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INVESTIGATING EFFICIENCY OF PCR METHOD IN DIAGNOSIS OF BRUCELLOSIS, COMPARING TO SEROLOGIC METHODS

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ABSTRACT

Brucellosis is one of the common diseases between human and animals that has worldwide outbreak. The mentioned disease is important due to its economic and health effects. Common diagnosis methods of brucellosis are always faced many limitations and problems in the laboratories. Hence, currently researchers have focused their attention on the molecular methods, especially PCR method. At the present study, sensitivity and efficiency of PCR method in diagnosis of brucellosis, comparing to serologic methods, has been evaluated. For this purpose, 30 blood samples suspected to brucellosis, along with their culture results, have been collected from the department of infectious diseases of Imam Khomeini Hospital Tehran. Then, common tests of serology such as Tube Wright, Rapid Wright, Rose Bengal, and 2ME tests have been conducted on all blood samples. DNA has been then extracted from all provided samples and then PCR test has been conducted using specific primers of genus. Perfect sensitivity of PCR method at the present study has proved high efficiency of the mentioned test in correct diagnosis of patients, especially endangered individuals.

KAYWORDS: Brucellosis, PCR Method, Serologic Methods, DNA Extraction

Brucellosis is a common disease between human and animals, which would be resulted from different species of gram-negative bacteria. The mentioned disease can cause numerous problems in terms of public health around the world, especially in Middle East and Iran. Success in diagnosing the mentioned disease is depended on using suitable and careful diagnosis methods. The Brucella genus has been composed of 6 classic species based on diversity of specific host including Brucella Abortus (cattle and buffalo); Brucella Ovis (sheep); and Brucella Suis (pig). Some species would be classified in different Biovars based on phenotypic properties. The number of species in the genus has been increased as a result of recent explorations about presence of the mentioned bacteria in marine mammals.

DIAGNOSIS OF BRUCELLOSIS

Golden standard method of diagnosing brucellosis is based on separating suspected colonies from samples such as body tissues of host, milk, or vaginal discharges on specific culture medium. The mentioned colonies would be then confirmed by bacteriologic diagnosis methods. The main defects of culture method can be firstly being time consuming since sampling time to exact detection of bacteria, which would take usually 2 weeks. During the period, rancher and livestock feed manufacturer would face many problems and hardships.

On the other hand, in human samples suspected to brucellosis, many efforts and hardships would be required till perfect diagnosis of the disease. Secondly, culture method has been relatively complicated and should be conducted in standard laboratories and by skillful individuals, since confirmation of many diagnosis properties of such bacterium like colony morphology needs specific skill and experience. Thirdly, common nature of the disease between human and livestock is a potential threat for laboratory personnel during culture and detection of the bacterium. Finally, because of large number of diagnosis properties and insignificant differences among different species and biovars and also occurrence of minor mutations, which would be resulted in unadjusted results and complicated interpretation of them. Hence, final results would face uncertainty. Serologic tests based on detecting anti-brucellosis antibodies, have better performance than culture technique; while their specific properties is less than culture method, especially in endemic regions or individuals involved with brucellosis. On the other hand, pseudo-positive results have been also observed in cases suffering from diseases such as Salmonelosis, tularemia, cholera, lupus erythematosus, and myeloma. However, there are also pseudo-negative results observable in primary stages of the disease or in cases of focal infections. Recently, different kinds of genetic methods

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have been designed for detection of brucellosis based on 16SrRNA or genetic locus of brucella's external layer proteins. The main objective of the mentioned methods is determining polymorphism in genus belonging to specific genetic locus using PCR method.

Presence of malt fever was confirmed for the first time by Pasteur Institute through separating Brucella Melitensis from culture of human blood in 1931. Dr. Kaveh (1944) separated Brucella Abortus from aborted fetuses of cattle in Tehran's cattle farms in the Razi Institution Iran. Accordingly, the affective factor in cattle abortion in Tehran was detected. Dr. Ansar (1948) separated the first case of Brucella Melitensis and later, Brucella Sius was separated also by Dr. Ebadi and Dr. Zoghi (1971). [1]

Contamination of livestock in 1948 was tested by complement fixation and sero-agglutination tests on 3647 blood samples of different animals by Dr. Tajbakhsh and Gutel. Obtained results from their examinations have been reported as follows: in human, the value was reported to 5.5% and since then till now, the disease has been progressed. [1]

Table 1: contamination of livestock

Name of animal	Contamination rate
Sheep	2.24
Goat	2.18
Cattle	12
Horse	0.73
Pig	17.6
Dog	4.87
Buffalo	5.5

Prevalence of the brucellosis in the world and Iran

Brucellosis, which has been existed in the domain of Mediterranean and Malt Island, North Africa, France, and Italy, is currently existed in most countries around the world. Except for several countries including Norway (1952), Swedish (1957), Finland (1960), Denmark (1962), Switzerland (1963), and Romania (1966) that have eradicated the mentioned disease, all remained countries are suffering more and less from the disease. In Canada, the disease has been controlled to some extent. In Bulgaria, Italy, Spain, Egypt, Cuba, England, Yugoslavia, and Greece, brucellosis is existed less and more. In Syria, Argentina, and Russia, the mentioned disease has been limited to specific regions and districts. In Mexico, Netherland, Austria, Luxemburg, and Ethiopia, the disease has been distributed in wide range. [1][2]

In general, the disease is existed in all regions of Iran, although its severity and weakness is varied in different zones. In some regions the contamination is more than other areas because of low class of hygiene and health culture and also high rate oflivestock density. [1][2]

HUMAN BRUCELLOSIS

Brucellosis has been existed for many years and has not been eradicated in most developed countries till now.High prevalence of the disease has been detected in some geographical regions; although it may be considered inconsiderable.

Contagion of brucellosis to human would be occurred through using non-pasteurized dairy products from animals or through direct contact with some parts of infected animals or through inhalation of infectedaerosol particles. [3]

CULTURE

Definite diagnosis of brucellosis needs separation of bacterium from the blood sample or tissue. Culture of blood is still a standard method and is often effective in acute phase of the disease [3]. Blood culture should be conducted several times, especially in febrile stage. Based on individual experimental methods and that how cultivars would be followed, sensitivity of blood culture would be varied. Percentage of cases with positive cultures can be from 15 to 70%. [4]

Bone marrow culture would be more successful than blood culture, especially in case of acute and chronic stages. Bone marrow culture would be considered as the golden standard for detection of brucellosis, since relatively high density of bacterium in the reticuloendothelial system can facilitate detection of microorganisms. In addition, removing bacteria from bone marrow is equal to microbial eradication; although sampling bone marrow for culture has been remained as an invasive and painful technique and its results have not been iterated around the world [5]. Urine culture should be conducted several times and in presence of lumbar puncture, CSF culture should be also conducted. All biopsy samples and Pus should be tested in terms of presence of brucella and medium should be stored for 4-8 weeks. Sometimes, brucella would be separated from

phlegm, bile, fetus twin, milk, vaginal discharges, and human seminal fluid. [6]

PCR

There is no doubt that among applied different methods of Genetic Engineering for rapid detection of infectious factors, PCR method has a specific position, which has been developed significantly over the years, being an emerging technique. The mentioned method has many applications in different domains of health and medical sciences, from which rapid detection of pathogenic factors (bacteria, viruses, parasites, and yeasts) and their toxins. [7]

Marston (1861) was the first person, who found about presence of brucellosis disease in the Malt Island and then named it under the title of Mediterranean or recurrent gastric fever. [8]

Zammit (1905) has found that goats are source of infection factor and would excrete microbes through milk and urine with no physical appearance and then would infect individuals. He also found that microbe source goats and their body would be prepared for growth of the microbes immediately after being pregnant. Afterwards, the goats will abort their fetuses as a result of activation of microbe in their body. In addition, he has referred role of goats in infecting human to malt fever and has found that blood of infected goats can contaminate and agglutinate factor microbe of fever i.e. agglutination response would become positive. [8]

Evans (1918) has found that structure of infectious microbes in human, cattle, pig, and buffalo are similar to each other. He has also indicated that human undulant fever can also cause abortion in cattle as a result of abortion microbe. Later during 1914-1918, it was found that mammals, dogs, cats, and birds are also sensitive to the mentioned disease. Shave and Mir (1941) named the mentioned bacteria as Brucella.

The study aims at investigating comparison of traditional diagnosis methods of brucellosis including culture, agglutination, and 2ME with specific PCR method on 30 human blood samples suspected to brucellosis. The study aims also at sensitivity evaluation of the samples, comparing to culture method as the golden standard for brucellosis diagnosis. Necessity of conducting such studies, especially in Iran, is clear due to prevalence of brucellosis disease in order to choose the best diagnosis methods and also design a suitable strategy in order to eradicate the mentioned disease.

METHODOLOGY

Common tests of serology such as Tube Wright, Rapid Wright, Rose Bengal, and 2ME tests have been conducted on all blood samples. DNA has been then extracted from all provided samples and then PCR test has been conducted using specific primers of genus. 30 blood samples were collected from suspected patients to brucellosis, along with results of blood culture, from infectious department of Imam Khomeini Hospital Tehran. The patients had some symptoms such as irregular fever, shiver, restlessness, insomnia, sweating, and muscular pains. In addition, time of contacting with livestock or consuming non-pasteurized dairy products was also provided.

All blood samples were stored in the laboratory under temperature of -20°C until time of conducting experiments.

Applied Brucella strains in this study included Brucella Abortus(S99), Brucella Melitensis (16M), and Brucella Suis (A/132). The mentioned bacteria have been cultured under 37°C and then have been typed and confirmed using standard methods. The mentioned strains have been used as positive control in PCR response. Additionally, extracted DNA from E. coli bacterium has been also considered as the negative control. In order to conduct exact investigation and also in order to confirm or reject correlation between PCR results and culture methods, chi square (X2) method has been applied.

DISCUSSION AND RESULTS

Results of culture

Obtained results from culture of 30 studied samples have been as follows: 10 items (33.33%) have been positive items. It should be mentioned that the results, along with the samples, has been transferred to Tehran's Pasteur Institute from Imam Khomeini Hospital.

Table1: obtained results from culturing 30 samples

Sample No	Culture	Sample No	Culture
1	Neg	16	Neg
2	Pos	17	Pos
3	Neg	18	Pos
4	Pos	19	Neg
5	Pos	20	Neg
6	Neg	21	Neg
7	Pos	22	Pos
8	Neg	23	Neg
9	Neg	24	Pos
10	Neg	25	Neg
11	Neg	26	Neg
12	Neg	27	Neg
13	Pos	28	Pos
14	Neg	29	Neg
15	Neg	30	Neg

Results of PCR test

22 out of 30 samples have been positive that the value is an equivalent for 76.66%.

Table 2: PCR results for 30 studied samples

Sample No	PCR test	Sample No	PCR test
1	Neg	16	Pos
2	Pos	17	Pos
3	Neg	18	Pos
4	Pos	19	Pos
5	Pos	20	Pos
6	Neg	21	Pos
7	Pos	22	Pos
8	Neg	23	Neg
9	Neg	24	Pos
10	Pos	25	Pos
11	Neg	26	Pos
12	Neg	27	Pos
13	Pos	28	Pos
14	Pos	29	Pos
15	Neg	30	Pos

Results of Rose Bengal test

Obtained results from the test on 30 blood samples have been as follows: out of 30 samples, 18 samples (60%) have become positive.

Table 3: obtained results from Rose Bengal test for 30 samples

Sample No	Rose Bengal test	Sample No	Rose Bengal test
1	Neg	16	Pos
2	Pos	17	Pos
3	Neg	18	Pos
4	Pos	19	Pos
5	Pos	20	Pos
6	Neg	21	Pos
7	Neg	22	Neg
8	Neg	23	Neg
9	Neg	24	Neg
10	Pos	25	Pos
11	Neg	26	Pos
12	Neg	27	Pos
13	Pos	28	Pos
14	Pos	29	Pos
15	Neg	30	Pos

Results of Rapid Wright test

Through conducting the test on studied samples, it was found that 19 out of 30 samples (63.33%) were positive. 8 samples that have negative culture become positive through this test and it could be because samples have been collected after acute stage of the disease from patients.

Table 4: obtained results from Rapid Wright Test for 30 samples

Sample No	Rapid Wright test	Sample No	Rapid Wright test
1	Neg	16	Pos
2	Pos	17	Pos
3	Neg	18	Pos
4	Pos	19	Pos
5	Pos	20	Pos
6	Neg	21	Pos
7	Neg	22	Neg
8	Neg	23	Neg
9	Neg	24	Pos
10	Pos	25	Pos
11	Neg	26	Pos
12	Neg	27	Pos
13	Pos	28	Pos
14	Pos	29	Pos
15	Neg	30	Pos

Results of Tube Wright test

19 out of 30 samples have become positive through conducting Tube Wright test (63.33%). Titers above 80 would be considered as positive samples.

Table 5: obtained results from Tube Wright test for 30 samples

Sample No	Rapid Wright test	Sample No	Rapid Wright test
1	Neg	16	Pos
2	Pos	17	Pos
3	Neg	18	Pos
4	Pos	19	Pos
5	Pos	20	Pos
6	Neg	21	Pos
7	Neg	22	Neg
8	Neg	23	Neg
9	Neg	24	Pos
10	Pos	25	Pos
11	Neg	26	Pos
12	Neg	27	Pos
13	Pos	28	Pos
14	Pos	29	Pos
15	Neg	30	Pos

Results of 2 ME test

Obtained results from this test on 30 samples have been as follows: 12 out of 30 applied samples (40%) have been positive. Titers above 40 have been considered as positive samples.

Table 6: obtained results from 2ME test for 30 samples

Sample No	Rapid Wright test	Sample No	Rapid Wright test
1	Neg	16	Pos
2	Pos	17	Pos
3	Neg	18	Pos
4	Pos	19	Neg
5	Pos	20	Neg
6	Neg	21	Pos
7	Pos	22	Neg
8	Neg	23	Neg
9	Neg	24	Pos
10	Neg	25	Neg
11	Neg	26	Neg
12	Neg	27	Neg
13	Pos	28	Pos
14	Pos	29	Neg
15	Neg	30	Neg

Table 7: different characteristics of serology and PCR methods based on obtained results fro, the present study and considering culture results

Test name	Sensitivity (%)	Characteristics (%)	Accuracy (%)	Spurious response (%)		Prediction power (%)	
				Neg	Pos	Neg	Pos
PCR	100	45	63.33	0	55	100	47.61
Rose Bengal	70	45	53.33	30	55	75	38.88
Rapid Wright	80	45	56.66	20	55	81.81	42.10
Tube Wright	80	45	56.66	20	55	81.81	42.10
2ME	90	45	86.66	101	15	94.44	75

In order to conduct exact investigation and also in order to confirm or reject correlation between PCR results and culture methods, chi square (X^2) method has been applied. Estimated X^2 value has been equal to 4.46. Desirable value of X^2 has been in confidence level of 0.05 and DF of 1. Distribution table of X^2 has been equal to 3.84. As a result, with low error possibility, correlation between obtained results from the two methods could be confirmed. In other words, statistically, the studied results would be significantly correlated and assumption lack of correlation would be rejected[13].

In the X^2 test, obtained value has been equal to 0.156. Hence, there has been no correlation between obtained results from PCR and Rose Bengal Tests.

The table of consistency between PCR results and Rapid Wright indicates uniform distribution of positive results of PCR in both positive and negative groups of Rapid Wright test. Through more exact assessment by X^2 test, the obtained value has been equal to 0.879. Desirable confidence level of X^2 has been 0.05 and DF has been equal to 1 in distribution table of X^2. Therefore, correlation between obtained results from PCR test and Rapid Wright test has been rejected.

In the X^2 test, value of X^2 has been obtained to 0.879. Hence, presence of correlation between results of PCR test and tube Wright test has been also rejected.

Using X^2 test, correlation between PCR results and 2ME results has been investigated. X^2 has been equal to 15.625. Since calculated X^2 value has been more than the desirable value for confidence level of 0.05 and DF of

1, correlation between PCR and 2ME results has been confirmed [13].

At the present study, PCR method has been evaluated, comparing to methods of Tube and Rapid Wright, Rose Bengal test, and 2ME. Affluence of infections without no specific symptom in ranchers and also presence of pseudo-positive items in the Wright test has been because of several reasons including auto agglutination, experience of vaccination, suffering from infectious diseases as a result of Pacteurella Tularensis, Yersinia enterocolitica, and Q fever agent, and also lack of titrating consumed antigen, along with international standard antiserum. On the other hand, pseudo-negative responses have been obtained through reasons including Prozone Phenomenon, infected by species Brucella Canis, low level of IgM in chronic situation of the disease, influence of imperfect immunoglobulin (Ig) in occupation of antigenic receptors, defect of anti chorea making system, low level of immunoglobulin, and lack of formation of specific antibodies at the early stages of illness. This would indicate that the test by itself can't have a decisive role in diagnosis of brucellosis disease and its results should be confirmed by obtained results form other blood tests. Additionally, in order to distinguish acute and chronic brucellosis and also reduction of negative responses of Wright test, 2ME test should be applied, since Wright test by itself would not be sufficient for this purpose. [9]

At the present study, obtained results from PCR test have been positive for 76.66% of samples and results from culture test have been positive for 33.33% of them. This can confirm that PCR method is prior to culture method for detection of brucellosis in laboratory. Moreover, due to optimization of all 3 pathogenic types ofBrucella in human, sensitivity of PCR method has been obtained more than culture method (100%). Hence, the ability of this method in correct detection of patients is significantly more than ability of culture method[15].

Through statistical investigation of studied methods, sensitivity of PCR, Rose Bengal, Rapid Wright, tube Wright, and 2ME have been obtained respectively to 100, 70, 80, and 90. Since sensitivity of PCR is more than other studied methods according to Elfaki et al (2005), it seems that this can be a reason for using an alternative for this method as diagnosis method in the laboratories. According to presented statistics in terms of determining specification of PCR test, it was found that the mentioned method has different specifications from other studied methods. In addition, since sensitivity of a test is more important than specifications of a method in order to detect a disease, it could be found that PCR method could be more applicable than other existed tests. However, specifications of 2ME method are prior to PCR test (90%). [10]

Existence of inhibitory compounds of hemoglobin in the blood sample is another reason for reduction of sensitivity of PCR test. Hence, using serum sample is better than using blood sample for this purpose. [7]

At the present study, replication of extracted DNA from species ofBrucella and also E. coli has indicated that piece 223 of base pair has been protected against all types of pathogenic Brucella bacteria including B. abortus, B. melitensis, and B. suis. Lack of replication of the piece in DNA samples, would prove specification of PCR designed as a professional genus test in molecular diognosis of Brucella through using combination of depended bacteria such as E. coli and its results have been in consistent with obtained results from other relevant studies. In other words, specifications of applied primers in detection of brucella are in high level. Since treatment of brucellosis resulted from different types of brucella would be done in same manner, commonness of primers for genus of brucella can be itself considered as an advantage for brucellosisdiagnosis [12].

Obtained results from the study indicate that there are many differences in applying PCR and serology for detection of brucellosis. In general, Rapid Wright test and Rose Bengal test can be applied as primary experiments for screening disease cases; although because of pseudo-responses, they can't be sufficient. Tube Wright test is one of the valuable experiments for medical diagnosis; although it includes pseudo-positive and negative responses. However, pseudo-negative responses would be removed through complementary test of Combos Wright and pseudo-positive responses would remain intact. Real responses would be achieved in PCR test, since the base of the mentioned test is direct diagnosis. Another advantage of the PCR method is its operating speed. [11]

CONCLUSION

At the present study, using specific primers of genus, PCR test caused replication of a 223 piece of base

pair from a 31kd protein (BSCP31) and also became positive in all standard types ofbrucella and also in all serum samples. While, culture of 33.33% of individuals (10.30) has been positive, results of tube agglutination, Rapid Wright, and Rose Bengal test have been obtained respectively to 63.33%, 53.33%, and 56.66%. Sample serum of 33.33% of individuals was also become positive in presence of 2ME. PCR test was obtained positive in 76.66% of sample individuals. Due to considering culture method as the golden standard, sensitivity and specification of PCR method in this study has been respectively equal to 100% and 45%. Perfect sensitivity of PCR method at this study has proved high efficiency of test for proper detection of patients, especially endangered individuals. At the present study, replication of extracted DNA from Brucella's genus types and E. coli has indicated that piece 223 of base pairs in all types of pathogenic brucella including B. abortus, B. melitensis, and B. suishave been protected.. Lack of replication of the piece in DNA samples, would prove specification of PCR designed as a professional genus test in molecular diognosis of Brucella through using combination of depended bacteria such as E. coli and its results have been in consistent with obtained results from other relevant studies. In this study, obtained results from PCR test have been positive in 76.66% of samples and results of culture test have been obtained positive in 33.33% of samples. This would confirm that PCR method is prior to culturemethod in laboratory diagnosis of brucellosis.

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