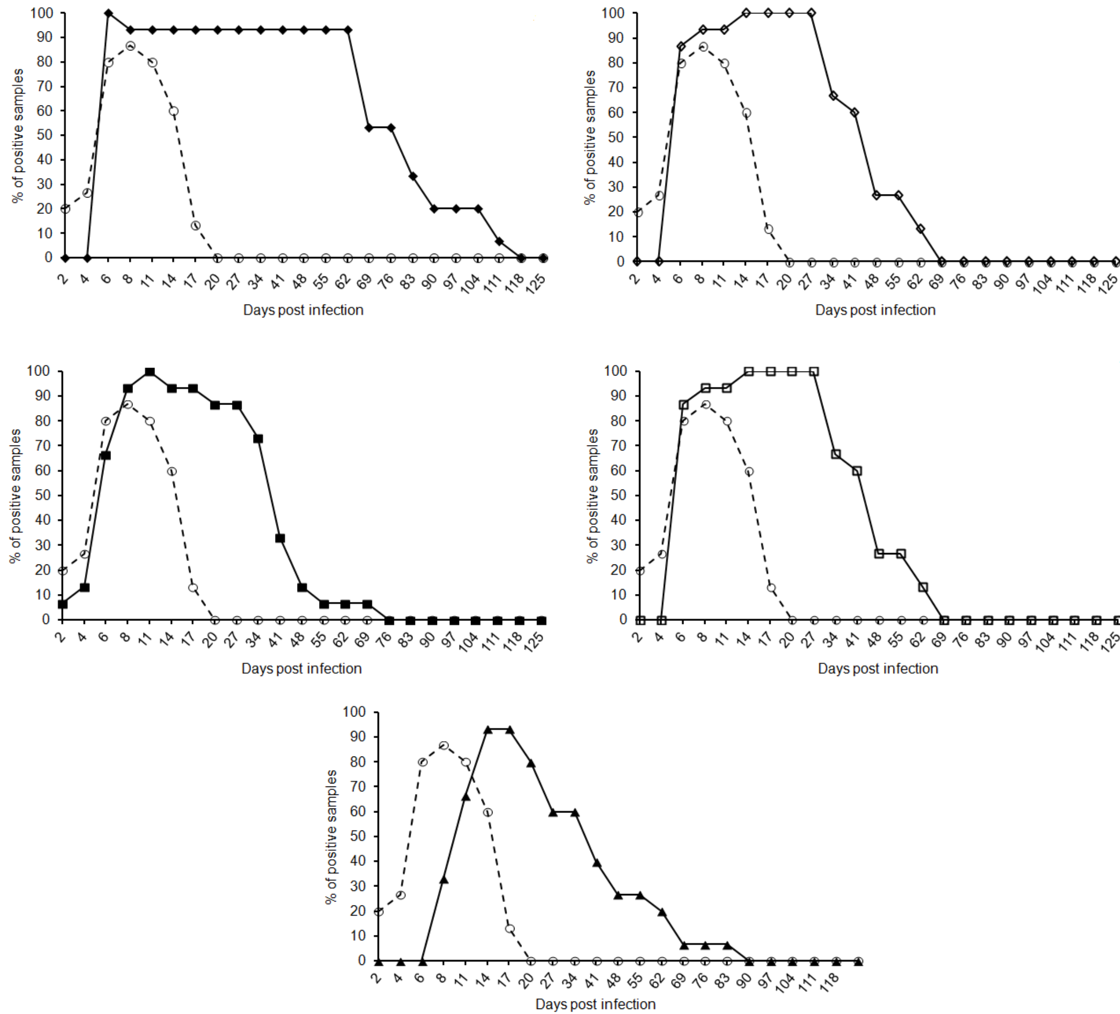

Fig. 2: Comparison of different serological tests for detection of caprine brucellosis induced by *B. melitensis* M16. (○) blood culture; (◆) Rose Bengal test; (■) Standard tube agglutination test; (▲) 2-mercaptoethanol test; (◇) Rocket immunoelectrophoresis test; (□) 2-dimensional immunoelectrophoresis test

Discussion

Along with tuberculosis and rabies, brucellosis is the most important bacterial zoonosis and with the exception of *B. ovis* and *B. neotomae*, all *Brucella* species can cause infections in humans (16). In this study, we evaluated and compared six routine methods, including BC, and five serological methods for diagnosis of brucellosis induced by *B. melitensis* M16 in experimentally infected sheep over a period of 125 days. Our finding demonstrated that RBT could detect samples that are more positive in a long duration of time in compare to all other tests BC is the poorest technique for Brucella diagnosis especially after passing a long time of initial infection.

Serological diagnosis of exposure to rough *Brucella* spp., such as *B. ovis*, *B. canis* or *B. abortus* is difficult owing to the absence of the immunodominant O-chain (O-PS) component of the lipopolysaccharide (LPS) antigen, which induces most of the antibodies detected by conventional serological tests for brucellosis (4). However, diagnosis of smooth phenotype of *Brucella* such as *B. melitensis* M16 is mainly based on the detection of antibodies directed to the O-PS, expressed on the surface of these microorganisms (17, 18). On the other hand, although PCR-based testing methods are fast and sensitive enough, but sensitivity, specificity and issues of quality control and quality assurance of the PCR assays vary between laboratories and no standardization of sample preparation, target genes and detection methods have been established yet (19). In addition, in patients with brucellosis, positive BC may be obtained in as low as 6% to as high as 92% of patients depending on a number of factors that include stage of the disease, prior antimicrobial therapy, the levels of *Brucella* titers, and the microbiological culture technique utilized (1). BC used in this study has a time limitation and only is detected *B. melitensis* in 2-17 DPI. This finding is in accordance with our previously unpublished studies that reported 3-13 DPI period for *B. melitensis* M16 identification in BC.

The speed, accuracy and simplicity of immunodiagnostic tests have led to the development of rapid techniques for the diagnosis of brucellosis. The serological tests include recombinant Omp31 based ELISA (rELISA), dot-ELISA, *B. melitensis* whole antigen based ELISA (pELISA), SAT, complement fixation test (CFT), RBPT and AGPT were used by Gupta and his colleagues for diagnosis of caprine brucellosis. Their founding demonstrated that rELISA and dot-ELISA combination is the best for detection of anti *B. melitensis* antibodies in goats (20). ELISA might be suitable for diagnosing *B. melitensis* infection in sheep, as it has a similar test performance compared to the RBT (21). Our finding is in agreement with these two reports and indicated the importance and usefulness of RBT in the diagnosis of caprine brucellosis. The SAT test, remains the most popular serological test used in the diagnosis of brucellosis, particularly in geographic regions that are endemic for the disease (22, 23). As well as, 2ME is cheap, easy to perform and can differentiate between IgM antibodies that appear during the acute stage and IgG antibodies that occur during chronic stage of brucellosis (22, 23). SAT can detect infection faster than 2ME (2 vs. 8 DPI, respectively) but the duration of positive detection in 2ME is longer than SAT (83 vs. 69 DPI, respectively).

On the other side of this study, we standardized and used two immunoelectrophoretic methods include RIE and 2DIEP in *B. melitensis* M16 serodiagnosis for the first time. There are no differences between these two immunoelec-trophoretic tests and the results obtained with these two tests are completely correlated. These two tests showed the highest sensitivity in detection of positive brucellosis samples about 18 days (17-34 DPI). Although there are no reports about using these two methods in brucellosis identification, but other researchers have emphasized on the importance of them in healthy and infected subjects (24-27). Based on our finding and features of these tests include quickly and easily performing, the use of them in *Brucella* diagnosis is highly recommended.

Conclusion

We standardized RIE and 2DIEP methods for identification of *B. melitensis M16* for the first time

and compare them with other conventional brucellosis diagnostic methods. In the absence of culture facilitates the diagnosis of brucellosis relies on agglutination tests. Despite having a plethora of serological tests for the diagnosis of brucellosis, none of these tests is 100% reliable or perfect. Therefore, serological test results should always be considered or interpreted in conjunction with patient history, clinical manifestations and other laboratory findings.

Ethical considerations

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

Acknowledgment

Authors are specifically grateful to different staff and sections of the Razi Vaccine and Serum Research Institute for assisting in this research. Moreover, this research was financially supported by a grant from Razi Vaccine and Serum Research Institute, Shiraz, Iran. The authors declare that there is no conflict of interests.

References

1. Al-Tawfiq JA, AbuKhamsin A (2009). A 24-year study of the epidemiology of human brucellosis in a health-care system in Eastern Saudi Arabia. *J Infect Public Health*, 2 (2): 81-5.
2. Vigeant P, Mendelson J, Miller MA (1995). Human to human transmission of *Brucella melitensis*. *Can J Infect Dis*, 6 (3): 153-5.
3. Mikolitch DJ, Boyce JM (1990). Brucella species. In: *Principles and Practice of Infectious Diseases*. Ed, GL Mandell, RG Douglas, JE Bennett. Churchill Livingstone, 3rd ed. New York, pp. 1735-42.
4. Adone R, Francia M, Ciuchini F (2008). *Brucella melitensis* B115-based complement fixation test to detect antibodies induced by Brucella rough strains. *J Appl Microbiol*, 105 (2): 567-74.
5. Nielsen K, Duncan JR (1990). *Animal brucellosis*. CRC press. USA.
6. Godfroid J, Cloeckaert A, Liautard J-P, Kohler S, Fretin D, Walravens K, Garin-Bastuji B, Letesson, J-J (2005). From the discovery of the Malta fever's agent to the discovery of a marine mammal reservoir, brucellosis has continuously been a re-emerging zoonosis. *Vet Res*, 36 (3): 313-26.
7. Diaz R, Maravi-Poma E, Rivero A (1976). Comparison of counter-immunoelectrophoresis with other serological tests in the diagnosis of human brucellosis. *Bull World Health Org*, 53 (4): 417-24.
8. Yagupsky P (1993). Detection of brucella in blood cultures. *J Clin Microbiol*, 37 (11): 3437-42.
9. Ozdemir M, Feyzioglu B, Kurtoglu MG, Dogan M, Dagi HT, Yuksekkaya S, Kesli R, Baysal B (2011). A comparison of immuncapture agglutination and ELISA methods in serological diagnosis of brucellosis. *Int J Med Sci*, 8 (5): 428-32.
10. Gomez MC, Nieto JA, Rosa C, Geijo P, Escribano MA, Munoz A, Lopez C (2008). Evaluation of seven tests for diagnosis of human brucellosis in an area where the disease is endemic. *Clin Vaccine Immunol*, 15 (6): 1031-3.
11. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951). Protein measurement with the Folin phenol reagent. *J Biol Chemistry*, 193 (1): 265-75.
12. Sepehrimanesh M, Aminlari M (2014). Arginase distribution in tissues of domestic avian species. *Comp Clin Pathol*, 23 (2): 353-6.
13. Bailey GS (1996). Ouchterlony double immunodiffusion. In: *The protein protocols handbook*. Ed, JM Walker. Springer, pp. 135.
14. Morgan WJ, Mackinnon DJ, Lawson JR, Cullen GA (1969). The rose bengal plate agglutination test in the diagnosis of brucellosis. *Vet Rec*, 85 (23): 636-41.
15. Walker JM (1996). Two-dimensional immunoelectrophoresis. In: *The protein protocols handbook*. Ed, JM Walker. Humana Press, pp. 763-8.
16. Spicic S, Zdelar-Tuk M, Racic I, Duvnjak S, Cvetnic Z (2010). Serological, bacteriological, and molecular diagnosis of brucellosis in domestic animals in Croatia. *Croat Med J*, 51 (4): 320-6.
17. Al Dahouk S, Nockler K, Scholz HC, Tomaso H, Bogumil R, Neubauer H (2006).

Immunoproteomic characterization of *Brucella abortus* 1119-3 preparations used for the serodiagnosis of *Brucella* infections. *J Immunol Meth*, 309 (1): 34-47.
18. Al Dahouk S, Tomaso H, Nockler K, Neubauer H, Frangoulidis D (2002). Laboratory-based diagnosis of brucellosis--a review of the literature. Part II: serological tests for brucellosis. *Clin Lab*, 49 (11-12): 577-89.
19. Yu WL, Nielsen K (2010). Review of detection of Brucella spp. by polymerase chain reaction. *Croat Med J*, 51 (4): 306-13.
20. Gupta VK, Shivasharanappa N, Kumar V, Kumar A (2014). Diagnostic evaluation of serological assays and different gene based PCR for detection of *Brucella melitensis* in goat. *Small Ruminant Res*, 117 (1): 94-102.
21. Garcia-Bocanegra I, Allepuz A, Perez JJ, Alba A, Giovannini A, Arenas A, Candeloro L, Pacios A, Saez JL, Gonzalez MA (2013). Evaluation of different enzyme-linked immunosorbent assays for the diagnosis of brucellosis due to *Brucella melitensis* in sheep. *Vet J*, 199 (3): 439-45.
22. Christopher S, Umapathy BL, Ravikumar KL (2010). Brucellosis: review on the recent trends in pathogenicity and laboratory diagnosis. *J Lab Phys*, 2 (2): 55-60.
23. Pabuccuoglu O, Ecemis T, El S, Coskun A, Akcali S, Sanlidag T (2011). Evaluation of serological tests for diagnosis of brucellosis. *Jap J Infect Dis*, 64 (4): 272-6.
24. Trollfors B, Taranger J, Lagergard T, Sundh V (2005). Reduced immunogenicity of diphtheria and tetanus toxoids when combined with pertussis toxoid. *Pediatr Infect Dis J*, 24 (1): 85-6.
25. Hu J, Li W, Wang T, Lin Z, Jiang M, Hu F (2012). Development of a label-free and innovative approach based on surface plasmon resonance biosensor for on-site detection of infectious bursal disease virus (IBDV). *Biosensors Bioelectronics*, 31 (1): 475-9.
26. Winther B, Gwaltney JM, Humphries JE, Hendley JO (2002). Cross-linked fibrin in the nasal fluid of patients with the common cold. *Clin Infect Dis*, 34 (5): 708-10.
27. Jorgensen CS, Schiodt FV, Dahl B, Laursen I, Houen G (2007). Comparison of rocket and crossed immuno-electrophoresis assays for determination of the level of actin complexing of Gc globulin. *Scand J Clin Lab Invest,* 67 (7): 767-77.