

Evaluation of designed IS711 primers and universal primers of B4 and B5 for detection of Brucella spp. in clinical samples

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Abstract

Background: Brucellosis as a global concern is a zoonotic infectious disease which affects a large number of individuals in developing countries. Microbiological, serological and molecular approaches are useful for detection and identification of *Brucella* spp. A confirmed diagnosis requires isolation of *Brucella* from clinical specimens that is the most sensitive method in the acute and sub-acute phases of the diseases. On the other hand, molecular diagnostic techniques are more sensitive and more specific than serological techniques, especially in chronic localized cases because of antigenic cross-reactions or antibody titers lower than 160. Until now different *Brucella* specific sequences like BCSP 31, IS711 and 16SrRNA have been amplified for detection of *Brucella* spp. In this study, the sensitivity and specificity of The B4-B5 primers and IS711 designed primers were evaluated for detection of of *Brucella* Spp. in the clinical samples. Results : Amplification of extracted DNA from serum of 49 suspected patients were tested with two sets of specific primers. The BCSP31 amplicon was 223 bp and all the 49 (100%) serum specimens were positive by B4-B5 primers, including 4 cases with negative 2ME test result. The designed IS711 primers amplified the IS711 product with 448 bp length and 46 of 49 (93.87%) cases were positive. The sensitivity of the applied primers (B4-B5 and IS711) was evaluated by using the serial dilutions of extracted purified DNA molecules of *B. melitensis* and *B. abortus* . The B4-B5 primers can detect the least number of both *B. melitensis* and *B. abortus* , 0.1 CFU/reaction. However, the designed IS711 set is able to detect 10 CFU/reaction. The B4-B5 primer and IS711 designed primer recognized 100% (49/49) and 94% (46/49) of the cases, respectively. Conclusion: This study indicated that the sensitivity of B4-B5 primer is 100%, while the sensitivity of the designed primer of IS711 is 94%. The laboratory experiment revealed that designed IS711 set is 1×10^2 times more sensitive than sensitivity of the other experiments for detection of IS711 target sequence in the specimens.

Background

Brucellosis is a zoonotic infectious disease caused by different species of *Brucella* and can be transmitted from infected animals to humans by animal exudates or the consumption of unpasteurized dairy products [1, 2].

About 500,000 patients with brucellosis are reported annually [3], while it is estimated that the number of patients is 10-25 times higher than the reported cases. The majority of reported cases belong to the Middle East countries, including Iran, Iraq and the Persian Gulf Region countries [4]. In Iran, the incidence of reported brucellosis is 34 per 100,000 [5].

Brucella is a non-motile, Gram-negative and intracellular coccobacillus including 4 human pathogenic species of *B. abortus*, *B. melitensis*, *B. suis*, and *B. canis* [2, 6]. Furthermore, there is high risk of infection in some professions such as veterinarians, cowhands, laboratory staffs and slaughterhouse workers [7]. These bacteria causing severe infection in humans with non-specific clinical symptoms which are similar to other febrile diseases like malaria, tuberculosis, and typhoid fever. A confirmed diagnosis is required to isolate the bacterial agent from clinical specimens like blood, bone marrow, CSF or tissues [8, 9].

Microbiological, serological and molecular approaches are useful for detection and identification of *Brucella* spp. and blood culture is known as the “gold standard” for *Brucella* spp. Nevertheless, the sensitivity of blood culture has been reported about 15% to 70%, often unsuccessful in chronic brucellosis and *Brucella* is categorized as biosafety level 3 pathogen [10, 11, 12].

Although the serological tests used for *Brucella* spp. include a variety of assays such as Rose Bengal, 2-Mercaptoethanol, Coombs and Serum Agglutination test [10], the specificity of these tests are low and are not suitable for endemic areas, particularly for patients with chronic brucellosis [13]. Besides, the specificity of serological assays could be affected by cross-reactivity with other Gram-negative bacteria, in the early stage of the disease [14, 15]. However, the molecular techniques such as DNA-based tests have proved to be fast (> 4 h) and DNA detection allows molecular typing of *Brucella* without exposure to the infective *Brucella* organism [16, 17]. The amplification of BCSP31 or IS711 enables the detection of *Brucella* DNA but does not identify the detected species (*B. melitensis*, *B. abortus*, etc.) [18]. Sequencing the whole genome of *B. abortus*, *B. melitensis*, *B. suis* has revealed high degree of genetic homology (up to 99.9%) in human pathogens and other species of *Brucella* [19], thus PCR as a nucleic acid amplification technique with high sensitivity and specificity can conquer the limitation of conventional methodology [20].

In the present study, the PCR technique is used to detect the bacterial cells of *Brucella* spp. in serum samples of suspected patients with brucellosis clinical symptoms, by two primer sets. Each primer set is evaluated by comparing with the other one. These primers include B4-B5 and the designed primer of IS711.

Methods

Area and time of study

The patient samples were collected from different endemic areas in Iran, including Babol, Shiraz, Mashhad, Borujerd, Urmia, Makoo, Khoy and Tabriz with 2, 2, 6, 4, 4, 3, 24 and 4 cases, respectively during a period of 10 months (from October 2017 to July 2018), The geographical distribution and the number of samples are shown in figure 1.

Case definition and data collection

Blood specimens were collected from 49 suspected cases of patients with brucellosis symptoms who were referred to diagnostic laboratories in different cities from northern (2 cases, 4.08%), southern (2 cases, 4.08%), western (39 cases, 79.59%) and eastern (6, 12.24%) provinces in Iran. The serum samples were processed on the same day as blood collection. Before blood collection, the written informed consent was obtained and the questionnaire was filled for each patient including age, sex, job, residence area and primary clinical symptoms. The significant statistical differences were tested with the Chi-square test.

Serological test:

Prior to amplification, the isolated specimens (sera) were tested by the serological assay of 2-Mercaptoethanol test (2ME). Then a positive 2ME titer was defined as either equal or greater than 1:80 and Coombs Wright titer was considered as either equal or greater than 1:80, in accordance with standard methods [21]. The studied samples were collected from suspected patients which their titer of the 2ME test was equal or higher than 1/20 and serial dilutions of serum samples were prepared as follows: 1/2, 1/10, 1/20, 1/40, 1/80, 1/160, 1/320, 1/640, 1/1280. Positive and negative controls were used to check the serological tests [21].

Bacterial DNA extraction

The viable bacterial samples of *B. melitensis* and *B. abortus* were provided from the microbial culture collection of Department of Microbiology, Faculty of Veterinary Medicine, University of Tehran. The genomic DNA was extracted by the commercial GTP kit, Gene Transfer Pioneers (www.irgtp.com, Tehran, Iran) according to the supplier manual. The quality and quantity of the DNA samples were evaluated by agarose gel electrophoresis and spectrophotometry. The purified DNA was stored at -20 °C until the day of amplification, followed by a serial dilution of the DNA samples. The DNA of *Escherichia coli* was extracted and used as the negative control.

Extraction of DNA from samples

The isolated patient samples were kept in two tubes, with and without sodium citrate. The DNA was extracted from the serum specimens of 200 µL volume, applying the commercial GTP kit (www.irgtp.com, Tehran, Iran) as mentioned above.

Bioinformatics analyses, primer design and DNA amplification

Two primer sets with different target genes were used followed by bioinformatics analyses:

First, B4 (5'-TGG CTC GGT TGC CAA TAT CAA-3') and B5 (5'-CGC GCT TGC CTT TCA GGT CTG-3'), with a target gene encoding a 31-kDa *B. abortus* antigen which is a conserved sequence in all species of *Brucella* [22]. The reaction was consisted of 12.5 µL 2X PCR master mix (Amplicon, Denmark), 5 µL DNA template, 0.5 µL of each primer and nuclease-free water up to 25 µL. Techne, touch gene gradient PCR machine, model: Techne TC-512 was used for the DNA amplification (www.techne.com). The thermo-cycler was programmed as follows: Initial denaturation at 95 °C for 5 min, 35 cycles of template denaturation at 94 °C for 1 min, 30 s for primer annealing at 60 °C and 60 s for primer extension at 72 °C with final extension cycle at 72 °C for 7 min.

Second, IS711 specific primer was designed based on the sequences of *B. melitensis* deposited in the GenBank, applying Codoncode Aligner software (V.7.1.2)). The designed primers, F (5'-CGC TCG CTG CCA TAC TTG CA-3') and R (5'-CTG AAC AAG CCG GGC CTG AT-3') amplified a 448 bp fragment which was a repetitive genetic element of IS711 of *Brucella* species. At least, one copy of this repetitive genetic

element may appear as a common locus in all species of *Brucella* [6]. The IS711 PCR assay was carried out in total volume of 25 µL. The gene amplification of the IS711 primer was programmed as follows: initial denaturation at 95 °C for 5 min. 35 cycles of template denaturation at 94 °C for 1 min, 60 s for primer annealing at 63 °C and 60 s for primer extension at 72 °C with final extension cycle at 72 °C for 7 min.

In each PCR assay, a positive control, extracted DNA from *B. melitensis* Rev. 1 and *B. abortus* S19 and negative control, extracted DNA from *E. coli* (ATCC 35218) were applied to control the cross-contamination. All the standard items were checked for prevention of any probable contamination [23]. The tests were carried out in two repeats. After the amplification process, the samples were run on 1% agarose gel (Sigma). The gel was stained by 1 µg/ml ethidium bromide and after destaining, the DNA bands were visualized in a Gel documentation UV chamber. The sequencing of amplified DNA revealed that the products are related to the *Brucella* specific gene sequences (supplementary file number 1-3).

Sensitivity assay

In the current study, for colony forming unit (cfu) estimation, a 48h incubated suspension of *B. melitensis* and *B. abortus* within sterile PBS was used for preparing serial dilutions from 10^{-1} to 10^{-10} . From each dilution, 0.1 ml was streaked onto the *Brucella* agar and was incubated at 37 °C for 72h. Then, the colonies of *B. melitensis* and *B. abortus* were counted [24] and the bacterial concentration was calculated to be about 5×10^8 cfu /ml for both *B. melitensis* and *B. abortus*. Then a serial dilution of extracted purified DNA of *B. melitensis* and *B. abortus* was prepared from 10^{-1} to 10^{-10} . Afterwards, five microliters of each dilution was used as template in the PCR process. No amplification was detected with *E. coli* DNA template. The tests were carried out in two repeats.

Results

Epidemiologic data

In this research, the geographical distribution of patients in different cities was as follows; Khoy: 48.97%, Mashhad: 12.24%, Tabriz and Urmia: 8.1%, Makoo, and Borujerd: 6.1%, Shiraz and Babol: 4.08 % (figure 2). In khoy area, a traditional cheese is consumed which is made with raw milk in ceramic vessel (Kozeh cheese) and they lack dairy industry.

Based on the collected data, 83.67% and 16.33% of the patients were men and women, respectively. The age of the patient ranged within 18 to 70 years old with the average of 33.02 years old. The Patient age groups consist of 1 patient under 20, 14 patients between 20-30, 27 patients between 30-40, 4 patients between 40-50, 2 patients between 50-60, and 1 patient over 60 years old (Figure 3).

The occupation of 32 (65.30%) patients was stockbreeding, 5 (10.20%) patients were dairy industries staff, 1 (2.04%) patient was the hospital nurse and 11 (22.44%) patients had miscellaneous jobs (Figure

4). The differences were statistically meaningful within groups with probability $p < .0001$ for sex, age and occupation.

All the patients had clinical symptoms representing brucellosis. The cases with positive 2ME test (45 cases) had complained of back pain, weightless, recurrent fever, fatigue for more than 6 months and the four cases with negative 2ME test recurrent fever, sweat, chills, headache, and myalgia, splenomegaly, spondylitis for less than 1 months.

Serological results of 2 Mercaptoethanol test (2ME)

In this study, 2ME test result revealed serum titers ranging from 1: 20 to 1: 1280. The result of the 2ME test in 49 serum samples was 3 (6.12%) patient with 1: 20 titer, 1 (2.04%) patient with 1: 40, 29 (59.18%) patient with 1: 80, 9 (18.36%) patient with 1: 160, 1 (2.04%) patient with 1: 320 and 4 (12.24%) patients with 1: 640 titer, 2 patients with 1/1280 (Figure 5).

Amplification with BCSP31-PCR

The amplification of the *Brucella* genus with B4 and B5 primers revealed an amplicon of 223 bp and all the 49 (100%) serum specimens isolated from patients were positive by B4 and B5 primers, including 4 cases with negative 2ME test result.

Amplification with IS711-PCR

Detection of *Brucella* in serum samples was performed by designed IS711 primers which were specific to *Brucella* genus. The amplicon of IS711 is 448 bp and 46 (93.87%) cases were positive among the 49 serum samples isolated from patients.

Evaluation of the sensitivities of studied primer pairs

The sensitivities of the applied primers (B4-B5 and IS711) were evaluated by using serial dilutions of extracted purified DNA molecules of *B. melitensis* and *B. abortus*. The B4-B5 primers were able to detect bacterial cells with the amount of 0.1 cfu/reaction for both *B. melitensis* and *B. abortus*, while the IS711 designed primer was able to detect bacterial cells with the amount of 10 cfu/reaction for both species.

Discussion

Generally, the *Brucella* culture remains the most specific diagnostic method for brucellosis, which is the most sensitive method in the acute and sub-acute phases of the diseases [25, 26]. The serological techniques lack sensitivity and specificity due to antigenic cross-reaction and may lead to erroneous diagnosis, especially when antibody titers are lower than 160. Molecular diagnostic techniques are more sensitive than culture for sub-acute or chronic localized forms of the disease. In these infections, the PCR as a reliable technique to gain accurate results within a short time, needs to be optimized. The

optimization has direct effects on the diagnostic outcomes [25, 26]. Furthermore, according to the previous reports molecular techniques such as PCR are known as a helpful option for detection of fastidious bacteria like *Brucella* [18]. Among different species of *Brucella*, *B. melitensis* is the main causative agent for human brucellosis in Middle East countries involving Iran, Iraq, Syria and India [27, 28].

The results of this study revealed that the difference between the infected number of individuals in men (83.67%) and women (16.37%), the age group of 30- 40 years (55.1%), stockbreeders (65.30%) are statistically significant with probability $p < .0001$.

In the current research, the PCR as a nucleic acid amplification techniques (NAAT) was used for detection of *Brucella* spp. isolated from serum specimens taken from patients with brucellosis in different geographic areas of Iran. For this purpose, 49 serum samples were isolated from patients with brucellosis. Four of the cases had 2ME titers of less than 1:80, three cases with 1:20 and one case with 1:40, all of four samples were positive with B4-B5 PCR test while, only one positive with IS711 specific primer set. The sequencing of the B4-B5 specific products (the three samples with negative with IS711 specific primer) revealed that all three samples have *Brucella* B4-B5 specific target sequences (supplementary file 1-3). The finding indicates that B4-B5 specific PCR test is able to detect *Brucell* spp. in cases with the 2ME titers of less than 1:80. The other publications indicate the 2ME titer of 1:80 is not denotative of active infection, especially in endemic regions [29, 30, 31]. The 2ME test could be used for prognosis [32] and also proved useful to follow up antibiotic therapy [33].

In different experiments, PCR protocols for detection of *Brucella* DNA in human blood or serum samples were evaluated using BCSP31 target sequence with the sensitivity of about 70 to 100% and specificity of 95 to 98.3%. As the most sensitive method it can detect extracted DNA of 15 cfu/ml water *Brucella*. [34, 35]. The specificity of the B4-B5 PCR assay, was confirmed according to previous similar results [22, 36, 37, 38]. Our results indicated that the PCR assay with designed primers of IS711 target detected a high number of samples containing *Brucella* in 2ME- positive and negative serum samples. The analytical sensitivity of the PCR tests targeting the BCSP31 and IS711 sequences of *Brucella* spp. has already been evaluated. It is expected that IS711 target is much more sensitive, since there are several copies of IS711 but a single copy of BCSP31 gene in the *Brucella* genome. The designed IS711 specific primer could detect at least 1×10^1 cfu/ ml bacteria in the samples, approximately 5×10^1 times more sensitive than the other reported IS711 primers that were used in the detection of this gene by Ciftciin, which could detect 5×10^2 cfu/ml bacteria [26]. These achievements confirm the PCR results that were reported by Khosravi [39] and Elfaki [40]. In these studies, a large number of samples containing *B. melitensis* DNA were detected by using the IS711 primers. However, our findings are significantly different from the reported results by Garshasbi, in which the sensitivity is low and a large number of *B. abortus* was detectable by using the IS711 primers [18].

In this PCR assay, the amplified DNA was purified from the serum sample instead of whole blood. The whole blood samples contain more inhibitors than serum samples. Besides, the DNA extraction from

serum sample is more efficient than the whole blood. Heparin is known as a PCR inhibitor that inhibits Taq DNA polymerase and EDTA acts by chelating Mg²⁺ ions. Sodium citrate is a good alternative of heparin or EDTA [38].

Moreover, the detection of *Brucella* is limited to 0.1 cfu/reaction by B4-B5 primers with 100% sensitivity, while the detection is limited to 10 cfu/reaction by designed IS711 primer with 93.87% sensitivity. The negative results for detection of *Brucella* in the aforementioned 3 samples, suggested that they had insufficient number of bacteria detectable by IS711 primer. It is worthwhile to mention that different amounts of template DNA were used, ranging from 1 to 5 µL to eliminate the possible reaction inhibition of the template DNA inhibitors.

Conclusion

Application of the IS711 and the BCSP31-based PCR assays in this survey is a promising method for detection and identification of *Brucella* spp, as routine clinical diagnostic procedure in microbiology laboratories, since they reduce the risk of working with infective microorganisms in the laboratories [18]. Our findings in this study have shown 100% sensitivity for B4-B5 specific PCR, while the sensitivity of designed IS711 sequence specific PCR was about 94%. The aim of this study was to develop a molecular method for detection of *Brucella* spp. especially for sub-acute or chronic localized forms of the disease. However, the gold standard diagnostic method for brucellosis, both in animals and humans is still based on the isolation of *Brucella* within the specimens [38].

Abbreviations

ATCC: American type culture collection; **BCSP:** *Brucella* cell surface protein; **CFU:** Colony forming unit; **CSF:** Cerebrospinal fluid; **DNA:** Deoxyribonucleic acid; **EDTA:** Ethylenediaminetetraacetic acid; **fg:** Femtogram; **IS711:** Insertion sequence711; **NAAT:** Nucleic acid amplification technique; **ng:** Nanogram; **PBS:** Phosphate buffer saline; **PCR:** Polymerase chain reaction; **WHO:** World health organization; **2ME:** 2 Mercaptoethanol.

Declarations

Ethics approval and consent to participate

The study was approved by the Faculty of Medicine, Tehran Islamic Azad University of Medical Sciences, Research Ethics Committee, with approval ID: IR. IUA. TMU. REC: 1397. 245.

The present study involved the clinical samples of suspected patients with brucellosis symptoms who were referred to diagnostic microbiology laboratories in the studied geographical areas.

The written informed consent was obtained prior to taking blood and the questionnaire which included age, sex, job, residence area, primary clinical symptoms was filled for each patient.

Consent for publication

Not applicable.

Availability of data and material

The accession numbers of *Brucella* used for the primers design in November 2017 are as follows:
AM040246 – AF036614 – AF047478 – DQ845343 – JF939171 – KF730265 – HM598413 – JN561159
– JN561158 – GQ443747 – GQ479519 – HM598412.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

PH and MRR conceived and designed the study; **PH and MRR** performed the experiments; **MS and AAS** advised the research; **PH, MRR and AAS** analyzed the data; **PH** wrote the manuscript; **MRR** revised the manuscript. All authors read and approved the final manuscript.

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Figures

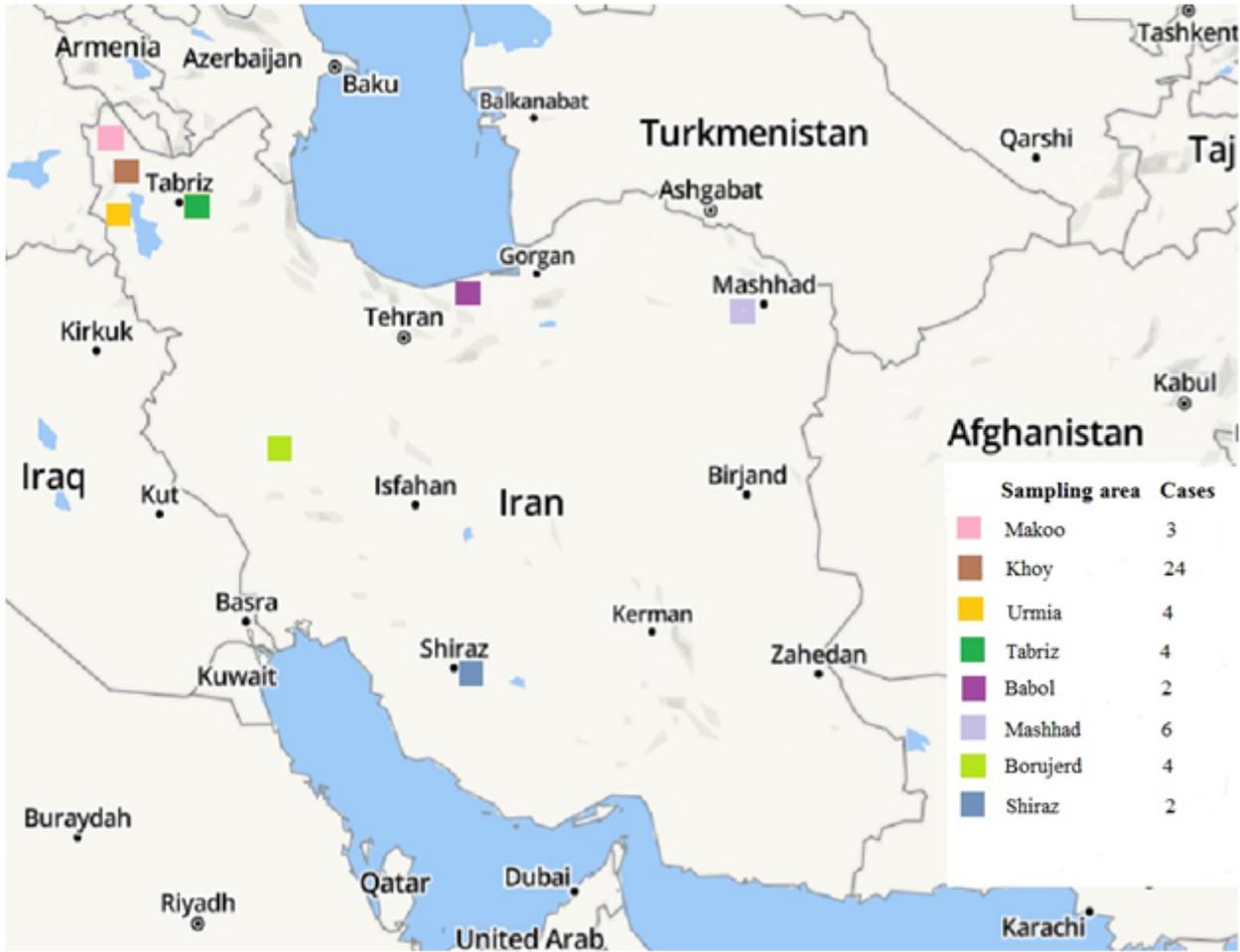


Figure 1

The map of sampling areas and the number of specimens.

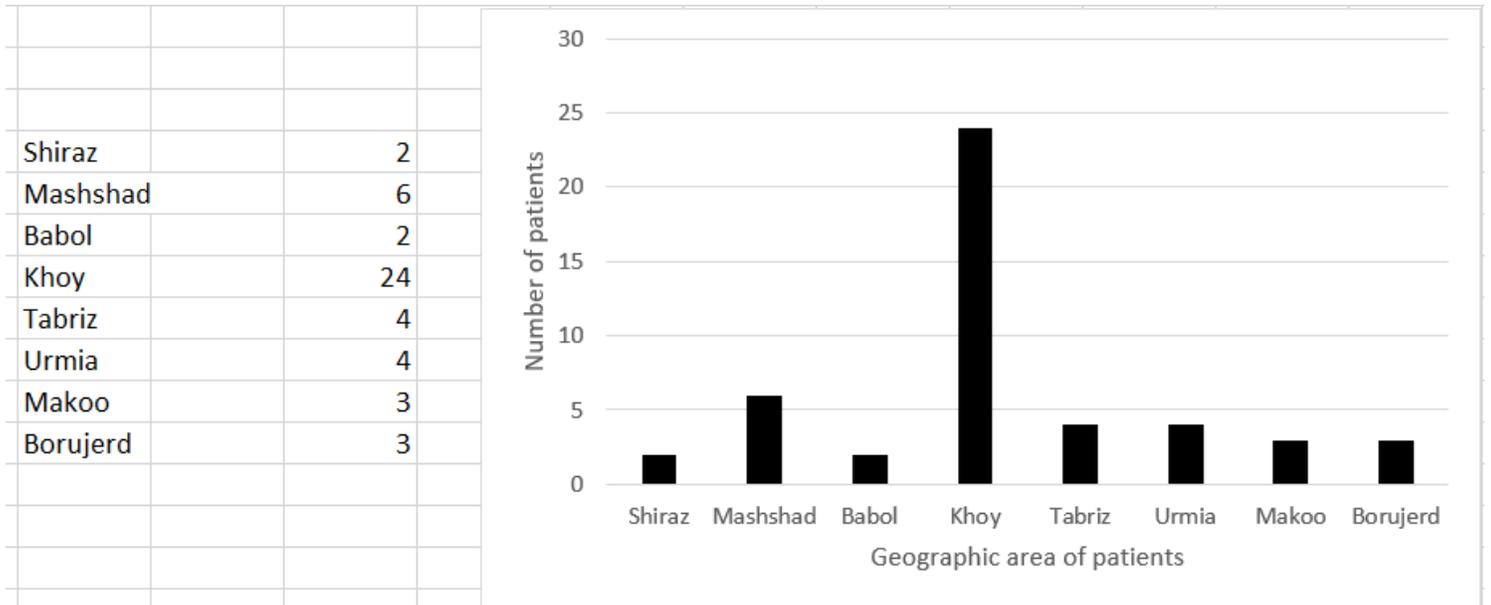


Figure 2

Distribution of studied patients in different cities. The total number of patients; 49. Shiraz:2, Mashhad:6, Babol:2, Khoy:24, Tabriz:4, Urmia:4, Makoo:3, Borujerd:3.

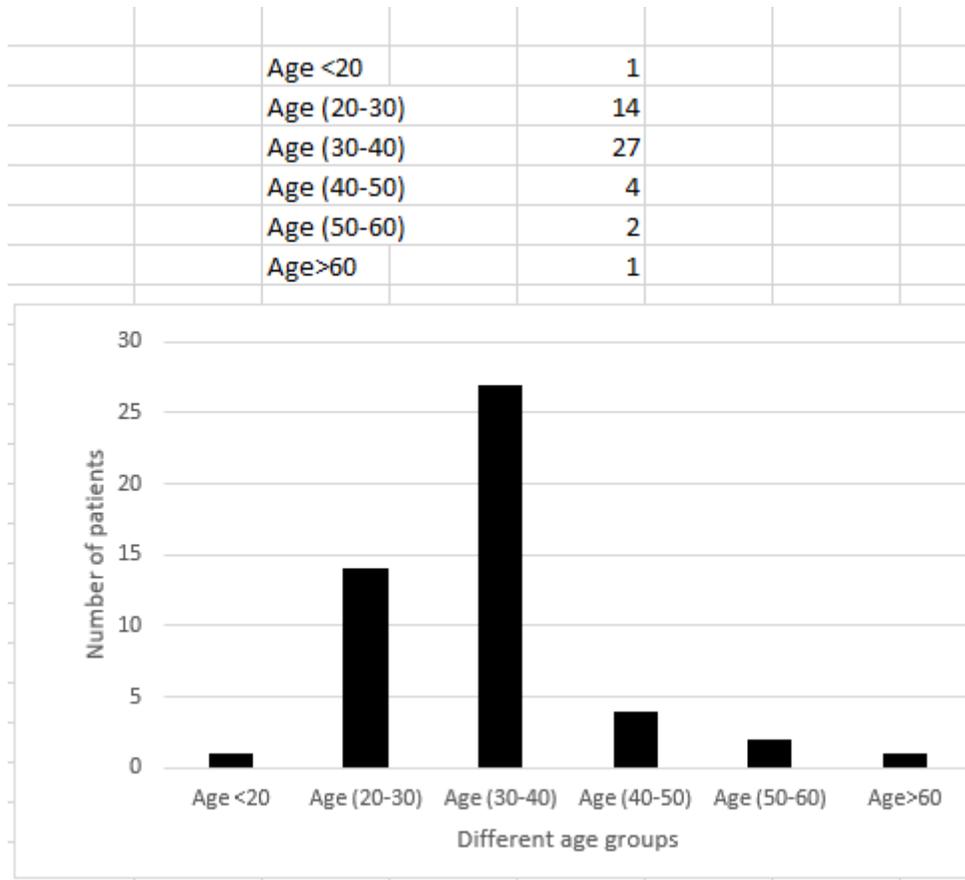


Figure 3

Age distribution of the studied patients. Total number of patients; 49, Age <20:1, Age (20-30):14, Age (30-40):27, Age (40-50):4, Age (50-60):2, Age > 60:1.

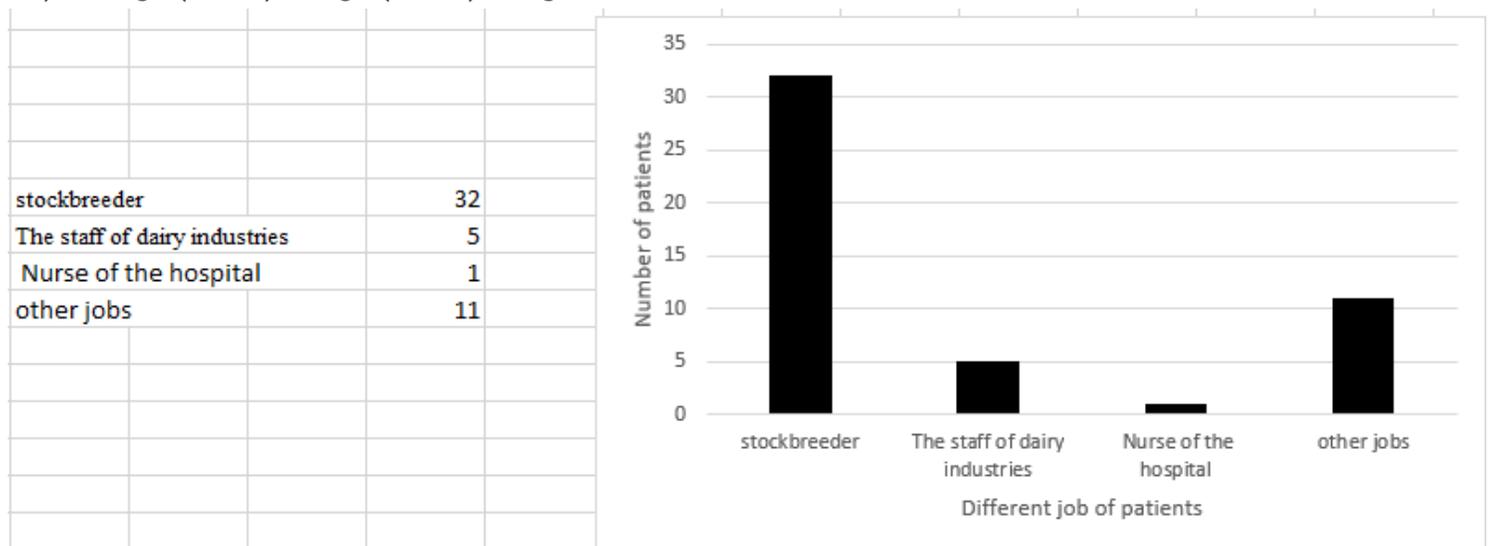


Figure 4

Occupations of the studied patients. Total number of patients; 49, stockbreeder: 32, The staff of dairy industries: 5, Nurse of the hospital: 1, other jobs: 11.

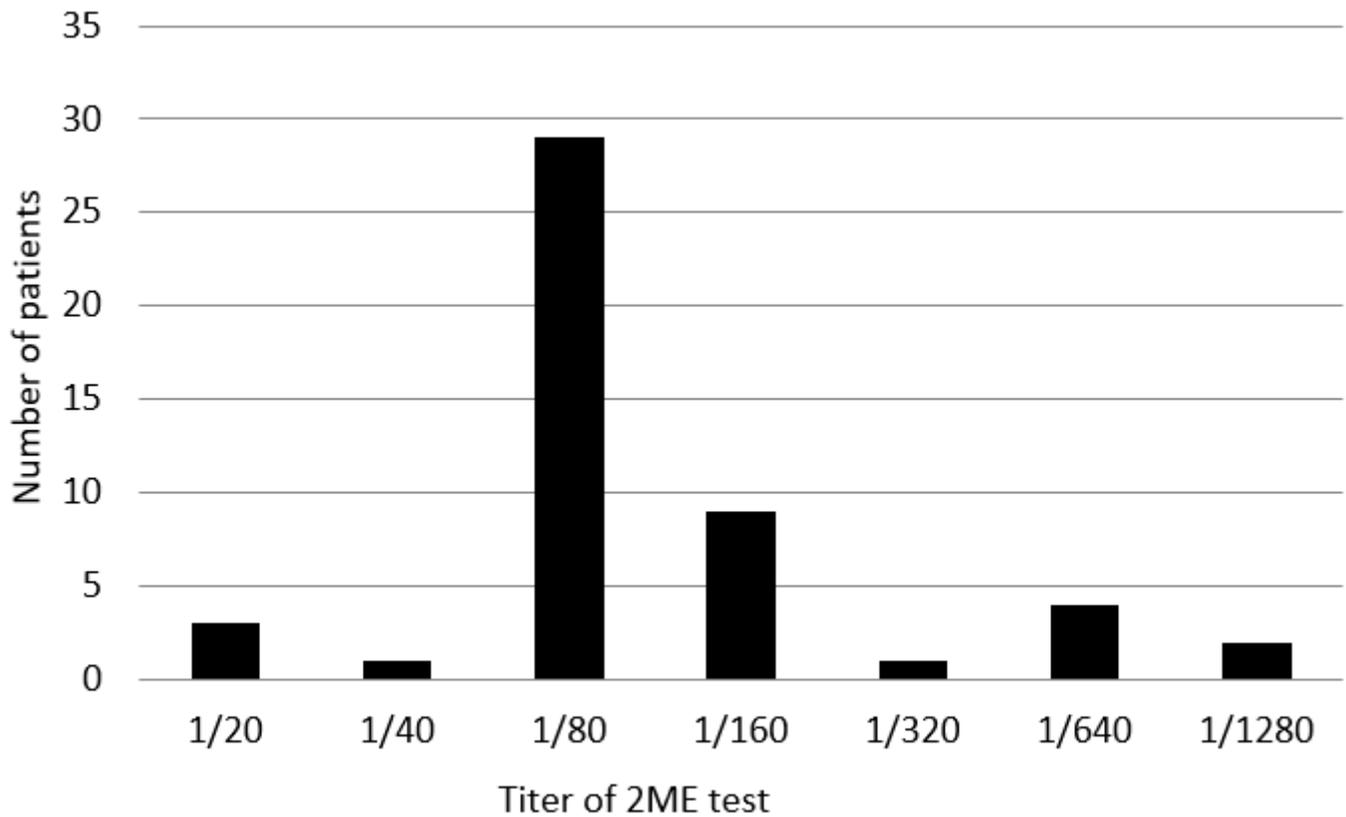


Figure 5

Results of 2ME test; Titer 1/20:3 patients, Titer 1/40:1, Titer 1/80:29, Titer 1/160:9, Titer 1/320:1, Titer: 1/640:6, Titer 1/1280:2.

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