Production of polyclonal antibodies for establishment of antigen detection test against brucella melitensis

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Abstract: Brucellosis is an infectious disease caused by the bacteria of the genus Brucella. The pathogenic strains of Brucella are primarily passed among animals and they cause disease in many different vertebrates. The study was designed to develop a serological, antigen detection test for Brucella Melitensis. For the purpose polyclonal antibodies against Brucella Melitensis raised in rabbits. The prime as well as booster doses of commercially available antigen of Brucella Melitensis was injected in rabbits at day 0 and days 14, 21 & 42, respectively. The hyper immune sera produced in rabbits were collected at day 56. The collected serum was checked for isotype of antibodies through IgG and IgM specific Enzyme Linked Immunosorbant Assay Technique. The serum titrated (Tube Agglutination Test) for titer of antibodies raised per ml using locally available commercial antigens. The titrated serum (having antibodies titer of 3200 agglutinating units of antibodies per ml of serum) was used for diagnosis of clinical samples through slide agglutination test. Different samples including serum, vaginal and milk were collected from Brucella positive patients admitted in different hospitals of the city. These samples were checked for presence of antigen through our raised sera. The sera successfully detected antigen in all three types of samples. The positive as well as negative samples were further confirmed through Polymerase Chain Reaction. The results indicated the test developed for antigen detection is although less sensitive than PCR but specific, confirmatory and cost effective than other slide and tube agglutination tests for the detection of antibodies available in the market.

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Key Words: Brucellosis, *Brucella Melitensis*, Tube agglutination test, Eliza, PCR, IgG, IgM.

Introduction

Brucellosis is an infectious zoonotic disease caused by the bacteria of the genus*brucella.* The pathogenic strains of *brucella*are primarily passed among animals, and they cause disease in many different vertebrates. Various *brucella*species *(B. abortus*and B. *melitensis)* affect sheep, goat, cattle, deer, elk, pigs, dogs, and several other animals. Humans become infected by coming in contact with animals or animals products that are contaminated with these bacteria (WHO, 1986).

Different techniques are available in the market for diagnosis of Brucella species. Among these Polymerase Chain Reaction and Enzyme linked immunosorbantassay are the most potent and sensitive but these are very expensive and a low income person barely can afford it. (Kohler *et al.*1975).Serological methods are extensively used in different laboratories for Brucella diagnosis. The most charming method used, in terms of cost, time and labor, are Slide and Tube Agglutination Tests (based on antibodies detection) despite of fact that these are less sensitive and specific methods and don't provide clear picture regarding active infection (Becker, 1969).

It has been observed that so many diagnostic kits of antibodies detection for B. *Melitensis* are available in the market but the availability of antigen detection test for *Brucella* strains is limited. There is a great need of locally developed antigen based detection test for Brucellosis to detect active infection, have good efficiency in labor & time and provides help in epidemiological surveys. Keeping in view the above scenario the following study performed for development of antigen detection test for B. *Melitensis*.

Material and Methods

In this study Polyclonal antibodies were raised against B. Melitensisin rabbits. The antigen of Laboratory Diagnostic Company, USA (Morganville, NJ 07751) was used for production of antibodies. A total of two Groups (A &B) of rabbits were taken having twenty and ten rabbits in respective group. Group A was injected with antigens of B. Melitensis while group B was kept as Control. The animals were kept in animal houses and were provided with fresh water, grass, vegetables, and fruits and feed supplements. After maintaining sterile conditions the Group A was injected with 0.5ml antigen of B.

Melitensisat Gluteal muscles (Fig 1) while group B was injected with normal saline. The following primary and booster scheduled of antigen injected was followed.

Day 0: Primary injection
Day 14: 1st Booster injection
Day 21: 2nd Booster injection
Day 42: 3rd Booster injection
Day 56: Serum collection

The blood samples were collected on day 0, 14, 21, 42 and 56.These blood samples were allowed to clot at room temperature for 2 hours and sera were collected with sterile syringes (Schunk *et al*. 2005).

The collected sera were checked for antibody titer through tube agglutination test.

Test Tube Agglutination Test

The collected serum was analyzed via test tube agglutination test as described by Spink et al., 1952.

The antigen and serum samples to be tested were brought to room temperature and normal physiological state. The normal saline solution (0.9%) was formed in a flask and 1.9ml of this solution added to first test tube and 1.0ml to the remaining nine test tubes. Serum to be tested (0.1ml) for B. Melitensis was added to first test tube, mixed and 1.0ml of it transferred to second test tube. The two fold serial dilution was made till ninth test tube and 1ml of it was discarded (Fig 2). The tenth tube was taken as a control. One drop of antigen was added to all tubes and all tubes were placed in incubator at 37^{o}C for 48hour. The results were taken after 48 hours.

The serum collected at day 56 was checked for isotype of antibodies through IgG and IgM specific Enzyme Linked Immunosorbant Assay Technique (Magee JT.1980).

Eliza For Isotyping Of IgG And IgM In Raised Sera

The ELISA method was based on the reaction of antibodies in the sample tested with the antigen adsorbed on the polystyrene surface. Unbound immunoglobulins were washed off. An enzyme-labeledanti-humanglobulin bonded to the antigen-antibody complex in a second step. After a new washing step, bound conjugate was developed with the aid of a substrate solution (TMB) to render a blue colored soluble product which turned into yellow after adding the acid stopping solution (Magee JT.1980).

Clinical Sampling And Diagnosis

Three different types of samples were collected (table2) from brucella positive patients admitted in five different hospitals of the district Peshawar (Table 1). These samples were checked for presence of antigen through our raised sera by applying SAT.

Slide Agglutination Test (SAT)

Before conducting the test the raised serum and collected clinical sample were brought to room temperature. A clear glass slide was taken and with the help of suitable pipette one drop of clinical sample were added. Further one drop of sera rose (having 3200 Agglutinating units of antibody per ml) was added and mixed with the help of applicator. The results were observed under indirect light and against dark background (Spink et al., 1952). The positive as well as negative samples were further confirmed through Polymerase Chain Reaction (PCR) to draw the final conclusion (Shanghai ZJ Bio-Tech Co., Ltd).

Results

The average antibody titer of the serum raised for B. Melitensis on day 0, 14, 21, 42 and 56 was 560, 1360, 2880, 4800, and 5760respectively.

The serum taken on day 56 was positive only for IgG Isotype of antibodies raised against B. Melitensis (Fig 5).

The serum having antibody titer of 3200 agglutinating units of antibody per ml was selected for the diagnosis/screening of clinical samples. Sero-Positive fifty Brucella patients from different Hospitals (Table 1) were included in this study. Different samples; fifteen milk, fifty bloodand fifty vaginal, were taken from fifty patients. It was foundeight milk, twenty five seraand thirty two vaginal samples werepositive out of total collected samples (Table 2).

All collected samples were confirmed further through Polymerase Chain Reaction (PCR) test (Fig 7). PCR provided eight samples positive in milk, twenty five samples in sera and thirty two samples invaginal secretions (Table 2). These results further indicated that the test sera raised gave 20%, 15% and 20% false negative results with milk, sera and vaginal secretion samples, respectively (Table 2). No false positive results were observed in the study.

Discussion

Brucella is a gram negative intracellular bacterium. It is one of the most widespread zoonosis in the world. Clinically the disease is diagnosed by several techniques such as isolation of the organism from the infected human, detecting the presence of antibodies in the serum of infected person through Slide Agglutination Test & Enzyme Linked Immunosorbant Assay and Molecular Techniques especially Polymerase Chain Reaction. Detection through Molecular Techniques is expensive and laborious. Most of the diagnostic laboratories prefer to use serology especially Slide Agglutination Test for screening purposes of Brucella species due to relative benefits in cost, time and labor. The available serological tests in the market are based on detection of humoral immune response which is always quite dubious in terms specificity and rarely help in exact diagnosis of active infection of Brucella. In addition, most of the kits available in the market for initial

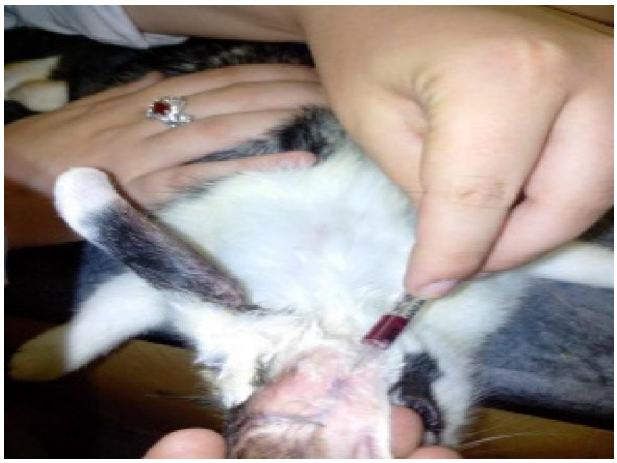
screening are imported from other countries and thus utilizing precious foreign exchange. Keeping in view these market needs and problems this study was designed to develop local antigen detection test for Brucella Melitensis so that it helps the diagnosticians in reduction in cost and time and diagnosis of active infection.

Multiple booster injection used in this study to raise hyper immune sera against B. Melitensis so that to have a higher concentration of Immunoglobulin G and negligible amount of Immunoglobulin M as the latter show more cross reactivity as compared to IgG. The same results described by the previous studies performed by (Corbel MJ. et al. 1985,Allan G et al. 1976, Nielsen K et al. 1984, Lamb V et al. 1979, Butler J et al. 1986).

As discussed antibody detection tests don't provide the final conclusive results. This study attempted to observe the results of antigen detection test in different tissues of Brucella patients instead of using antibody detection test for diagnosis. This study returned quite good results while treating the raised antibodies with different clinical samples. Previous studies claimed blood/serum as a choice for serology (Bundle DR et al. 1987, Corbel MJ et al 2006) but in this study good result obtained even with milk and vaginal samples.

Out of 50 sero-positive patients 18 patient's sera samples reacted with raised antibodies. Similarly, out of fifty vaginal samples 22 samples give agglutination with the raised antibodies while 5 milk samples agglutinated out of 15 samples, showed the presence of antigen in these samples and in turn true positive cases. The study clearly depicted that the blood is not only a sample of choice (Poester FP et al. 2010) but other tissues and body secretion can also be used for diagnostic purposes in conjunction with other confirmatory tests as compared to relying only on presence of antibodies detection tests that are less specific or less sensitive (Allan G et al. 1976, Nielsen K et al. 1984, Lamb V et al. 1979, Butler J et al. 1986).

Furthermore, all samples (Milk, Serum and Vaginal) were further confirmed with Polymerase Chain Reaction to have an idea about false positive and false negative cases. It showed 8, 25 and 32 positive samples of milk, serum and vaginal secretions respectively showing false negative results of 20%, 15%, and 20% with milk, serum, and vaginal secretions in turn (Table 2) through antigen detection test developed. It is in agreement with (Maher, A.M., 2010) that PCR is more sensitive technique as compared to serological tests. Meanwhile no false positive test was observed. That further increase the reliability of the test developed.



A

B

Fig 1. Slide A and B shows collection of blood from rabbits and the booster's injection of antigen respectively.

Figure 2. Serum dilution from test tube 1-9.

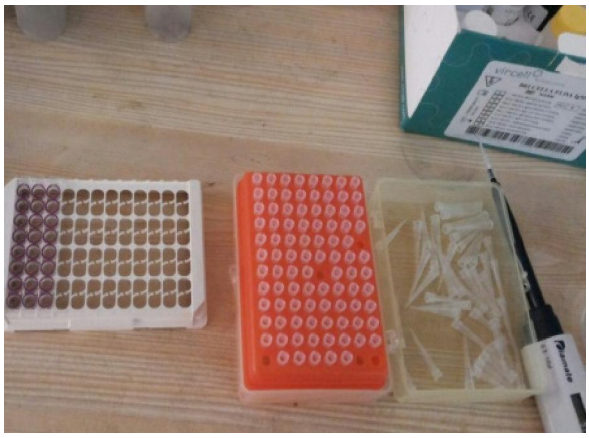


Fig. 3. Eliza kit for IgG and Ig Misotype detection.

Table 1. Name of Hospitals

S.NO.	Name of Hospitals
1	Hayatabad Medical Complex
2	Khyber Teaching Hospital
3	Johar Maternity Hospital
4	Lady Reading Hospital
5	Govt. Maternity Hospital Hashtnagar

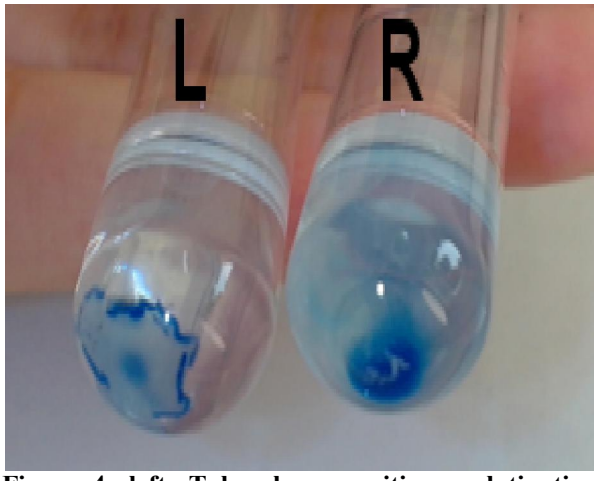


Figure 4: left: Tube shows positive agglutination reaction. Right: Tube shows negative agglutination reaction (control).

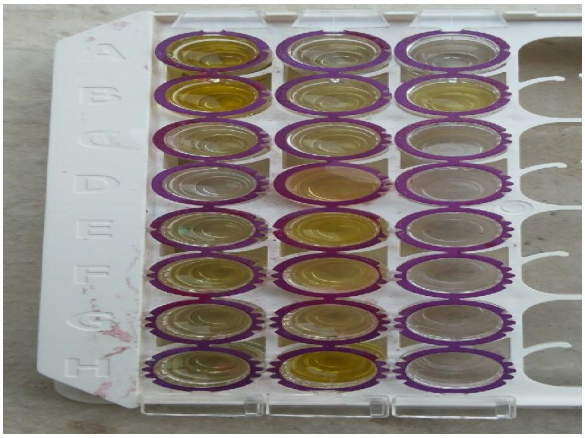


Figure 5. ELIZA for isotypeing of IgG in raised sera.

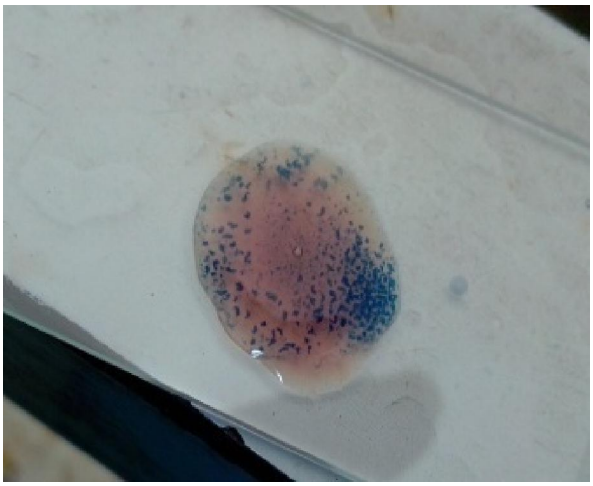


Fig. 6. Positive Slide agglutination test.

Table 2(Sampling)

Sample identity (n)	Milk sample (15)	Serum sample (50)	Vaginal swab (50)
Positive with sera developed	5	18	22
Positive with PCR	8	25	32
Percentage of false negative samples	20%	15%	20%

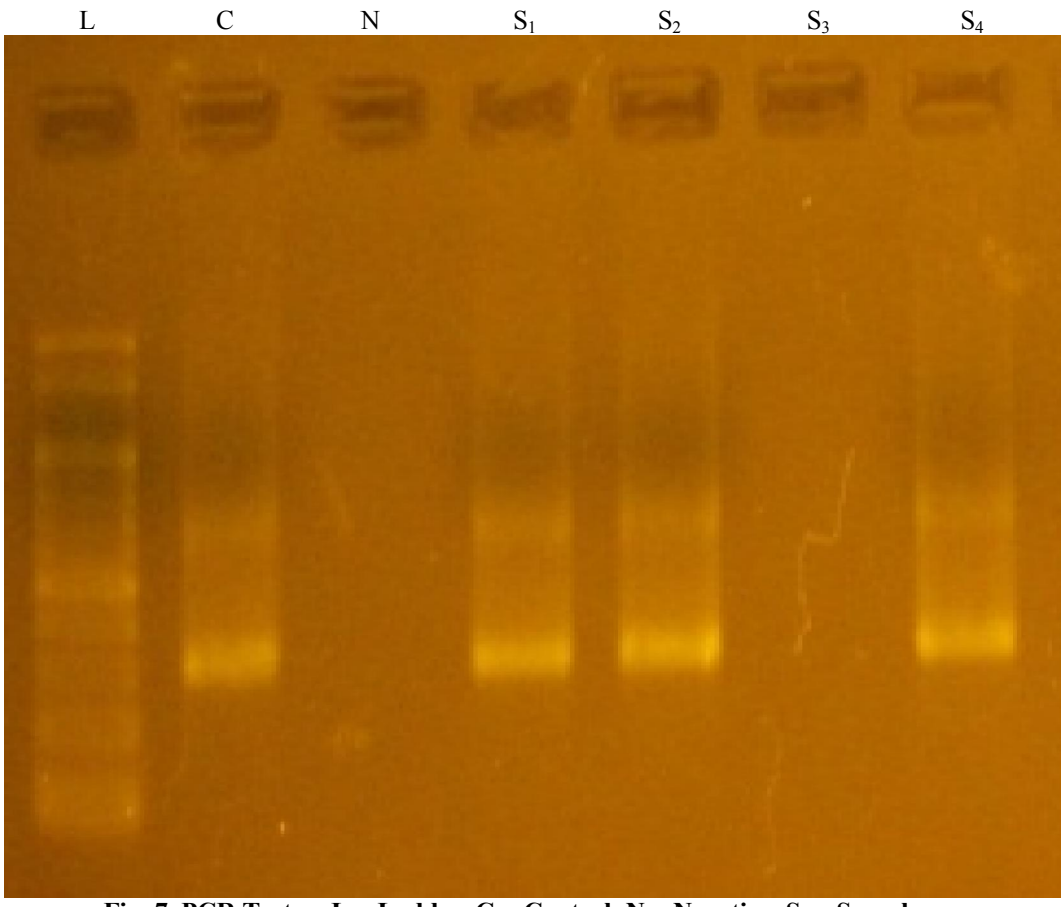


Fig. 7. PCR Test. L = Ladder, C = Control, N = Negative, S_n = Sample no.

Conclusion

In conclusion the antigen detection test through raising Brucella specific antibodies is a good alternative to antibody detection test in the market although it has sensitivity issues, but still relatively more reliable because of being detecting antigen, less costly and providing flexibility to check diverse samples as compared with antibody detection tests.

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