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Culture of *Borrelia persica* and its Flagellar Antigen *in vitro*

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Abstract: *Borrelia persica* is a strain seen only in the Middle East and responsible for relapsing fever. These spirochetes are notable for multiphasic antigenic variation of polymorphic outer membrane lipoproteins, a phenomenon responsible for immune evasion. Diagnosis of the disease is a problem and requires a fixed antigen like the flagellar antigen. *In vitro* culture of *B. persica* was carried out for the first time and flagellar antigen was purified from culture. 10% SDS was added to the mixture to dissolve the cell wall and then the solution was sheared in an Omni mixer. Electron microscopy confirmed the purity of a 42 KDa periplasmic antigen as revealed by SDS-PAGE. Indirect haemagglutination kits were designed using the pure flagella and tested for cross reactivity with another relapsing fever spirochaete *Borrelia microtii* positive serum. The kit showed 98% sensitivity and 95% specificity.

Key word: *Borrelia persica*, relapsing fever, indirect hemagglutination kit

INTRODUCTION

The genus *Borrelia* comprises the arthropod vector-transmissible spirochetes. These organisms are responsible for two groups of human disease: lyme borreleosis, which is transmitted by the hard *Ixodes* ticks and relapsing fever; most of which are transmitted by the soft ticks (*Agrasidae*), the exception being a louse borne relapsing fever which is transmitted by *Pediculus humanus* (Barbour *et al.*, 1983). *Borrelia hermsii*, *Borrelia parkeri*, *B. turicatae* and *B. duttoni* are etiological agents of louse-borne relapsing disease (Burgdorfer and Schwann, 1999). In Iran, the species which cause relapsing fever are *Borrelia persica*, *Borrelia latschywii*, *Borrelia microttii* and *Borrelia balthazardi*, each of which is responsible for spread of the disease in certain areas of the country. *Borrelia persica (B. persica)* was discovered in Iran by Dschunkowsky in 1913 in the northeast region of Ardebil. Its vector is the tick *Ornithodorous thollazoni* (*O.thollazoni*) (Karimi *et al.*, 1979).

Relapsing fever is characterized by fever followed by septicemia. The high fevers of infected patients spontaneously abate and then recur. This characteristic pattern of remission and relapse not only gives relapsing fever its name but also allows it to be differentiated clinically from other febrile illnesses as it has since the 1840 sec (Dworkin *et al.*, 2008).

The relapse phenomenon occurs because of genetically programmed shifting of outer surface proteins of the *Borrelia* that allows a new clone to avoid destruction by antibodies directed against the majority of the original infecting organisms (Porcella *et al.*, 2005). Thus, the person clinically improves until the new clone multiplies sufficiently to cause another relapse.

Between bouts, no *Borrelia* is perceived in the blood, but it may be identified in the spleen, liver or the intestines. Humoral immunity rather than cellular immunity seems to play a part in recovery from and immunity to relapsing fever (LaRocca and Benach, 2008). Diagnosis of Borrelia is carried out by a number of methods: Direct smear is the only specific method for diagnosis of *Borrelia* in blood (although only 70%of the patients have *Borrelia* in their blood at any given time). *In vivo* culture of blood in experimental animals can be also used for diagnosis (Stoenner *et al.*, 1982). Only guinea pigs and rabbits get fever due to *B. persica* and not mice and rats which are used for *B. microtti* another Iranian strain; these animals are used for *in vivo* diagnosis (Schwann and Hinnebush, 1998), but, serology is now the main method of diagnosis (Wojciechowska-Koszko *et al.*, 2011).

Surface targets of host immunity and potential virulence factors are the outer membrane proteins. The characterization of these rare outer membrane proteins in an organism that undergo antigenic variation is important

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for understanding the pathogenesis of relapsing fever and potentially relevant to the development of an efficacious vaccine. But, for serological diagnosis a fixed antigen is required (Lopez *et al.*, 2009, 2010). The flagella on the surface of the pathogen are one of the first antigens against which antibodies are formed (Hansen *et al.*, 1988). Flagella has been identified as the first antigen to which IgM respond if formed and is used for diagnosis in Lyme disease, IgG levels to other antigens do not show any change after treatment but antibody level to flagellin show a distinct decrease after successful treatment and hence can be used as a marker for termination of Lyme borreleosis (Panelius *et al.*, 2010; Burbelo *et al.*, 2010). *In vitro* culture of different Borrelia has been reported but *Borrelia persica* has proven to be difficult as reported by Farrah *et al.* (2010).

A culture of *B. persica* was set up for the first time and the flagellar antigen purified by the technique of Assmar *et al.* (2002b) and used for preparation of a serological kit involving an indirect haemmaglutination assay.

MATERIALS AND METHODS

Spirochetes: Iranian strains of the spirochetes *B. persica* and *B. microti* strains were maintained in *O. thollazoni* and *O. erraticus* ticks. Inoculation of guinea pigs was carried out by tick bites of *O. thollazoni*. *B. microtti* was introduced in a similar manner in white mice. Infected animals suffered from 2-3 bouts of fever of 1-2 day duration, interspersed with 3d without fever, a typical picture of relapsing fever. In guinea pigs, when the number of *Borrelia* reached 20-30 per field (dark field microscopy (X40) (Assmar *et al.*, 2002a) blood was collected and injected into other guinea pigs for *in vivo* maintenance.

In vitro cell culture: Cells were cultivated *in vitro* in BSK medium (SIGMA, USA) 7% gelatin and 10% sera. An important component is 10% rabbit serum which was replaced with either fetal calf serum or guinea pig serum. The cells were incubated (35°C for 5 d) in 18×80 cm tubes with Teflon lids, filled 2 cms from the top of the tube. Counts were carried out at different times and the generation period for each one calculated.

Infected sera: Were obtained from blood of 60 guinea pigs immunized with *B. persica* and 60 mice immunized with *B. microtti*. Sera from normal non immunized mice and guinea pigs were used as negative controls. Sera were preserved at -70°C (Assmar *et al.*, 2002b).

Sonicated antigen: *Borrelia* was grown in 500 mL BSK medium and a 3 d growth culture of Borrelia. Cells were

harvested by centrifugation at 7,000 g for 20 min and then suspended in 10 mL physiological saline. The suspension obtained was sonicated on ice at 90 Hz sec for 5 min and then centrifuged at 10,000 g for 30 min at 4°C. The supernatant was designated as antigen of *B. persica* and stored at -70°C (Assmar *et al.*, 2002b).

Purification of flagellar antigen: *Borrelia* cells (3×10^{10}) obtained from 3l of BSK media were centrifuged at 7,000 g for 20 min at 20°C and washed 4 times with sterile physiological saline and then suspended in 50 mL normal saline. Sodium dodecyl sulphate (SDS-Merck, Germany) concentrations (0.005-0.04%) were added to the cells, which were then maintained for 20 min at room temperature. They were then centrifuged at 25,000 g at 4°C. The suspension obtained contained outer envelope protein and was filtered using a 0.22 µ filter and SDS removed by dialysis for 18 h in PBS containing 30 mM MgCl$_2$ (MERCK Germany) and stored at -70°C and named as outer envelope fraction.

Flagella: The precipitate acquired for the technique described above was suspended in 50 mL sterile normal saline and the suspension sheared for 5 min in an Omni mixer at 16,000 g and then centrifuged at 25,000 g for 40 min at 4 °C and the supernatant collected each time in the same way, concentrated using PEG 6000 (Merck, Germany) and then dialyzed against 20 1 of PBS. The protein obtained was designated as periplasmic flagellar protein (pf protein).

SDS-polyacrylamide gel electrophoresis: was carried out by Laemmeli, 1972,s technique 1972, Standard molecular markers of 29, 34, 66 and 210 KD were used (Sigma, USA). Samples (20 µL) were mixed with buffer containing 2-mercaptoethanol and SDS and boiled for 2 min; they were electrophoressed on 13X10 mm² gels containing 5% stacking gel and 12% resolving gel, using 18 mA for the stacking gel and 24 mA for the resolving gel. Gels were stained with silver (Blum *et al.*, 1987). Protein was measured throughout using Bradford (1976).

Electron microscopy: Was done by the method of (Garon 1981). The 3 µL of different samples were placed on 300 mesh grids, treated with parlodin (Sigma, USA) for 30 min at room temperature, washed with distilled water and then stained with 1% Uranyl acetate (Sigma, USA). Samples were visualized using Hitachi electron microscope.

Counter immunoelectrophoresis: Was carried out using purified flagellar antigen and antibody positive serum by using barbiturate buffer (Merck, Germany, pH 8.2) and 50 mA current for 90 min (Sherris *et al.*, 2004).

Dot blotting: Purified flagellar protein was blotted on nitrocellulose and then incubated with positive serum then with secondary anti guinea pig peroxidase conjugated antibody. Bands were visualized with DAB and hydrogen peroxide (Merck, Germany) (Sherris *et al.*, 2004).

Indirect hemagglutination assay: (IHA) Sheep red blood cells were washed and sensitized with tannic acid and coated with sonicate (700 mg mL^{-1}) or flagellar protein (400 mg mL^{-1}). The test was carried out on 60 serum samples from mice immunized with *B. persica* and 60 mice immunized with *B. microtti* as test samples and 60 serum samples from non-immunized mice as controls (Herbert 1978).

Statistical analysis: The geometric mean reverse titre (GMRT) and student's t test were used for IHA-sonicate and flagella.

RESULTS

The best results were seen in BSK medium with 10% guinea pig serum (Fig. 1, 2). The lag phase of *B. persica* ranged from 18 to 24 hours and was followed by the log phase of 24-96 h and then a stationary phase of 96-112 h then followed by cell death. The number of cells obtained in this medium was 140 million mL^{-1} and the generation time was 10.97 h.

As guinea pig serum was difficult to obtain, fetal calf serum, which showed only a little difference between the time and number of cells produced was used instead for large production of *Borrelia* proteins. Rabbit serum did not give good results (Fig. 1). Bacteria collected in this way were used for flagellar purification. The outer envelope of bacteria was removed with SDS. Flagella on the outer surface of the bacteria were revealed after 5 min treatment with SDS and electron microscopy clearly showed flagella on the outer surface of the cells (Fig. 3). An outer envelope protein showed 2 bands at 31 and 34 KD on SDS-PAGE and by increasing the detergent concentration from 0.005 to 0.04% a larger amount of protein from the outer envelope of the cell was seen. There was an obvious relationship between SDS and outer envelope protein where at concentrations >0.04% the 42 KDa flagellin contaminated the outer envelope fraction. The concentration of SDS was standardized to 0.3% as it exhibited the greatest amount of flagellin in the protoplasmic cylinder fraction (Fig. 4); higher concentrations resulted in destruction of the protoplasmic cylinder fractions, as detected by electron microscopy.

Protoplasmic cylinders which had settled in the precipitate and the supernatant contained outer envelope fractions. SDS PAGE showed that the protoplasmic cylinder fractions contained a large amount of 42 KDa flagellin (fig.4 lane D). Electron microscopy revealed flagella at this stage (Fig. 4) and the concentrated precipitant showed a lot of flagella (Fig. 4 lane B). In this study, 3.3 mg of flagellin was obtained from 5×10^{10} cells.

Serology: Counter-immunoelectrophoresis and dot blotting was carried out showing a high sensitivity of the flagellar protein for the antibody positive serum. The IHA flagella kit was tested on 50 *B. microtti* positive sera, 50 *B. persica* positive sera and 60 normal sera that were used as negative controls (Table 1). GMRT of anti Borrelia sera was calculated to obtain specificity and sensitivity of kit (Fig. 5). The kits showed 98% sensitivity and
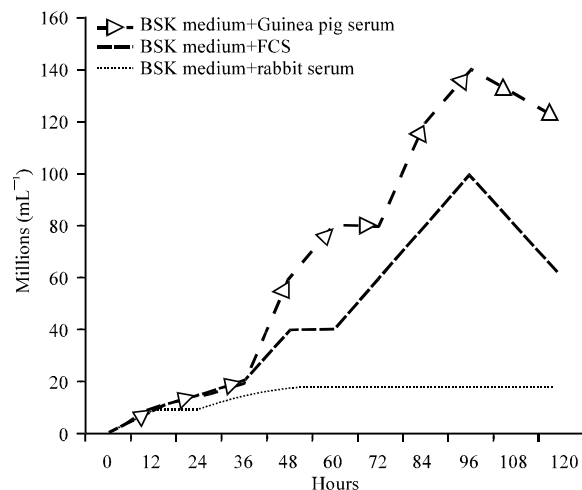

Fig. 1: Growth curve of *B. persica* in BSK medium containing different animal sera
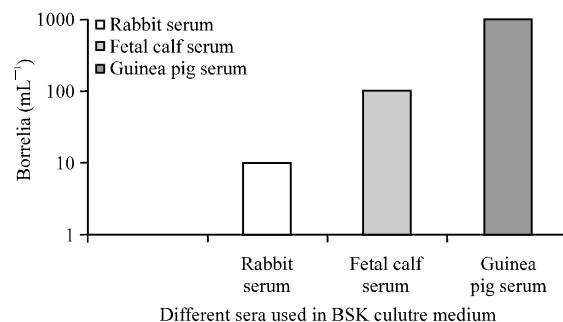

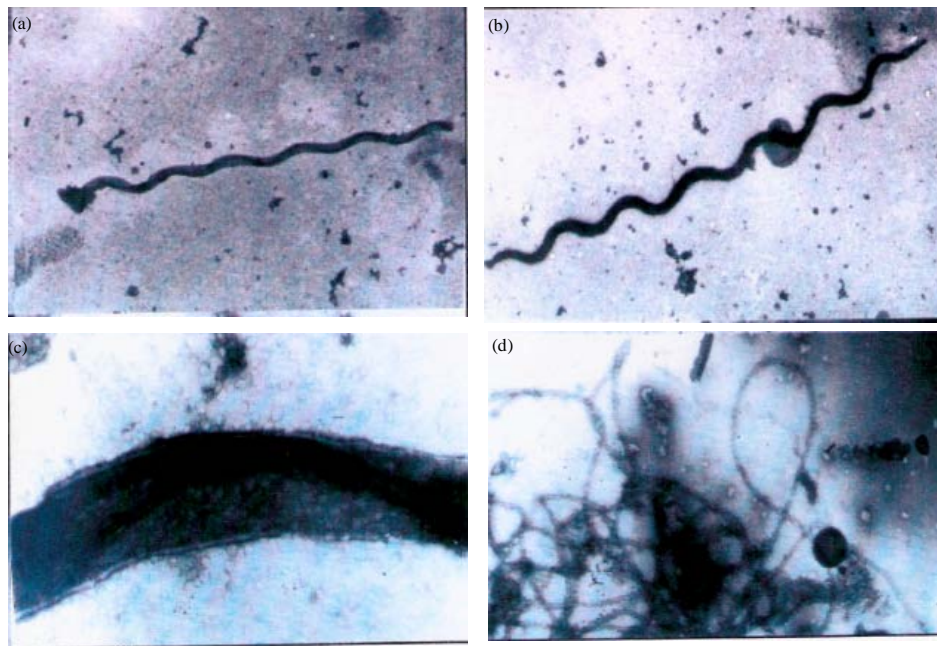Fig. 2: Effect of different sera in BSK medium on *B. persica* growth

Fig. 3(a-d): Electron microscopic photographs of different stages of Borrelia persica periplasmic flagellar purification (a) *B. persica* original magnification (OM) X15,000, (b) *B.persica* after 5 min treatment with 0.03% SDS (OM×15,000), (c) *B. persica* after 10 min treatment with 0.03% SDS (OM×20,000) and (d) Pure flagella of *B. persica* (OM×250,000)
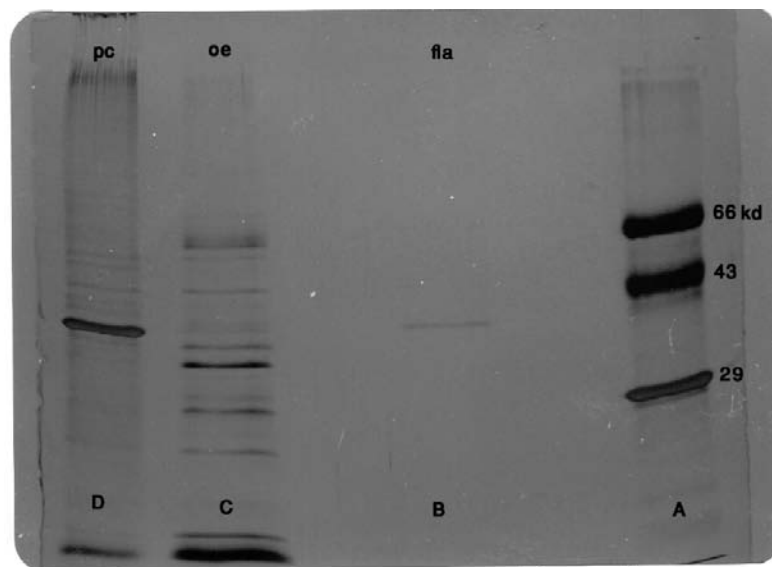

Fig. 4: 10% SDS-PAGE of *Borrelia persica* using silver staining: Lane A: Standard molecular weight markers, Lane B: Pure periplasmic flagellar antigen Lane C: Outer protein extract purified periplasmic flagellar protein Lane D: protoplasmic cylinder

Table 1: Cross reactivity of flagellar antigens of *B. presica* and *B. microtti* using *B. persica* IHA flagellar kit

| Inoculation | No. of samples | No. of positive | No. negative | Antibody titre | | | | | | GMRT[b] |
				1/2	1/4	1/8	1/16	1/32	1/64	
B. persica	50	45	5	1	2	5	8	25	4	45.3
B. microttii	50	29	21	16	9	3	1	0	0	3.25
Non immunized animals	50	12	38	6	5	1	0	0	0	3.25

[a]No. of positive samples, [b]A significant (p<0.001) difference in GMRT was observed using Student's t-test
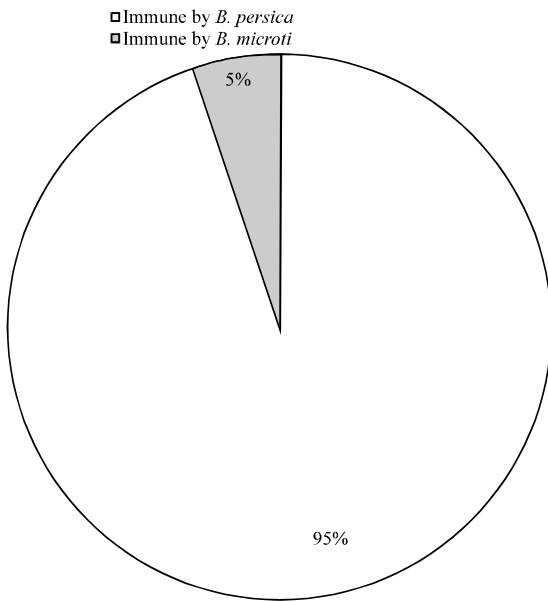

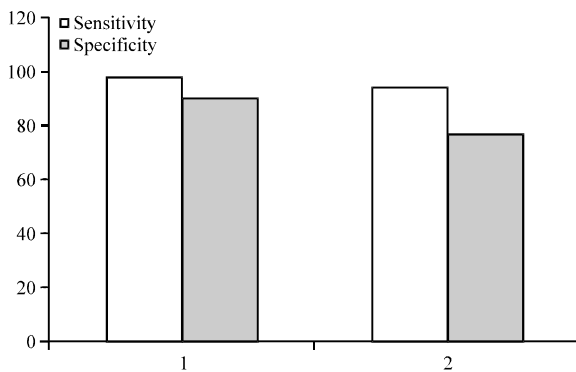Fig. 5: GMRT of anti *Borrelia* sera detected by IHA-flagella

Fig. 6: Comparison of sensitivity and specificity of IHA-flagella with IHA-sonicate

94% specificity for IHA flagella and 90% sensitivity and 76% specificity for IHA-sonicate kit (Fig. 6).

DISCUSSION

At present, the best medium for growth of *Borrelia* is BSK (Barbour *et al.*, 1983).It is comprised of an enrichment medium along with albumin and BSA. Albumin added alone or with animal serum provides long chain fatty acids as *Borrelia* are unable to cause elongation of stored fatty acids by beta oxidation. The cholesterol present in cell culture medium is required for cell structure and glucose outside cells with phospholipids are used for building cells.

BSK medium was used with 7% gelatin and 10% serums of different animals were tested to obtain enough *Borrelia* for periplasmic flagellar purification (Barbour, 1984).

To date, nobody has reported the *in vitro* culture of *B. persica*, but we have carried it out for the first time using a modification of BSK medium. Different animal sera were used. The best results for *B. persica* were seen with guinea pig sera and not with others. Gelatin increases the density of the medium which helps in the spiral like growth of the bacteria, but as obtaining this serum is difficult, fetal calf serum which is easier to get was tested and the results though not as good were commendable. Growth time was reached in 11 h. Rabbit serum did not provide appropriate results (Fig. 1, 2). Aeration of the medium was important, culture was carried out in tubes so that the CO_2 produced by the spirochete remains in the medium and CO_2 is not abundant.

The 42 KD flagellar proteins purified from *B. presica* was strain specific and could distinguish between the two species of relapsing fever in Iran. Its molecular weight which was similar to the one purified from *B. microtii* (Assmar *et al.*, 2002b) and also close to the 41 KD flagellum obtained from *B. burgodorferi* (Coleman and Benach, 1989). However, very little cross reactivity was seen between antibody positive serums of the two strains of relapsing fever causing Borreleosis. Recently, Lopez *et al.* (2010) have identified a 57 KD antigen that can discriminate between relapsing fever and Lyme borreliosis but our flagella was species specific distinguishing between relapsing fever strains.

For over a decade, ultrastructural analysis, biochemical isolation and characterization have indicated that the periplasmic flagellar filaments of *B. burgdorferi* differ from those of other spirochetes, being composed primarily of a 41 KD protein. However, a flaA homologue

of 37 KD of *B. burgdorferi* has been found, it is present at amounts one tenth that of flaB and is used for diagnosis of Lyme disease. A three step detergent treatment (1% triton X) followed by shearing in an omnimixer and cesium chloride gradient ultracentrifugation was used for its purification. (Brahmasha and Greenberg, 1989).

Sarcosyl has also been tested with poor results for flagella purification but SDS was used in this study for the first time with notable results. To date, all work has been carried out on *B. burgdoferi* responsible for Lyme disease and the only report on borrelia responsible for relapsing fever was in by Assmar *et al.* (2002b).

For diagnosis of Lyme disease, the first two bands (to appear) is reported to be 41 KDa followed by the outer protein c (ospc) band and / or the one at 39 KDa, the last two being highly species specific while the former is not. For detection of IgG, ELISA using flagellar antigen for coating of plate was effective, increasing both specificity and sensitivity of the kit. For detection of IgM, coating with flagellar antigen alone, only increased specificity having no effect on sensitivity (Gabriel *et al.*, 1996).

Some recombinant chimeras containing key sequences from a number of *B. burgdorferi* proteins have been generated containing OspA, OspB, OspC, Fla and p93 (Panelius *et al.*, 2002, 2003). Flagellin Fla or p41 and p93 are two good candidates for serodiagnosis of Lyme disease. This region of p41 used alone is reported to be very sensitive but not specific enough for detection of Lyme disease. As a component of chimeric constructs, however, it adds significantly to the diagnostic performance of the chimeric antigens (Gomes-Solecki *et al.*, 2000).

Recently, using immunoproteomics, a surface antigen was recognized on *Borrelia hermsii* which could distinguish between Lyme disease and relapsing fever. One candidate that was identified is a hypothetical protein with a molecular mass of 57 kDa that was designated Borrelia immunogenic protein A (BipA). This protein was further investigated as a potential diagnostic antigen for *B. hermsii* given that it is absent from the *B. burgdorferi* genome (Lopez *et al.*, 2010). It is very interesting to note that the flagellar antigen purified by us was highly specific and sensitive to *B. persica*, that means it could distinguish between two strains of relapsing fever, upto 98% sensitivity and 94% specificity. The IHA kits prepared from the sonicate also could distinguish upto 76% specificity, between the two strains, but the flagellar protein was 94% specific. We do not have Lyme disease in Iran, otherwise, kits made from this flagella could easily distinguish between the two strains.

The advantages of this antigen, its stability, strain specificity, the fact that it is the primary one to be recognized by the immune system in recurrent fever and a marker for recovery all of which make it a very good one for diagnosis and prognosis. With the advent of new technologies like proteomics and metabolomics, an analysis of glycosylation of *B. persica* flagellins has been carried out by mass spectrometry, a similar study using 1HNMR for study of its metabolome can be carried out to get a clearer picture about the dynamics of relapsing fever spirochetes especially *B. persica* (Zamani *et al.*, 2011; Arjmand *et al.*, 2010).

CONCLUSION

An *invitro* culture of *Borrelia persica* was standardized using different sera with BSK medium. A 42 KD flagellar antigen was purified and an IHA kit was prepared and tested on *B. persica* and *B. microtti* immunized sera. The kit was seen to be 98% sensitive and 94% specific and could easily distinguish between the two close strains of relapsing fever causing Borrelia.

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