Evaluation of ELISA Using Different Antigens in the Discrimination between Brucella Vaccinated and Infected Cattle

Amin. M. M. 1, Ahmed, S. A. 1, Zaki, H. M 2, and Ismail, R. I 2.

1Dept.of Vet.Medicine &Inf. Diseases, Faculty of Veterinary Medicine, Cairo University, Egypt.
2Department of Brucellosis Research, Animal Health Research Institute, Dokki, Giza, Egypt.
dr.mahmoudamin43@gmail.com

Abstract: The main objective of the present study was the diagnosis and differentiation between Brucella infected and vaccinated cattle. The animals included in this study were 180 naturally infected non vaccinated cows in governmental farm (group 1), 125 brucella free cows in which strain 19 vaccination had never been practiced (group 2) and 530 strain 19 vaccinated cows (group 3). Sera from these animals were examined for brucellosis using Immunoblot and iELISA using LPS or CPE as coating antigens. The highest values of the ability of serological tests to differentiate S19 vaccinated animals from those infected ones were detected in iELISA using CPE as coating antigen and lowest values were seen in iELISA using LPS as coating antigen.

Keywords: Brucellosis, Serodiagnosis, ELISA, Immunoplot.

1. Introduction

Brucellosis is a zoonotic world wide infectious disease of animal that is caused by a number of host adopted species of gram negative intracellular bacteria of the genus brucella (Ochali et al., 2005) also it is one of the major diseases that is responsible for reproductive failure in animal livestock (Xavier et al., 2009).

Brucellosis eradication programs are based exclusively on serological screening of cattle as well as sheep herds to detect and remove infected animals and using attenuated vaccines such as Brucella abortus S19 for cattle and Brucella melitensis Rev1 vaccine for sheep and goats.

The presence of smooth lipopolysaccharides (S-LPS) with an o-chain explains the appearance and persistence of antibodies in serum following administration of these vaccines is consider one of the most difficult tasks in the serological diagnosis of bovine brucellosis is the discrimination of infected from vaccinated animals (Nielsen et al., 1989).

Indeed most standard serological tests, i.e. serum agglutination and complement fixation use whole smooth cell preparations as antigen for diagnosis of brucellosis (Magee, 1980; De Klerk and Anderson, 1985).

Since S-LPS is the major exposed antigen of the cell surface (Debbabhet et al., 1995) as the antibody response to the S-LPS that is usually measured. In addition, these techniques do not distinguish clearly infected from vaccinated animals, and also other gram negative bacteria may cross react with smooth brucella species such as Yersinia enterocolitica 0:9.

Consequently, the specificity of LPS-based assays is low Anti-LPS antibodies may persist for more than a year after acute brucellosis (Almuneef and Memish, 2003). It cannot differentiate vaccinated from infected animals by most of the LPS-based assays (Samartino et al., 1999). Therefore, the serological diagnosis of brucellosis is still a challenge in animal diseases. The development of LPS-free protein-based diagnostic may be the key to overcome all of these challenges.

Thus it is reasonable to assume that the determination of the humeral response against Brucella proteins could help to circumvent those undesired reactivities although the internal antigen may be considered as an excellent antigen for its specificity as cytoplasmic proteins induce a higher antibody response than outer membrane proteins (Letesson et al., 1997). Additionally, the production of antibodies directed against proteins may be host specific, but this practical use seems to be limited since the antibodies cannot detected in early stages of infection (Srikawat et al., 1989). Therefore, the aim of this study was the extraction and purification of lipopolysaccharides (S-LPS) and cytoplasmic protein extract (CPE) antigens and characterization of these antigens using sodium Dodecyl sulfate polyacrylamide gel Electrophoresis (SDS – PAGE).

Apply immunoblot analysis on examined sera against extracted antigens to detect the immunogenic bands. Evaluation of indirect ELISA using lipopolysaccharides(LPS) or cytoplasmic protein extract (CPE) as coating antigens in diagnosis as well as estimation ability of applied tests in differentiation of brucella infected from vaccinated cows.
2. Material and methods

Animals:

Naturally infected cows: A total of 180 naturally infected non vaccinated cows in governmental farm where *Br. melitensis* is endemic. These cows had a history of abortion and reproductive troubles (group 1).

Brucella free cows: A total of 125 animals from brucella free areas and strain 19 vaccination had never been practiced (group 2).

Strain 19 vaccinated cows:A total of 530 cows, these were negative to serological tests at the time of vaccination. The animals were vaccinated between 3 to 8 months of age with a dose of 3-8x10^9 CFU. They were bled at 2 weeks post vaccination and every 2 weeks until 24 weeks post vaccination (group 3).

Samples:

Serum samples were collected from all animals for immunoblot and indirect ELISA. Blood samples were allowed to clot and the sera were separated by centrifugation and stored at at -20°C in the deep freezer for serological tests.

2.1. Antigen Preparation

The lipopolysaccharides (LPS) of *Brucelal melitensis* biovar 3 (field strain) was extracted and purified from proteins and other contaminant by phenol-chloroform-petroleum ether method described by Galanose et al. (1969) and modified by Qureshi and Takayama (1982).

Extraction of cytosoluble antigen of *Brucelal melitensis* biovar 3 (field strain) as described by Chin and Turner (1990) and Quantitation of the protein content by (Lowry et al., 1951).

2.2. Polyacrylamide gel electrophoresis (SDS-PAGE):

It was performed according to the methods described by Laemmli (1970)

Samples were heated at 100°C for 5 min. before they were loaded onto 10% (wt/vol) running gel with a 4%(wt/vol) stacking gel. Electrophoresis was carried out at a constant voltage until the dye had migrated to the bottom of the gel.

2.3. Immunoblots

The extracted antigens (LPS or CPE) were separated by SDS-PAGE and transferred to nitrocellulose sheets at 60V overnight with cooling following the method of Towbinet al. (1979).

2.4. ELISA

It was carried out according to the methods described by Bassiri et al. (1993). Coating: Disposable polystyrene microtiter plates with flat bottom were coated with either LPS diluted in carbonate buffer at 0.5 µg/ml (100 µl/well) (Kittelberger et al., 1998) or CPE preparation at final concentration of 20 µL/ml each per well in100 µl of carbonate buffer (Weynants et al., 1996) and incubated overnight at 4°C. The coated plates were incubated at 37 °C for 1 hour then over night at 4°C followed by three times of washing using washing buffer (200 µl/well). Blocking was performed by adding the blocking buffer with 200 µl/well of a solution of PBS-Tween (20 mM phosphate buffer(pH 7.4) containing 0.05% Tween20), 3% casein for one hour in incubator then washed different times. Two fold serial dilution of serum samples were added (100 µl/well) and then incubated for one hour, washed different times using washing buffer. Alkaline phosphatase labeled antihovine IgG conjugate (Sigma Chemical Co) was added (100 µl/well) at 37 °C for 1 hour then washed three times. Substrate indicator mixture (100 µl/well) was added and the plates incubated at room temperature in dark place. Stopping of the reaction occurred using stopping buffer (50 µl/well) then reading at 492 nm wave lengths (versa max apparatus USA). Control positive and negative sera were included in each time at work. ELISA titers were expressed as the reciprocal of the highest serum dilution that gave an OD reading of at least two times the OD for negative serum.

3. Result and Discussion

Extracted LPS preparation from *Brucelal melitensis* biovar 3 was obtained in almost pure form and results obtained in Table (1) and Figure (1) indicated that 4 bands in SDS-PAGE were detected after staining with silver nitrate method.

The molecular weight of these bands ranged between 6.79 to 11.32 KDa which is similar to that reported by Bogardet al. (1987). These bands represent the core region and clusters of polysaccharide side chains. Similar observation was recorded by Grain-Bastujiet al. (1990). It has been demonstrated that cytoplasmic proteins are the common internal antigens of smooth and rough strains of *brucella* (Baldiet al., 1999). Therefore, it was assumed that the cytoplasmic proteins of genus *brucella* are specific for the genus and most of them are common to its entire species.
Table (1): Sodium dodecyl sulfate polyacrylamide gel electrophoresis pattern of lipopolysaccharides extracted from \textit{Brucellamelitensis}\textsubscript{biovars}3.

<table>
<thead>
<tr>
<th>Lanes bands</th>
<th>Marker M.W(KDa)</th>
<th>Amount</th>
<th>LPS M.W(KDa)</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>200</td>
<td>4.90</td>
<td>11.32</td>
<td>59.8</td>
</tr>
<tr>
<td>2</td>
<td>97.40</td>
<td>6.35</td>
<td>9.10</td>
<td>9.08</td>
</tr>
<tr>
<td>3</td>
<td>68</td>
<td>13.8</td>
<td>8.21</td>
<td>16.0</td>
</tr>
<tr>
<td>4</td>
<td>29</td>
<td>32.1</td>
<td>6.79</td>
<td>15.2</td>
</tr>
<tr>
<td>5</td>
<td>14.30</td>
<td>23.1</td>
<td>--</td>
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</tr>
<tr>
<td>6</td>
<td>6.20</td>
<td>19.7</td>
<td>--</td>
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</tr>
</tbody>
</table>

M.W : Molecular weight  
K Da : Kilo Dalton

Fig (1): Sodium dodecyl sulfate polyacrylamide gel electrophoresis pattern of lipopolysaccharides of \textit{Brucellamelitensis} stained by silver stain.

Lane (1) Lane (2)

The SDS-PAGE of CPE extracted from \textit{Brucellamelitensis}\textsubscript{biovar} 3 stained by (Coomassie blue) Table (2) Figure (2) revealed 8 protein bands ranging from 10.28 to 80.80 KDa. This result agrees with that recorded by \textit{Debbarhet et al. (1996)} who found CPE bands in the molecular weight mass ranging from 10 to 80 Kda.

Table (2): Sodium dodecyl sulfate polyacrylamide gel electrophoresis pattern of cytoplasmic protein extracted from \textit{Brucellamelitensis}\textsubscript{biovar} 3.

<table>
<thead>
<tr>
<th>Lanes bands</th>
<th>Marker M.W(KDa)</th>
<th>Amount</th>
<th>CPE M.W(KDa)</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>97.4</td>
<td>6.98</td>
<td>80.88</td>
<td>36.1</td>
</tr>
<tr>
<td>2</td>
<td>58.1</td>
<td>23.4</td>
<td>58.07</td>
<td>29.6</td>
</tr>
<tr>
<td>3</td>
<td>39.8</td>
<td>22.8</td>
<td>38.33</td>
<td>7.81</td>
</tr>
<tr>
<td>4</td>
<td>29</td>
<td>14.8</td>
<td>36.14</td>
<td>3.1</td>
</tr>
<tr>
<td>5</td>
<td>20.1</td>
<td>14.5</td>
<td>27.08</td>
<td>9.37</td>
</tr>
<tr>
<td>6</td>
<td>14.3</td>
<td>17.5</td>
<td>25.71</td>
<td>5.38</td>
</tr>
<tr>
<td>7</td>
<td>--</td>
<td>-</td>
<td>18.01</td>
<td>3.18</td>
</tr>
<tr>
<td>8</td>
<td>-</td>
<td>-</td>
<td>10.28</td>
<td>5.49</td>
</tr>
</tbody>
</table>

Fig. (2): Sodium dodecyl sulfate polyacrylamide gel electrophoresis pattern of cytoplasmic protein of \textit{Brucellamelitensis} stained by coomassie stain.

Lane (1) CPELane (2) Marker

Lane (1) Lane (2)

Lane (1) Lane (2)

Lane (1) Lane (2)
Immunoblotting analysis using LPS, the antigen antibodies reaction of sera of brucella infected cows was typically similar to sera of S$_{19}$ vaccinated cow. As shown in Figure (3). In fact, the reactivity of brucella infected cows cannot be distinguished from that of cows vaccinated with S$_{19}$ against LPS antigen.

These results are similar to that recorded by Marin et al. (1999) who suggested that the similarity in the S-LPS response hampered diagnostic effort to differentiate vaccinated from infected animals. Also Baldi et al. (1996) reported that S$_{19}$ vaccinated animals developed anti LPS IgG level that overlapped with those developed by brucella infected cattle.

In this study immunoblot technique was used to analyze bovine antibody responses to CPE of Brucella melitensis biovar 3 to determine whether there are antigen specific difference in serologic response of S$_{19}$ vaccinated and naturally infected animals. Figure (4)

![Immunoblot analysis using LPS antigen](image1)

Lane (1) Lane (2) Lane (3) Lane (4) Lane (5) Lane (6) Lane (7) Lane (8)
Fig. (3): Immunoblot analysis using LPS antigen.
Lane (1): S$_{19}$ vaccinated cow sera (4 weeks post vaccination) against LPS antigen.
Lane (2): S$_{19}$ vaccinated cow sera (6 weeks post vaccination) against LPS antigen.
Lane (3): S$_{19}$ vaccinated cow sera (8 weeks post vaccination) against LPS antigen.
Lane (4): S$_{19}$ vaccinated cow sera (10 weeks post vaccination) against LPS antigen.
Lane (5): S$_{19}$ vaccinated cow sera (12 weeks post vaccination) against LPS antigen.
Lane (6): S$_{19}$ vaccinated cow sera (16 weeks post vaccination) against LPS antigen.
Lane (7): S$_{19}$ vaccinated cow sera (20 weeks post vaccination) against LPS antigen.
Lane (8): Brucella infected cow sera against LPS antigen.

![Immunoblot analysis using CPE antigen](image2)

Lane (1) Lane (2) Lane (3) Lane (4) Lane (5) Lane (6) Lane (7) Lane (8)
Fig. (4): Immunoblot analysis using CPE antigen.
Lane (1): Brucella S$_{19}$ vaccinated cow sera against CPE (4 weeks post vaccination).
Lane (2): Brucella S$_{19}$ vaccinated cow sera against CPE (6 weeks post vaccination).
Lane (3): Brucella S$_{19}$ vaccinated cow sera against CPE (8 weeks post vaccination).
Lane (4): Brucella S$_{19}$ vaccinated cow sera against CPE (10 weeks post vaccination).
Lane (5): Brucella S$_{19}$ vaccinated cow sera against CPE (12 weeks post vaccination).
Lane (6): Brucella S$_{19}$ vaccinated cow sera against CPE (16 weeks post vaccination).
Lane (7): Brucella S$_{19}$ vaccinated cow sera against CPE (24 weeks post vaccination).
Lane (8): Brucella infected cow sera against CPE.
By using peroxidase rabbit anti bovine IgG conjugate, sera from naturally infected cows showed strong antibody reaction against 80.88, 58.07, 38.33, 36.14, 27.08, 25.71, 18.01 and 10.28 KDa bands. However, sera from S19 vaccinated animals 4 weeks post vaccination showed week immunoglobulin reactivity against 80.88, 58.07 and 38.33 KDa bands while, no reaction was observed against 18.01 KDa bands. Sera from vaccinated cows 6, 8, 10 and 12 weeks post vaccination developed week antibody reaction against 80.88 and 58.07 KDa only.

So, our results confirm the finding reported by Debbrah et al. (1995) who found that vaccinated animals not developed anti 18 KDa protein response. Also Hemmen et al. (1995) could not find by competitive ELISA, any anti – 18KDa protein response in S19 vaccinated cattle.

Enzyme linked immuno sorbent assay using purified antigens and/or monoclonal antibodies have developed in order to eliminate the problem of low specificity (Onsel, 2005). This technique has been evaluated for many years for their diagnostic performance to detect serum antibodies to brucella species.

Indirect ELISA have several advantages being that the antibodies to be detected react with the antigen without performing secondary function as agglutination precipitation or activation of complement (Nielsen and Kwok, 1995).

A variety of Brucella melitensis surface antigens contribute to the diagnosis of infection in cattle. Although the internal antigen may be considered as an excellent antigen for its specificity, its practical use seems to be limited since the antibodies cannot detected in early stages of infection (Serikawa et al., 1989).

It has long been recognized that the LPS is the major antigen of the surface of smooth brucella (Zygmunt et al., 1994). Naturally infected animals have continuous exposure to antigens because of the persistence of pathogenic strains and produce LPS antibody response (Nielsen et al., 1989).

In this study the results of antibody reactivity of sera of suspected cows and non-reactors (brucella free cows) are shown in Table (3).

Non specific cross reacting antibodies in uninfected animals against brucelalipopolysaccharidies (LPS) have been shown due to several pathogens including Escherichia coli, Yersinia enterocolitica and Pseudomonas salanacearum (Nielsen and Duncan, 1982).

Cytosplasmic proteins are the predominant components of the soluble Brucella species fraction from both smooth and rough strains. Thus they are relatively easy to obtain and it has been reported that both humoral and cellular immune responses against them occur (Goldboumet al., 1992).

Results showed in Table (4) revealed that 131 (72.8%) of suspected cows developed IgG against CPE and all non reactors (brucella free cows) showed negative reaction against CPE.

These results agree with Debbrah et al. (1995) who added that the antibody response against cytosoluble proteins however was always less intense and more heterogenous than antibody response against LPS but it is more specific.
Results of antibody reactivity of sera of vaccinated cows using LPS as coating antigen in indirect ELISA was shown in Table (5) revealed that 100% of vaccinated cows 4 weeks post vaccination showed IgG anti LPS antibodies. This may attributed to the presence of large amount of smooth LPS on vaccinal Brucella strains induce a high level of anti-LPS antibody response (Jimenez de Bagues et al., 1992).

The usefulness of antigen other than LPS for differential diagnosis of bovine brucellosis has been poorly investigated.

Table (5): Antibody reactivity of sera of vaccinated cows using LPS as coating antigen in indirect ELISA.

<table>
<thead>
<tr>
<th>No. of examined animals</th>
<th>Time of examination (weeks)</th>
<th>Titers</th>
<th>Total reactor</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>20</td>
<td>40</td>
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<tr>
<td>530</td>
<td></td>
<td>No.</td>
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<tr>
<td>2</td>
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<td>164</td>
<td>30.9</td>
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<tr>
<td>4</td>
<td></td>
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<td>26.4</td>
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<td>6</td>
<td></td>
<td>125</td>
<td>23.6</td>
</tr>
<tr>
<td>8</td>
<td></td>
<td>114</td>
<td>21.5</td>
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<tr>
<td>10</td>
<td></td>
<td>99</td>
<td>18.7</td>
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<tr>
<td>12</td>
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<td>110</td>
<td>20.7</td>
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<td>93</td>
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<tr>
<td>24</td>
<td></td>
<td>11</td>
<td>2.1</td>
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</tbody>
</table>

The result of antibody reactivity of sera of vaccinated cows using CPE as coating antigen in indirect ELISA was shown in Table (6) and revealed that only 4.2%, 3.2% and 0.9% of vaccinated animals were positive with low titer 4, 6 and 8 weeks post vaccination, respectively.

Table (6): Antibody reactivity of sera of vaccinated cows using CPE as coating antigen in indirect ELISA.

<table>
<thead>
<tr>
<th>No. of examined animals</th>
<th>Time of examination (weeks)</th>
<th>Titers</th>
<th>Total reactor</th>
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<td>No.</td>
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</table>

The marked difference anti-CPE levels between vaccinated and infected cattle is probably explained by differences in the duration of the exposure to brucella antigen.

In the case of infection with virulent strains the exposure to brucella antigen persists for longer periods, producing a stronger antibody response. This fact indicates the potential usefulness of protein antigens for diagnosing active brucellosis (Baldiet al., 1996).

It is obvious that an iELISA test for determining antibodies directed to cytoplasmic proteins of Br. melitensis could constitute a specific and sensitive test for diagnosis of bovine brucellosis (Goldbaum, 1992).

Also Baldiet al. (1996) reported that no cross reactivity of reported to take place between Br. melitensis cytoplasmic proteins and proteins from other genera.

From the results showed in Table (7), it is clear that indirect ELISA using LPS cannot able to differentiate S19 vaccinated animals from infected ones.

Table (7): Ability of different serological test to differentiate S19 vaccinated animals (ADV) at different intervals post vaccination.
In similar approach Fensterbanket al. (1982) showed that S19 vaccination produce an intense serological response as measured by standard serological tests which do not permit distinction between vaccinated and infected animals. Jacques et al. (2007) has also reported that S19 vaccine is known to induce antibody response in vaccinated animals indistinguishable by current conventional serological tests from those observed in challenge animals.

Conventional serological methods such as agglutination tests and primary binding assays such as ELISA, principally measure antibody S-LPS either as presented on intact bacterium or immobilized on a plastic matrix. The antibody response of animals to S-LPS from smooth vaccines or field strains decrease by time but antibody titers persist longer in naturally infected animals. So they have a limited ability to discriminate vaccinated from naturally infected animals (Crastaet al. 2008).

When Brucella infection is diagnosed by measuring anti LPS antibodies, at least two interfering groups exist: animals recently vaccinated with S19 and those infected with crossly reacted bacteria. Several authors have proposed the use of antigenic components different from LPS as means to improve the diagnosis of bovine brucellosis (Cloekaert et al. 1992; Gold baum et al., 1994 and Hemmenet al., 1995).

In the present study we have been performed to measure the humoral immune response of vaccinated cattle against CPE.

As Limetet al. (1988) suggested that antigens not present in S19 should be used to differentiating vaccinated from infected cattle. Another possibility however, is that because of transient exposure to brucella antigens in S19 vaccinated cattle, poorly immunogenic component fails to significantly impact the immune system.

In deed ELISA with CPE antigen able to differentiate antibody responses of S19 vaccinated cow from those of suspected animals Table (7). This suggest that the antibody of cow infected with virulent Brucellamellitensis differ qualitatively from those of S19 vaccinated cows by their specificity for cytosoluble proteins antigen (Debrahet al., 1995).

As far as, we know in this study is applied to follow up the humoral immune response of vaccinated to cytoplasmic proteins of brucella. As shown here, this antiprotein response is measured by immunoblot or ELISA, could be useful for differentiating vaccinated from infected animals. In contrast detecting anti-LPS IgG antibodies would not be useful for testing cattle post vaccination.

While S19 vaccinated animals developed anti-LPS IgG level that overlapped with those developed by brucella infected cattle, a significant anti-18-KDa-protein antibody response was detected only in brucella infected cattle.

In conclusion, the antiprotein(especially 18-kd protein) humoral reactivity could be potentially useful for differentiation of Brucella infected cattle from those vaccinated with S19.

Corresponding author
Amin. M. M.
Dept.of Vet. Medicine &Inf. Diseases, Faculty of Veterinary Medicine, Cairo University, Egypt.
dr.mahmoudamin43@gmail.com

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