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Trial to increase the sensitivity of Brucella antigens treated with Binary ethylene imine as inactivated agent

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The diagnosis of brucellosis is mainly based on the detection of anti-LPS antibodies. High temperature kills Brucella cells by causing lysis of the membrane, so the phenol-heat killed brucella antigen may lake specificity as a result of destruction the majority of proteins in the cell wall. Accordingly, attention was directed to produce antigen using binary ethylene imine as an inactivator. The produced antigen showed high specificity in detecting *Brucella abortus* and *Brucella melitensis*-infected animals, but sensitivity was not affected in comparison with the standard Rose Bengal antigen. In Enzyme immunotransfer blot (EITB), phenol–heat killed brucella cells showed only 3 bands (37.375, 23.47 and 7.83 kDa) that denotes denaturation for at least 6 bands whereas binary inactivated brucella cells showed similarity with non-treated ones.

Brucellosis is an important infectious disease that mainly affects cattle, sheep and goats. The disease in cattle is most commonly caused by *Brucella abortus* whilst brucellosis in sheep and goats, is caused by *Brucella melitensis*, a very important zoonotic agent (Joint, 1986; Sangari *et al*., 1996; Garin-Bastuji *et al*., 1998; MoriyÓn and Gamazo, 1998; Schurig *et al*., 2002).

Isolation of brucella organisms from the suspected animal is the diagnostic method known to produce the best results in terms of specificity. However, this method has a limited sensitivity, expensive and cumbersome and has the added difficulty of being unpractical to apply at a large scale in control campaigns. Accordingly, the indirect diagnosis of disease based on serological tests is of choice in the eradication programmes. The standard Rose Bengal (RB) and complement fixation (CFT) tests are the main serological tests used to detect antibodies against *Brucella abortus and Brucella melitensis* infections. Both tests have been used for several decades, proving to be successful for eradicating bovine brucellosis in some countries, and are the official tests currently used in the European rules for the eradication of *B. melitensis* infection in small ruminants. Nevertheless, there is evidence that both tests are significantly less effective for the diagnosis of brucellosis in sheep and goats than in cattle. (Joint, 1986; Garin-Bastuji *et al*.,1998; Nielsen 2002).

The EU rules consider that the best strategy for diagnosing sheep and goat brucellosis is the combined use of Rose Bengal as the screening test and the complement fixation (CF) test as the confirmatory test. However, it has been reported that a relatively high proportion of sheep and goats from *B. melitensis* infected flocks showed negative results in the standard Rose Bengal test but positive in the CF test, questioning the sensitivity of the former as a screening test (Blasco *et al*.,1994). Thus, the simultaneous use of both is recommended to obtain a maximal sensitivity (Garin-Bastuji *et al*., 1998). Therefore, the aim of this work was based upon, trying to increase the sensitivity of Rose Bengal by using binary ethylene imine as an inactivator, comparing between standard Rose Bengal (SRB) and Binary Rose Bengal (BRB) by Enzyme linked immunotransfer blot (EITB).

Material and methods

Brucella strains. Virulent *Brucella abortus* strain 2308 (S-2308) was obtained from Vet. Lab., New How, Surry, K.T.15 England. This strain was used for experimental infection of guinea pigs. Virulent *Brucella melitensis* strain 16 M was obtained from Vet. Lab., New How, Surry, K.T.15 England. This strain was used for experimental infection of guinea pigs.

Animals and Experimental design. One hundred and fifty guinea pigs (300-350 grams body weight) were divided into 3 equal groups, the first was injected with virulent *Brucella*

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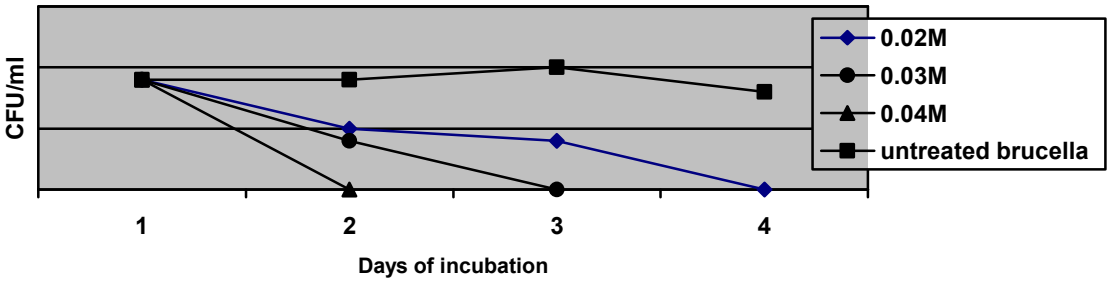
Fig (1):Inactivation curve of Brucella living cells (S99) with different concentrations of BEI 0.02M,0.03M and 0.04M



abortus strain 2308 (S-2308) ($5X10^3$ viable count) , the 2nd group was injected with virulent *Brucella melitensis* strain 16 M ($5X10^3$ viable count) and the 3rd was control non-infected.

Serum samples. Sera were collected after inoculation of 2 groups of guinea pigs with virulent *Brucella abortus* strain 2308 and *Brucella melitensis* strain 16 M. All animals were tested for brucella antibodies weekly for 3 months post infection. All sera were tested against the antigens using standard Rose Bengal and binary Rose Bengal. Sera from control non-infected guinea pigs were also collected.

Binary ethylene amine (BEI) preparation. It was prepared through cyclization of 1 M 2-bromoethylamine hydrobromide in previously wormed 0.2 N sodium hydroxide in water bath 37°C, till pH falls to 7.2. The solution was immediately used as inactivating agent (Girard and Mary, 1990).

Antigens preparation.

i. Whole cell antigen preparation. *B. abortus* biovar 1 (S 99) was cultivated onto potato agar. The bacteria were harvested in phenol saline from their respective growth media and heat killed for 2 h at 95°C. Rose Bengal antigen was prepared according to (Alton, *et al.,*1988)

ii. Binary ethylene amine Rose Bengal antigen. Brucella cells (S 99) were collected in sterile saline and the viable count was adjusted to $9X10^9$ CFU in three aliquots. BEI was added with different concentrations in each vial (0.02 M, 0.03 M and 0.04M). The three vials were incubated at 37 °C, and the viable count was determined periodically. (Hassanien, 1992).

Preparation of Hyperimmune serum using *Brucella abortus* S (99). Ten guinea pigs (300-350 g. body weight each) were injected intraperitoneally with 1 ml of a suspension containing 10^9 organisms/ml of heat killed, phenol preserved *B. abortus* S (99) in PBS in days 0, 7, 21, 28. After 21 days, guinea pigs were boostered for two times by 3 weekly intraperitonial injections of 10^9 heat killed *B. abortus* cells. (Shohreh Farshad, *et al*., 2002)

Enzyme immunotransfer blot (EITB). Different Brucella antigens (10ug each) were electrophoresed using 10% SDS-PAGE under reducing conditions (Lammli, 1970).The fractionated antigen was electrically transferred onto nitrocellulose (NC) membrane. NC sheets were blocked by 5% BSA in PBS for 2 h. on a rocker platform. Hyperimmune sera prepared in guinea pigs were diluted at 1:100 in BSA/ PBS–T incubated with the fractionated Brucella NC strips for 2 h. on a rocker platform. Following washing, protein-A-peroxidase (as conjugate) diluted at 1:1000 (Sigma Co.) in PBS–T was added to NC strips for 1 h. on a rocker platform. The chromogen 3- amino, 9-ethyl cabazole (AEC) substrate (Sigma) was added to NC strips and allowed to develop for 30 min. The reaction was visualized by the naked eye.

Results and Discussion

The world Health organisation (WHO) laboratory biosafety manual classifies Brucella in Risk group III, as brucellosis is readily transmissible to humans causing acute febrile illness - undulant fever - which may progress to a more chronic form and can also produce serious complications affecting the musculo-skeletal, cardiovascular, and central nervous systems. Infection is often due to occupational exposure and is essentially acquired by the oral, respiratory, or conjunctival routes, but ingestion of dairy products constitutes the main risk to the general public. There is an occupational risk to veterinarians and farmers who handle infected animals and aborted fetuses or placentae (WHO, 1997; European Commission, 1999).

The diagnosis of brucellosis is mainly based on the detection of anti-LPS antibodies. Due to substantial similarity of the O-polysaccharide of brucella LPS to that of various other Gram-

Table (1): Comparison between standard and Binary inactivated Rose Bengal in detection of experimentally infected guinea pigs' sera.

Group	Guinea pigs number	Standard Rose Bengal		Binary Rose Bengal (0.02M)	
		+ve	-ve	+ve	-ve
I- experimentally Infected guinea pigs with *Brucella abortus* 2308	50	50	0	50	0
II-experimentally infected guinea pigs with *Brucella melitensis* M 16	50	45	5	48	2
III-non-infected guinea pigs	50	1	49	0	50

Table (2): Whole cell protein analysis of different *Brucella* antigens using Enzyme immunotransfer blot.

Lanes: Rows	Marker (mol.w.)	Lane 1 (mol.w.)	Lane 2 (mol.w.)	Lane 3 (mol.w.)	Lane 4 (mol.w.)	Lane 5 (mol.w.)
r 1	175					
r 2		128.355	128.355			
r 3	83					
r 4	62	60.286	60.286		60.286	
r 5		54.577		54.577	54.577	
r 6			52.679	52.679		
r 7	47.5	49.959	49.959	49.959	49.959	
r 8				44.594	44.594	
r 9		41.313	41.313			
r 10				37.375	37.375	37.375
r 11	32.5	32.160		32.160	32.160	
r 12			30.825	30.825	30.825	
r 13				28.641	28.641	
r 14		26.748	26.748	26.748	26.748	
r 15	25					23..47
r 16	16.5		17.775		17.775	
r 17		13.723		13.723	13.723	
r 18			10.38	10.38		
r 19		7.83				7.83

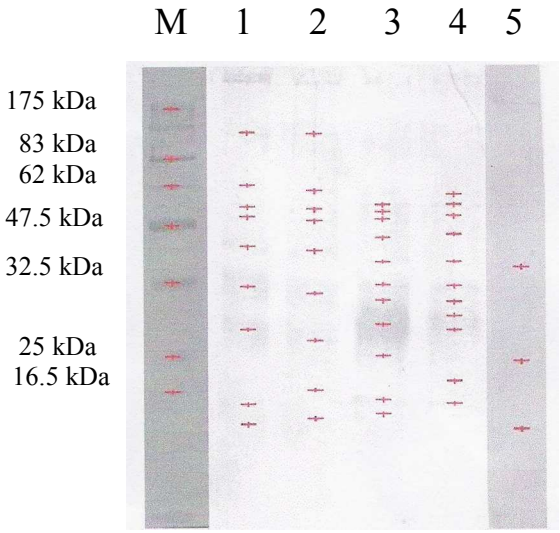
Lane (1): untreated Brucella, Lane (2): Brucella treated with 0.02M of BEI, Lane (3): Brucella treated with 0.03M of BEI, Lane (4): Brucella treated with 0.04M of BEI, Lane (5): Heat killed Brucella.

negative bacteria, serological tests of samples containing high amounts of LPS lack specificity. Hence, the development of assays based on more specific protein antigens is an essential subject in brucellosis research. The aim of this study was proteomic characterization of various antigen preparations from the diagnostic reference strain; *Brucella abortus* (S99) and the identification of immunogenic proteins suitable for serological assays (Nöckler, *et al*., 2006).

The Brucella cell envelope is a three-layered structure in which an inner or cytoplasmic membrane, a periplasmic space, and an outer membrane can be differentiated. Brucella cell walls consist of a peptidoglycan (PG) layer strongly associated with the outer membrane. The outer membrane contains LPS, proteins and phospholipids. A lipoprotein covalently linked to PG has been described as a major OMP (Farshad *et al*., 2002).

The procedures most frequently used for the sero diagnosis of brucellosis are the rapid slide

Fig (2): Enzyme immunotransfer blot of different Brucella antigens against crude hyperimmune serum prepared in guinea pigs (Brucella S99 antiserum).



M=broad range biolab pre-stained marker, Lane (1): untreated Brucella, Lane(2): Brucella treated with 0.02M of BEI, Lane(3): Brucella treated with 0.03M of BEI, Lane(4): Brucella treated with 0.04M of BEI, Lane(5): Heat killed Brucella

agglutination test (RSAT), standard tube agglutination test (SAT), 2- mercaptoetanol tube agglutination test (2ME), complement fixation (CF) test and enzyme-linked immunosorbent assay (ELISA). One major problem in sero diagnosis is the cross reactions that occur between brucellae and other bacteria. A second problem is that a diagnosis of brucellosis cannot be established on the antibody titer alone.

This article describes production and characterization of *Brucella abortus* S (99) surface antigens. These antigenic structures may be employed in serodiagnostic tests which currently utilize the whole cells bacteria, and consequently it may increase the specificity and decrease cross-reactions between *B. abortus* and the other bacteria.

The prepared phenol-heat killed bacteria was compared with S 99 inactivated with binary ethylene imine (BEI) in different concentrations (0.2 M, 0.3 M and 0.4 M). BEI inactivated brucella cells over a period of 3 days at 37°C, and the viable count was determined periodically till complete inactivation. 0.04 M, 0.03 M and 0.02 M concentration of BEI inactivated brucella living cells (9 X 10^9 CFU) after one day, 2 days and 3 days respectively as shown in Fig (1).

The produced antigens (0.02M, 0.03M and 0.04M BEI) were standardized using standard antisera in comparison with the commercial antigen, the Rose Bengal plate test was applied (Alton *et al*., 1988). Binary inactivated antigen (0.02M) gave the best results as follows: coarse clumbing, definite cleaning in less than 1 minute with the control (+++) positive antisera. Fine agglutination, definite rim with the control (++) positive antisera. Little agglutination, some rim with the control (+) positive antisera. No agglutination, no rim formation with the control (-) negative antisera

The produced antigen (0.02M BEI) was compared with standard Rose Bengal by testing experimentally infected guinea pigs with *Brucella abortus* 2308 and *Brucella melitensis* M 16 (virulent stains). All animals were tested for the antibody response of experimentally infected guinea pigs by Rose Bengal test, over a period of 3 months post infection (PI). The antibody response appeared at 10 days post infection (PI), gradually increased till 7 weeks PI then declined gradually till 14 weeks PI.

Using a Bayesian approach, test sensitivity and specificity were estimated and compared as mentioned by Ronald, (1991), no significant differences were detected in sensitivity among various antigens used. However, binary inactivated antigen showed better specificity, and detected guinea pigs infected with *Brucella melitensis*, as shown in Table (1).

Enzyme immunotransfer blot (EITB) was applied to analyse the antigenic structure of the different antigens. Lane 1 (untreated Brucella cell) showed 9 protein bands ranging from 128.355 kDa to 7.83 kDa, lane 2 (Brucella cells treated with 0.2 M binary) showed 9 protein bands ranging from 128.355 to 10.38 kDa, lane 3 (Brucella cells treated with 0.3 M binary) gave 11 protein bands ranging from 54.577 to 10.38 kDa, lane 4 (Brucella cells treated with 0.4 M binary) gave 11 protein bands ranging from 60.286 to 13.723 kDa, and lane 5 (heat-killed Brucella) showed only 3 bands ranging from 37.375 to 7.83 kDa. as shown in Table (2) and Fig (2). These results revealed similarities between lanes 1 & 2 i.e. binary ethylene amine (0.2 M) did not show bad affect on living *brucella* cells during inactivation, while differed slightly from lanes 3 & 4. These results agreed with Hassanien, (1992) who recorded that binary ethylene imine inactivated AHV virus (virulent type-9 strain) without detection of residual virus. A concentration of 0.03 M of the inactivating agent is recommended where no changes in viral antigenic properties were noticed in complement

fixation test. On the other hand phenol–heat killed brucella cells showed only 3 bands, this denotes denaturation for at least 6 bands. This result agreed with Hansel et al. (1994) who recoded that proteins travel as a 140 kDa complex if subjected to SDS-PAGE without heat denaturation, but they collapse into 50 kDa monomers if heated in SDS. Farshad *et al*., (2002) mentioned that the major *B. abortus* outer membrane proteins (OMP) have the molecular masses of 36-38 and 25-27 kDa. They are also called group 2 porin proteins and group 3 proteins respectively. Cell wall of *B. melitensis* contains another major protein of molecular mass 31-34 kDa, which is minor in *B. abortus* strains. A lipoprotein covalently linked to PG has also been described as a major OMP. Other OMP identified so far are minor species with molecular masses of 10, 16.5, 19 and 89 kDa. OMP 10, 16 and 19 share antigenic determinants with bacteria of the family Rizobiaceae. The 89 kDa OMP is probably a protein of group 1 with a molecular mass of 88-94 kDa which agreed with the results obtained in the current study. In conclusion, the best method for preparation of Brucella antigen is by adding 0.02 M BEI, because it gave nearly the same result of non-treated *Brucella abortus* (S 99) with hyperimmune sera prepared from crude Brucella antigen as shown in Fig (2) and Table (2).

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محاولة لزيادة حساسية أنتيجينات البروسيلا بإستخدام بينارى إثيلين أمين كمثبط

د.كمال الدين حسين ، د.سلوى سعد عوض

تشخيص مرض البروسيلا يعتمد أساساً على الكشف عن الأجسام المناعية المضادة للبيوبولى ساكريد. و لأن درجات الحرارة العالية تقتل البكتريا عن طريق تكسير الغشاء الخارجى للميكروب ، فإن أنتجين البروسيلا المحضر يقتل البكتريا حرارياً يفتقد إلى النوعية. لذلك اتجهت الأنظار لاستعمال البينارى إثيلين أمين كمثبط فى إنتاج أنتجين للكشف عن مرض البروسيلا. و أظهرت النتائج أن الأنتجين المنتج يتميز بنوعية عالية فى الكشف عن البروسيلا أبورتس و مليتينسيس و لكن درجة الحساسية لم تتأثر بالمقارنة مع أنتجين الروز بينجال العيارى. بإستخدام الإختبار المناعى الإنزيمى التلونى وجد أن أنتجين البروسيلا المحضر يقتل البكتريا حرارياً أظهر ثلاث بروتينات فقط أوزانها الجزيئية تساوى 37.375, 23.47 and 7.83 kDa و أنه فقد ستة بروتينات أخرى. بينما الأنتجين المحضر بإستخدام البينارى إثيلين أمين كمثبط لم يتغيرو كان مشابهاً لخلايا البروسيلا غيرالمثبطة.