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STUDY ANIMAL CENTERS WHICH INFECTED WITH BRUCELLA BACTERIA AND DETERMINE COMMON SPECIES OF BRUCELLA BY PCR METHOD IN THE CITY OF ZARANDIEH FROM MARCH 2012 AND JUNE 2013

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ABSTRACT

Brucellosis is one of the most common diseases and is considered as zoonosis a common disease between animals and human) and city of Zarandieh which is a part of province of Markazi is an endemic area. The aim of this study is to determine brucellosis disease Seroepidemiologic in the livestock of this area and also determine the species and infected biotypes in human and animal isolated This is a cross-sectional – descriptive study and 340 blood samples were collected from suspected animals (cattle - sheep - goat) at least one year from the time of abortion and 2 years after vaccination passage, along with this study, 23 positive blood samples were taken from cattle in terms of serology and 12 blood samples of human from patients who referred to hospital with clinical signs, at Zarandieh Shodaha hospital. Each sample was inoculated on Castaneda's first and then was incubated for 14 days under conditions of 37 ° C. For the detection of grown bacteria, stained Gram, biochemical tests were used. In the next stage, PCR test was used with amplification of omp2a and omp2b fragments which amplified with special primers to identify Brucella species .Of the 35 samples taken from brucellosis patients and suspected Livestock which are serologically positive, 18 cases were confirmed to be infected with Brucella in terms of molecular testing with positive culture and PCR Omp2a gene segments and omp2b all human and animal isolates have respectively bp1100 and bp1200 sizes ,also banding pattern created by PCR ,diagnosed similar with of Brucella Melitensis biotype 2.This study shows that the prevalence of brucellosis caused by Brucella Melitensis biotype 2 in the city is high. And effort to eradicate these bacteria should be performed in the city Zarandieh.

KEYWORDS: Brucellosis, Brucella Melitensis, omp2a-omp2, Zarandieh

INTRODUCTION

Brucellosis is a zoonotic disease in humans and animals and is created by Kokobasil bacteria, negative Gram that are found in normal flora in the urinary tract of livestock, such as domestic animals such as cattle, goats, sheep, dogs and pigs (1) There are four species of bacteria, including Brucellosis, kanis, abortus and Melitensis which are matter and causing disease in humans, the most common species of Brucella are Melitensis. However, this bacteria has aerobic growth, But some varieties of this species, in the case of 10% CO2 may have more growth in sheep and goats. Melitensis species have been observed mostly in sheep and goats, Brucella abortus in Cows, Switzerland species in pigs and Brucella kanis in dogs.

(Infections created in human recognized by various species of Brucella in brucellosis disease infection (Brucellosis) that are created through exposure of humans orally, respiratory and through skin with the infected animals and their products. The disease is endemic in Iran and the high prevalence of infection has reported as following: Hamadan 5/107, Kurdistan 5/83, West Azerbaijan 4/71, Zanjan Province 1/67, 66 At Markazi in every 100,000 people of human population (8). Annually, 500000 new conflicts are reported due to this disease in the world, according to the WHO. This has reported that this number of the true prevalence of the disease is insignificant, since the disease has different symptoms. Diversity and non-proprietary and non-typical clinical symptoms of Brucellosis will highlight the need for a diagnosis based on laboratory findings. Common symptoms at brucellosis patients could be fever, shivering, night sweating, loss of appetite, fatigue, and weight loss; as Brucellosis illness is not generally fatal if not recognized and grows chronic, but can cause serious problems for patients (1). Due to Lack of specific symptoms of this disease and its symptoms similarity to other diseases, as well as solid growth of Brucella, the diagnosis is will become difficult. in brucellosis disease from Brucella species the Diagnosis is occurred when the bone marrow is seperated from blood or other tissue (8). Several common methods used for the diagnosis of this disease_ include blood culture which is varied in range of 90-53 percent. In the absence of Serological culture of PCR, ELISA is used. A variety of Agglutination test and Rose Bengal tests (Rose Bengal other methods) are usually used to recognize Brucellosis. Rose Bengal tests (Rose

Bengal) is one of the serological diagnosis of brucellosis. Which its being positive will be confirmed by applying agglutination tests and Bengal.

This test is based on antibodies response with smooth Brucella lipopolysaccharide (1). This 2ME Komos Wright antibody was available in patient's serum for a long time and the diagnosis of disease is vital to investigate spread of the disease in endemic areas. antibody generated against Smooth Brucella LPS tends a lot to connect to other negative-gram bacteria such as Yersinia Interkolitika, Vibrio cholera ,E. coli , Fransiska Toloransys bacteria And this enhances binding or (Cross reaction) that causes the false Reply.

1. To prevent cross-reactivity of antibodies IgM, 2ME test is used to measure specifically IgG antibodies agglutination and for chronic patients, test Coombs is used which is much more sensitive. (6,1). To identification of Brucella species, classical methods are used , such as :urease , need to CO2, growth at Tionyn and fuchsine environment, Production of H2S, agglutination by antiserum and talking about phage lysis methods and molecular methods , PCR Real time can be cited(1). Most of the known genes of Brucella were studied to achieve these targets , but some of them specifically , molecular typing of Brucella species and biotypes of Brucella were considered noticeably(4) sometimes, too much similarities between the DNA of Brucella's has led to consider all the species belong to the genus Brucella DNA polymorphism Therefore, the identification of Brucella DNA polymorphisms and application of molecular classification (determine the type) - molecular typing- of Brucella, can define the differences between the variation of these techniques. The molecular identification of Brucella by PCR is quite feasible (10, 9). The outer membrane proteins (OMP) of Brucella species have recently attracted attention. And we can show the difference between the types of Brucella infection and some types biotypes.

An Omp2 gene places in an assimilated position with slight differences among species of Brucella and biotypes, this gene is composed of two parts under these names: omp2a and omp2b (3.13) Purine- KDa360- is encoded by omp2 locus.This locus is composed of two genes which are very close and under the names of omp2a and omp2b. (3.11).

MATERIALS AND METHODS

Study and study population:

Laboratory work with Brucella is very dangerous and to avoid personnel protection detailed protective rules should be applied during the Bacterial isolation from aborted fetuses and cultures and laboratory tests should be performed in equipped Laboratories and after isolating Brucella bacteria and determine if it is Brucella bacteria, extraction will be performed with extraction kits of bacteria genome.

In the first stage of this descriptive cross-sectional study which was examined for 15 months, to assess Seroepidemiology of brucellosis disease in animal populations in the city Zarandieh, 340 blood samples in the amount of 10 ml, have bled from jugular vein and veins of suspicious animals (cattle -sheep-goat) which passed at least one year of their abortion and 2 years of their vaccination to assess the prevalence of the disease to test the prevalance rate. Sample taken from the animals were sent to the microbiology laboratory of Shohada Zarandieh Hospital to be investigated. A. The prepared samples in a laboratory were centrifuged for 10 minutes at round of rpm1500 and the serum was separated from blood clots and put under serological tests (Rose Bengal test-Wright tube -2ME). In this study Samples with antibody 80/1 and upper were considered as positive cases of livestock brucellosis.

In the second phase of the study to determine the common species and biotypes of Brucella bacteria which infected cattle and human were performed. During this stage 35 blood samples(in amount of 10ml), 23 cases from livestock - 12 human cases with clinical symptoms of the disease includes fever, chills, night sweats, loss of appetite and weight loss after obtaining consent from patients) were bled and serologic tests Rose Bengal test, Wright test, 2 ME and Coombs were taken on.

Blood cultures and confirmatory tests for Brucella bacteria:

At the beginning of the study, prepared blood samples in the amount of 10ml from suspect animals and suspected human cases of the disease were injected. In the blood culture, diphasic of Castaneda (prepared by Bahar Afshan Co.) was incubated in the incubation device for 14 days at 37 ° C. After this time samples in Brucella agar medium (prepared from oxide Company) were incubated at 37 ° C for 3 days. From 35 cultured samples only 18 Brucella colonies were isolated and isolated colonies were investigated under Gram stain (negative-Gram Kokobasil) and

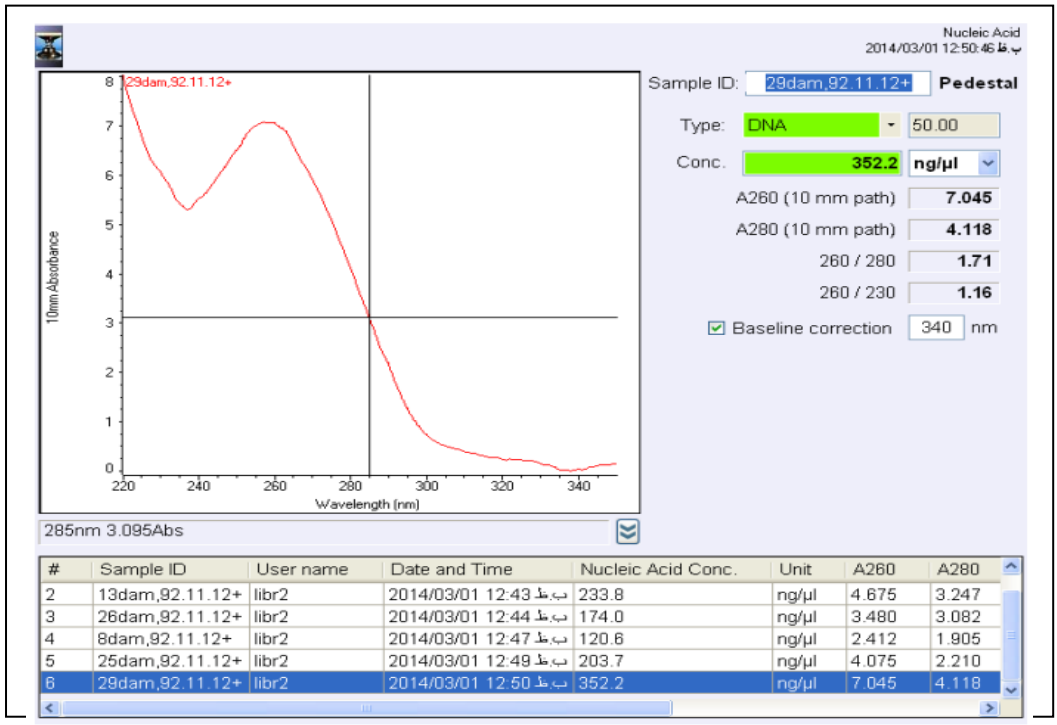
biochemical tests (urease, produced H2S, catalase, oxidase) ,. Samples were cultured negatively were incubated for 2 weeks for confidence. (7, 14)

Brucella DNA extraction:

Brucella DNA extraction was performed using chloroform method; so that Brucella agar medium containing colonies washed with 5ml of phenol saline to kill bacteria and after collecting bacteria, we put it at a temperature of 68 ° C for 2 hours and centrifuge it at 5000rpm for 20 minutes and then deposits with 4 mg / ml of lysozyme solution was placed put at 4 ° C for 30 min . In the next step, 200 microliter SDS (5/0 percent) and proteinase K (mg / ml200) were added at 37 ° C for 1 hour and after purification of DNA, cells proteins are lysed complete with Phenol chloroform ISOMIL alcohol and once with isoamyl alcohol, extraction grid were and once complete.

To increase the concentration and purity of DNA , it was precipitated with 70% ethanol and air dried .To recognize samples' quality and quantity, again dissolved it in TE buffer (PH = 8 EDTA = 1m, Tris-Hcl 50mM). And was determined by Nanodrop In the presence of radiation uv, Concentration (320OD) (Fig. 1) .Also electrophoresis sample was performed in 1.5% gel with a buffer with these features: TEA (PH: 8) (EDTA 1Mm Tris-acetat 0.5mg / ml) and the ethidium bromide was used to stain and the DNA was kept at 4 ° C until used (4)

Figure 1: The determination of the amount of used DNA for PCR testing using Nano drop device:



#	Sample ID	User name	Date and Time	Nucleic Acid Conc.	Unit	A260	A280
2	13dam,92.11.12+	libr2	2014/03/01 12:43 ب.ظ	233.8	ng/µl	4.675	3.247
3	26dam,92.11.12+	libr2	2014/03/01 12:44 ب.ظ	174.0	ng/µl	3.480	3.082
4	8dam,92.11.12+	libr2	2014/03/01 12:47 ب.ظ	120.6	ng/µl	2.412	1.905
5	25dam,92.11.12+	libr2	2014/03/01 12:49 ب.ظ	203.7	ng/µl	4.075	2.210
6	29dam,92.11.12+	libr2	2014/03/01 12:50 ب.ظ	352.2	ng/µl	7.045	4.118

Amplification of DNA by PCR:

By using specific primers of omp2a, omp2B, fragment genes OF PCR determined to apply to amplify target gene, with designed device (By Sina gene Co) .Primers arrangements which used are the same for all target Brucellas according to reference number 4, will be as follow:

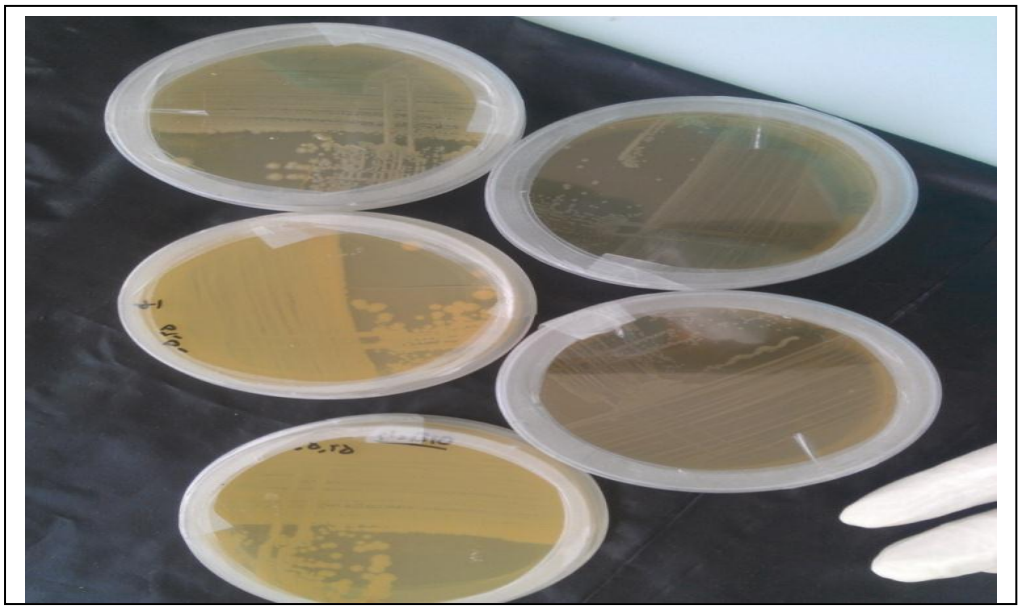
Omp2aF5-GGCTATTCAAAATTCTGGCG-3
Omp2aR5-ATCGATTCTCACGCTTTCGT-3
Omp2bF 5-CCTTCAGCCAAATCAGAATG-3
Omp2bR5-GGTCAGCATAAAAAGCAAGC-3

Solutions required to PCR amplification include:
Buffer of Reaction 10X PCR = 2 / 5ml, DNA: 352ng / ml, mgcl2: 50Mm, 10Mm from each dNTPs, 1PM from each of the oligonucleotide primers, 1 unit from Tag DNA Polymeras (Cinnagen Co). (0.2= Mm ,kcl = 50, (w / v) TritionX100 (1/0 percent),, 1.75mM Mgcl2, 10Mm Tris-HCL, mg / ml of BSA (4)

Timesheet of PCR device:
Timesheet of PCR
Step 1: initial denaturation at 95 ° C for 45 seconds
Step 2: The second denaturation at 95 ° C for 1 minute
Step 3: Annealing Step for primers to bind to the DNA for 2 min at 58 °
Step 4: the extension at 70 ° C to 3 minutes.
Then repetition of Step 2-4 for 30 cycles and a final extension Step at 70 ° C for 10 minutes was performed.

Electrophoresis on Agarose gel:
Agglutination test:
10 microliters of DNA from PCR product is transferred on agarose gel 1. 5 percent and then with 100 ml 1XTBE parts were painted and after, by using a UV Trans laminator with radiation UV, generated bands image became visible and findings have been discovered. IN this method antiserum of M & A were used for the detection of isolated bacterial species and in this study cutie of Wright test is 1.80 that equals four positive which is more than 200 international units of antibodies, was evaluated as positive test for patients. (14, 15).

Figure 2. View grown Brucella colonies on Brucella agar location



FINDINGS
The results of confirmatory tests:
After 10 days from blood cultures, suspected Brucella colonies (picture number 2) have been observed and also isolated after testing positive Gram stain, catalase, oxidase and urease Brucella.

Table 1: shows the results of tests conducted to determine the species of grown Brucella isolated fro human and animal samples

Tests to determined species Brucella	H2S productio n test	catalase	Oxidase	Urease	CO2	Growth dilution Tayonyn	Growth in different diluted fuchsin Melitensis
	-	+	+	-	+	+	+

Serological findings of livestock disease of brucellosis infection:
Brucella infection in 340 blood samples taken from suspected animals suspected of having brucellosis (animals that had abortiontill a year ago and animals which have passed up to 2 years since the vaccination of livestock) showed that by using serological tests (Rose Bengal - wright tube -2 ME) 83 samples (24%) became positive in terms of serologic tests and 257 specimens; namely 75.5 samples were negative. From 340 domestic samples which prepared, 21,179, 97 and 43 samples belonged to cows, sheep, rams and goats, which respectively 28.5% of the cow sample, 33.4% of the sheep, 4/13% of the rams, 9/13 % of the pork became positive. 257 samples equivalent 5.75 samples were reported negative.

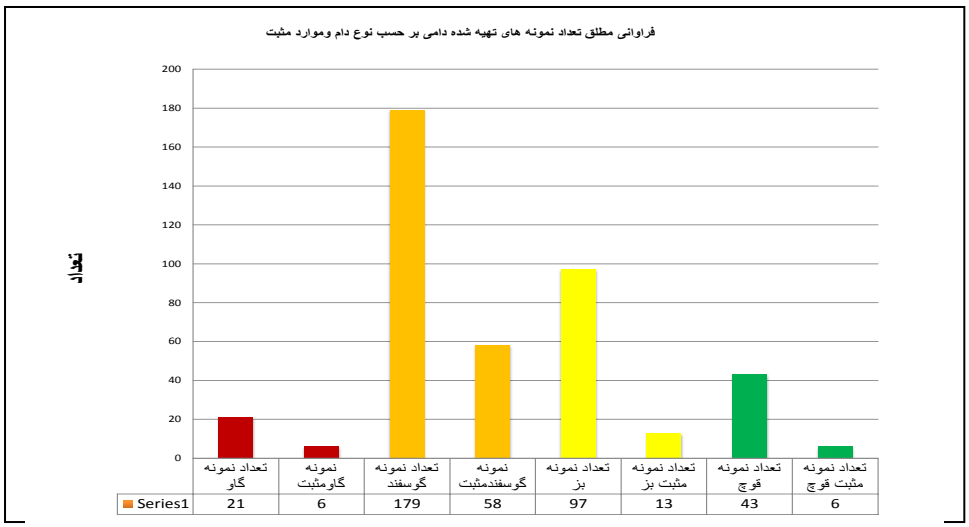


Diagram **1: Diagram of brucellosis infection in livestock city of Zarandieh from date March 2012 and June 2013**

Table 2: Results of serologic tests for livestock, for example:

The number (percentage)		Test
Negative results	Positive results	
(75.5) 257	(24.4) 83	

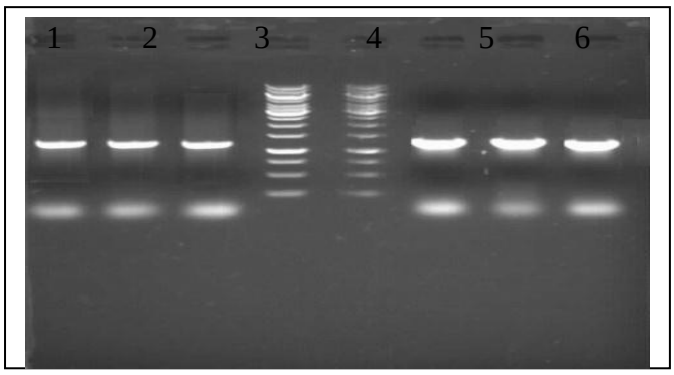
The results of PCR and biochemical techniques to determine the species of Brucella:
After performing PCR, the results indicate the detection of Brucella isolated from patients can be seen in Figure 3:

RESULTS
Animal and human infection with Brucella were approved and identified in all 35 samples used in this study. Brucella with agar culture and performing on biochemical and serological tests, culture showed that from 35 samples, 18 samples (51%) infected with B. Melitensis biotype 2 And 17 samples equals to 5/48 were reported negative, DNA were extracted from each of the above examples by PCR and electrophoresis on agarose gel electrophoresis of two pieces in the size of 1200bp, 1100bp, respectively which are related to omp2a parts and omp2b respectively.

Table 3: results of the PCR test for human and animal samples:

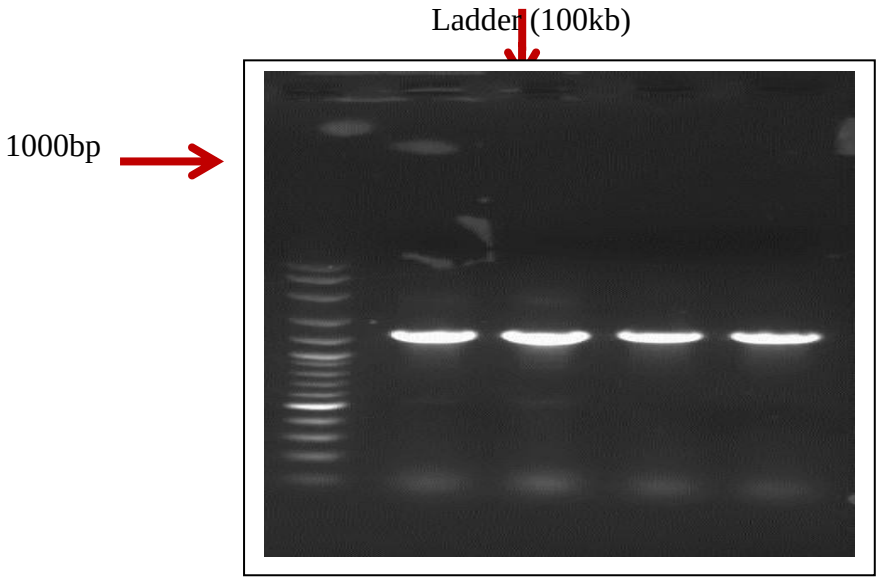
Negative results	Positive results (percent)	Test
(48.5%) 17	(%51) 18	PCR

Figure 3: The arrangement of wells from left to right in the animal model is as following:



Wells No. 1: positive control on omp2a, wells No. 2: omp2a positive, wells No. 3: omp2a positive, well no 4: Well No. 5: ladder 1, Wells No 6: Positive control mp2b, wells No 7 omp2 b is positive. wells No 8: Ladder (100bp)

Figure 4: The arrangement of wells from left to right in human subjects is as following:



Well No. 1: ladder (500kb), wells no 2: positive control omp2a, Well No. 3: omp2a positive, Well No. 4 positive control omp2b, Well No. 5: omp2b positive

Amplified fragments of Omp2a and omp2b by Using Forward primers and Reward were sent for arrangement to Sina Colon Co in the city of Karaj) interface co, an English company in Iran. Sequencing fragments with the of Sanjar method which is accurate method for DNA analysis, was performed and the result of sequencing parts (Sequencing) was sent to Iran. Forward and the Reward sent sequences were copied separately in the software Bioedite and the both primer sequences blast together to determine the complete sequence of the genome of each sample (Figure 5 and Figure 6). Then complete sequences for each sample were compared separately on NCBI (Gen bank) and were observed ,every 18 positive samples in terms of bacterial culture (13 animal - 5 the human) on the site B. Melitensis viciae 2 (M5-90, M28, ATC23457) had hundred percent homology . (Figure 7)

Figure 5: omp2a genomic sequencing for all 18 samples tested positively With the PCR in Software Bioedite

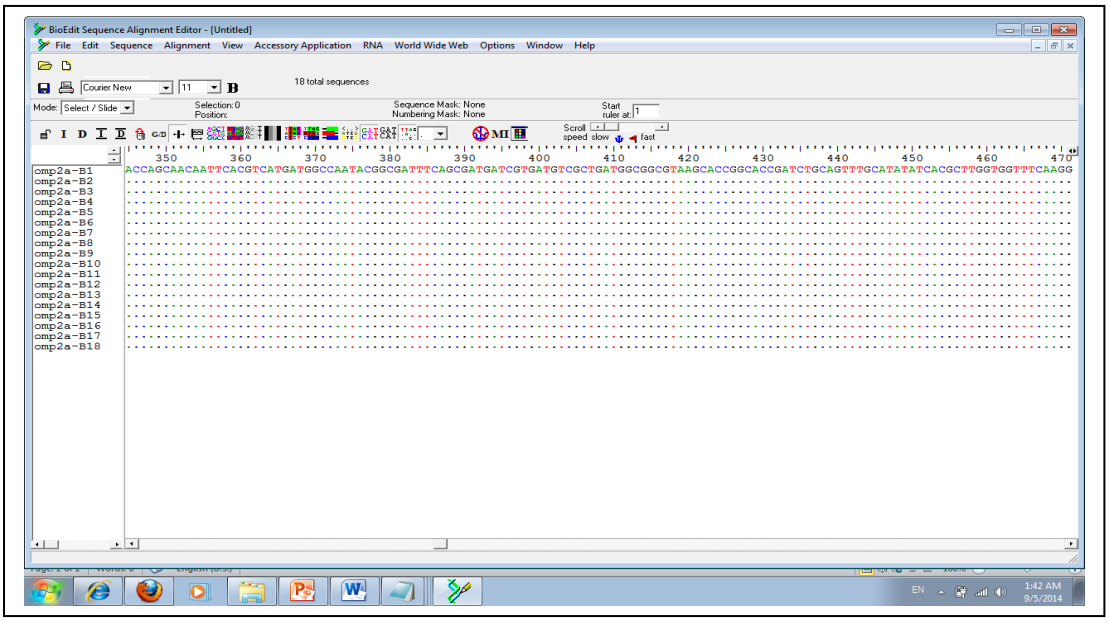


Figure 6: mp2b genomic sequencing for all 18 samples tested positively With the PCR in Software Bioedite

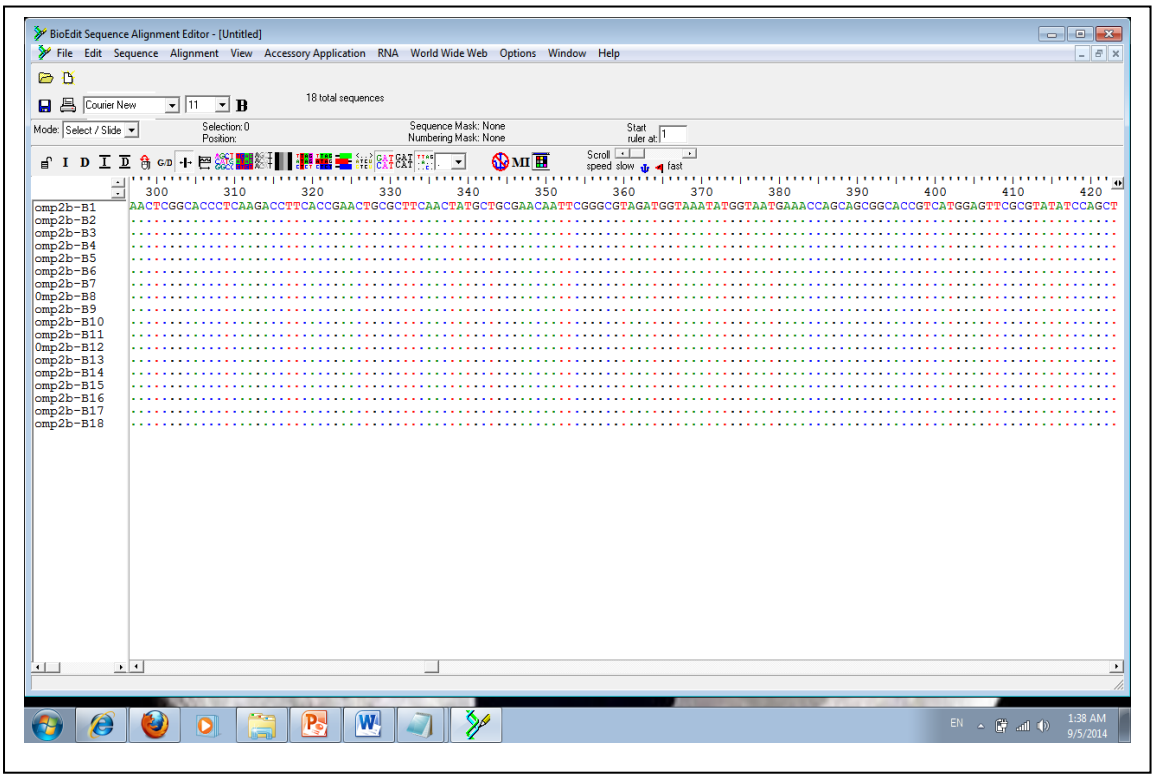
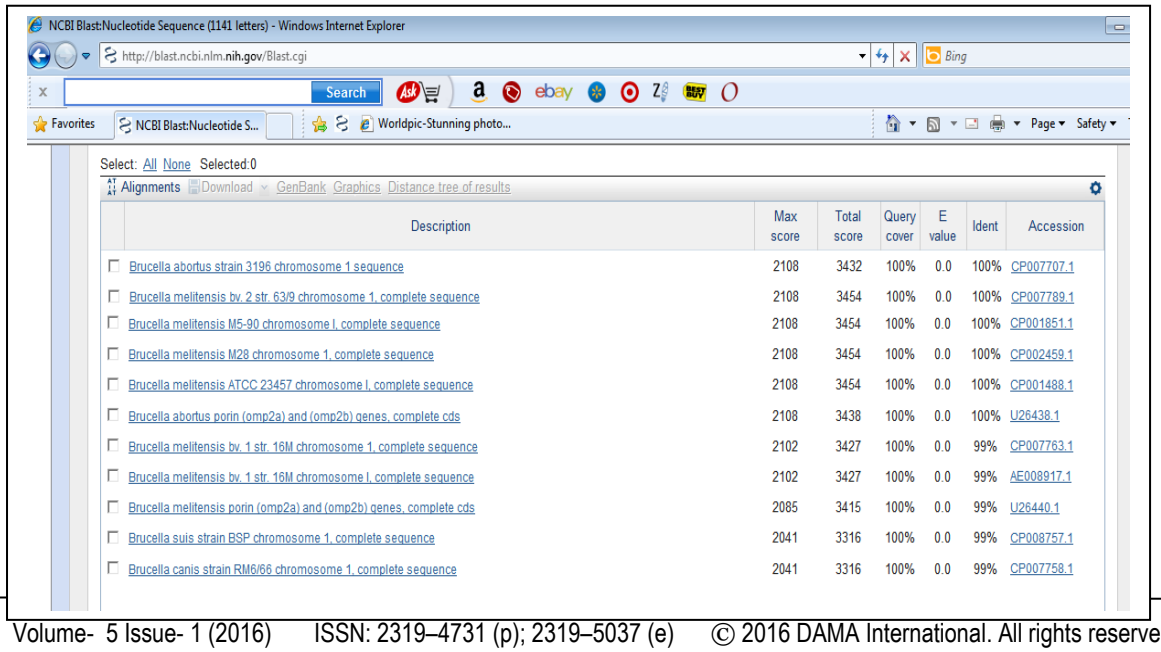


Figure 7: compares the complete genome sequence of each sample on NCBI (Gen bank) and observe100% of homology with Mlitynsis Brucella biotype 2



Description	Max score	Total score	Query cover	E value	Ident	Accession
Brucella abortus strain 3196 chromosome 1 sequence	2108	3432	100%	0.0	100%	CP007707.1
Brucella melitensis bv. 2 str. 63/9 chromosome 1, complete sequence	2108	3454	100%	0.0	100%	CP007789.1
Brucella melitensis M5-90 chromosome I, complete sequence	2108	3454	100%	0.0	100%	CP001851.1
Brucella melitensis M28 chromosome 1, complete sequence	2108	3454	100%	0.0	100%	CP002459.1
Brucella melitensis ATCC 23457 chromosome I, complete sequence	2108	3454	100%	0.0	100%	CP001488.1
Brucella abortus porin (omp2a) and (omp2b) genes, complete cds	2108	3438	100%	0.0	100%	U26438.1
Brucella melitensis bv. 1 str. 16M chromosome 1, complete sequence	2102	3427	100%	0.0	99%	CP007763.1
Brucella melitensis bv. 1 str. 16M chromosome I, complete sequence	2102	3427	100%	0.0	99%	AE008917.1
Brucella melitensis porin (omp2a) and (omp2b) genes, complete cds	2085	3415	100%	0.0	99%	U26440.1
Brucella suis strain BSP chromosome 1, complete sequence	2041	3316	100%	0.0	99%	CP008757.1
Brucella canis strain RM6/66 chromosome 1, complete sequence	2041	3316	100%	0.0	99%	CP007758.1

DISCUSSION

1).Brucellosis is a zoonotic disease of domestic and wild animals which can also be transmitted to humans directly and indirectly (1) in the diagnosis of brucellosis, it cannot be based on clinical symptoms, as could be confused by other diseases such as malaria, typhoid ; So to detect microorganisms by culture, serological and the molecular methods is needed . This study was performed on 340 samples, infection percentage in sheep, cattle, goats and sheep were positive in following amounts: 4/32 and the 5/28 and the 8/13 and the 4/13 respectively. Compared with the study that doctor Massoud Ghaffari performed in 1382, on 4651 cows and the 6380 sheep , respectively 66 samples of cow and 21 sheep sample a were positive. In this study, 35 positive samples of animal and the human cases which their being positive was approved through serology (Rose Bengal-Wright Wright -Kombus -2ME) were studied. Strains of Brucella were separated only from 18 samples. And the process of separation took place with culture methods and biochemical tests and PCR. Brucella Melitensis species which were diagnosed, compared to a study in Mexico done by Rosemary and partners in 2012 to identify Brucella species by using 153 samples of human and cow and dairy products, which were carried out by using a PCR test, As a result, 101 samples of Brousella Mlitynsi and 51 samples B. abortus and a sample of Brousella Switzerland were reported.

In this study from 35 blood samples from the suspected animal and human cases and Wright titration 80/1 along with clinical signs of brucellosis, 18 samples of Brucella bacteria with their expansion in culture of Castaneda were isolated and then cultured on Brucella agar and in 51 results were positive, in this regard, studies conducted by researchers including Abolfazl Gholipour and colleagues in which 100 samples of aborted fetuses of sheep with Brucella Melitensis with Wright titr more than 80/1 , Only 5 samples of Brucella bacteria Melitensis has isolated from human and bacteria Brucella Mlytinsis sample was isolated from aborted fetuses animals in which only 15% of cases were positively serologic and 12% of bacterial cultures become positive. With more than 80/1 Wright titr, Only 5 samples of bacteria Brucella Melitensis has isolated from human and bacteria Brucella Mlytinsis sample was isolated from aborted fetuses' animals in which only 15% of cases were positively serologic and 12% of bacterial cultures become positive.
that lower degree of results of the Brucella Melitensis isolation from human towards the study can raise 3 reasons: (1) it is likely that an increasing number of suspected cases seen in study of Mr. A. Gholipour be in the chronic stage of brucellosis disease. And at this stage, separation is too difficult and probably more cases of Prepared samples have been in the acute phase of the disease.
2). In endemic areas, patients who are serologically positive to the disease will face high prevalence. Markazi province and city Zarandieh belong to endemic areas, Patients suspected to brucellosis may also suffer from other chronic diseases and only have positive serum for brucellosis, the Samples with 320/1 titr and a higher likelihood of isolation of Brucella , are more. In a study carried out in Turkey in the city of Kars By Mr. Erdogan and Atabay woked on aborted Fetus of animals, using PCR-RFLP testing with gene amplification of omp2a from 37 samples, 14 samples of B. Melitensis were isolated (12) Compared to Present study which has done with the proliferation of genes omp2a and omp2b, on 35 of animal and human samples, it has found that Mlitinsis Brucella biovar 2 occurs in the city Zarandieh.

CONCLUSION

In this study, 35 blood samples of Animal and human which have positive serologic tests for Brucella; Only 18 samples, which were cultured on Brucella agar, have been confirmed. Test results determined the species by Biochemical and PCR methods and all isolates of Brosa Melitensis are classified in biotype 2 and Brucella Melitensis biotype 2 is a common species in city of Zarandieh. By supplying blood from animal and human ill cases, and in order to being informed from arrival of new species and strains of Brucella, in the city Zarandieh, Applying culture method should be periodically, Results of this study indicates the PCR method sensitivity as well as its higher speed than traditional serological methods and culture, and also the risk of infection to staff, will reduced into least rate..

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