COMPARISON OF DIFFERENT SEROLOGICAL ASSAYS FOR THE DIFFERENTIAL DIAGNOSIS OF BRUCELLOSIS

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Abstract

COMPARISON OF DIFFERENT SEROLOGICAL ASSAYS FOR THE DIFFERENTIAL DIAGNOSIS OF BRUCELLOSIS.

Two indirect and two competitive Enzyme-linked immunosorbent assays (I-ELISA102, I-ELISA103, C-ELISA1 and C-ELISA2 respectively) have been evaluated in comparison with traditional test such as Radial Immunodiffusion (RID), Complement Fixation (CF), Rose Bengal Agglutination (RB) and Rivanol agglutination (RV). The sera analysed included 1018 sera obtained from non-vaccinated bovines, 848 sera from brucellosis free herds calf vaccinated with Strain-19, 295 sera obtained from brucellosis free herds adult vaccinated with Strain-19 and 665 sera from Brucella abortus biotype 1 (field strain) infected herds. Cut-off off values calculated by ROC analysis were established for each ELISA. Although all ELISAs fulfilled the requirements for sensitivity and specificity, in our hands C-ELISA2 performed slightly better than the other assays for differentiating infected from vaccinated bovines. The specificity of this test was similar to that of RID assay which is known to have high specificity for differentiating adult vaccinated from infected bovines. The kappa value among the different tests was good and within the limits of reproducibility and performance expected for the different assays.

From the different immunoenzymatic assays, the C-ELISA2, which uses LPS as antigen and a monoclonal antibody against the C/Y epitope as competing reagent, seems to be the most promising of the ELISAs and therefore can be recommended for screening a large number of serum samples on a laboratory basis.

1. INTRODUCTION

Brucella species (B. abortus, B. melitensis and B. suis, B. ovis and B. canis) are responsible for brucellosis in animals and humans causing severe economic and public health problems [1]. There is clear evidence that Brucella species are capable of surviving and multiplying within cells [2,3]. This fact could explain the marked tendency of the disease to result in focal involvement, forms with long evolution, and frequent relapses. There is also indication that LPS and related polysaccharides (NH), which are the most important antigens of Brucella, are implicated in the pathogenesis of these bacteria [4,5]. An upgraded model of the outer membrane of smooth Brucella displaying the most conspicuous antigens is presented in Figure 1.
FIG. 1. Schematic representation of S Brucella spp. OM. 2-deoxy, D-manno octulosonic acid (Kdo), lipid A (LA), free lipoprotein (LP), bound lipoprotein (B-LP) lipopolysaccharide (LPS), native hapten polysaccharide (NH), lipid bound NH (NH-L), phosphatidylcholine (PC), hydroxylated C_{28:0} fatty acid (OH-C_{28:0}), ornithine lipids (OL), OM proteins (OMP), OM proteins group 3 (OMP3), and porin (PO).

The physical and chemical characteristics of Brucella LPS and antigenically related polysaccharides (NH) have been extensively documented [4,5,6,7]. The antibody response elicited against Brucella LPS and the antigenically related native hapten polysaccharide (NH) during infection is by far the strongest when compared to those induced by other molecules of this microorganism [8]. Consequently, Brucella LPS has been considered the most important antigen during the immune response in brucellosis and the target for many serological and immunological studies [4,5,8]. In contrast to enterobacterial LPS and other polysaccharide molecules, Brucella LPS is capable of inducing strong IgG and IgM antibody responses.

A total of 11 epitopes (Figure 2) have been recognised in Brucella LPS [9,10]. Four of them (A, M, C/Y and C) have been identified in the O polysaccharide chain. The C and C/Y epitopes are found in all smooth type LPSs. The A epitope is characteristic of Brucella abortus (biotype 1) whereas the M epitope is found in B. melitensis (biotype 1) species. In some cases both the A and M epitopes can be found in the same bacterial strain as in the case of some strains of B. suis (biotype 4). Two epitopes are found in the core oligosaccharide (R1 and R2), three in the lipid A (LA1, LA2 and LA3) and two more in the lipid A associated peptide (LAOmp3-1 and LAOmp3-2). Most of the serum antibodies from infected or immunised animals are directed against the C/Y epitope present in the O-antigen and NH polysaccharide. Antibody responses against other epitopes located in these polysaccharides, as well as in the core oligosaccharide and lipid A moieties, although perceptible are produced in minor quantities (Figure 3).
FIG. 2. Schematic representation of Brucella abortus (biotype 1) lipopolysaccharide. The different epitopes recognised by sera from infected bovines and monoclonal antibodies are indicated. The geometric diagrams indicate the degree of reactivity.

A broad spectrum of activities concerning antibodies against Brucella LPS and NH have been described. For instance, it has been shown that antibodies against LPS and NH epitopes produced during infection are of higher affinity than those produced during vaccination or immunisation with purified molecules [8]. Moreover, most of these antibodies correspond to the IgG1 isotype, suggesting a T dependent response. Opsonising and complement fixing antibodies facilitate
phagocytosis and intracellular destruction of ingested *Brucella* [11]. Several experiments have demonstrated that passively transferred polyclonal or monoclonal antibodies in mice can protect against challenges by pathogenic bacteria [12]. Antibodies of the IgG class directed against the C/Y epitope seem to be the most protective of all [8,12]. Finally it was proved that passively transferred antibodies against O chain and NH from infected animals into mice and rabbits were capable of inducing a strong type I hypersensitivity reaction after injection of minimal quantities of these polysaccharides [13]. For all these reasons the detection of IgG1 antibodies against C/Y epitopes located in the O chain polysaccharide of LPS in serological test has been considered a key factor in the diagnosis of brucellosis [8,14].

2. MATERIAL AND METHODS

Two indirect and two competitive Enzyme-linked immunosorbent assays (I-ELISA102, I-ELISA103, C-ELISA1 and C-ELISA2 respectively) have been evaluated in comparison with traditional test such as Radial Immunodiffusion (RID), Complement Fixation (CF), Rose Bengal Agglutination (RB) and Rivanol Agglutination (RV). All the ELISA kits and their respective protocols and computation analysis were supplied by the Joint FAO/IAEA Division, Vienna, Austria and carried out as described in previous works [14,15,16]. The traditional serological assays were developed and performed as described elsewhere [17]. The sera analysed included 1018 sera obtained from non-vaccinated bovines, 848 sera from brucellosis free herds calf vaccinated with Strain-19, 295 sera obtained from brucellosis free herds adult vaccinated with Strain-19 and 665 sera from *Brucella abortus* biotype 1 (field strain) infected herds. The data obtained from the analysis of the samples of vaccinated and infected bovines was plotted in frequency histograms and the diagnostic sensitivity and specificity calculated as described elsewhere [16] using complement fixation (1/40) as standard test. Receiver operator analysis (ROC) for determination of cut-off value was performed with modifications as described elsewhere [18,19].

3. RESULTS

The frequency distribution of infected and vaccinated bovines for the different ELISAs is presented in Figure 4, 5, 6, 7. Even though a moderate overlapping between the infected and vaccinated animals is detected, a clear cut-off off value calculated by ROC analysis was established for each ELISA assay (Figure 8). Although all ELISAs fulfilled the requirements for sensitivity and specificity (Table I and II), in our hands C-ELISA2 performed slightly better than the other assays for differentiating infected from vaccinated bovines. The specificity of this test was similar to that of RID assay which is known to have high specificity for differentiating adult vaccinated from infected bovines. The kappa value among the different tests was good and within the limits of reproducibility and performance expected for the different assays (Table III).
FIG. 4. Frequency distribution of infected and vaccinated bovines in the indirect ELISA (I ELISA102).

FIG. 5. Frequency distribution of infected and vaccinated bovines in the indirect ELISA (I ELISA103).
**FIG. 6.** Frequency distribution of infected and vaccinated bovines in the competitive ELISA (C-ELISA1).

**FIG. 7.** Frequency distribution of infected and vaccinated bovines in the competitive ELISA (C-ELISA2).
**FIG. 8.** Receiver operator (ROC) curves for each ELISA assay. The arrow shows the cut-off point.

**TABLE I. SENSITIVITY AND SPECIFICITY OF THE DIFFERENT ELISAS**

<table>
<thead>
<tr>
<th>Assay</th>
<th>Sensitivity</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>IELISA102 (70%)</td>
<td>100% (99.44 to 100)</td>
<td>95.65 ± 1.37%</td>
</tr>
<tr>
<td>IELISA103 (65%)</td>
<td>99.34% (96.66 to 100)</td>
<td>93.54 ± 1.65%</td>
</tr>
<tr>
<td>CELISA-1 (21%)</td>
<td>95.39 ± 3.33%</td>
<td>94.36 ± 1.52%</td>
</tr>
<tr>
<td>CELISA-2 (21%)</td>
<td>94.08 ± 3.75%</td>
<td>96.83 ± 1.18%</td>
</tr>
</tbody>
</table>

For sensitivity positives defined: RB/CFT[-], N =152  
For specificity positives defined: RB/CFT[-], N =851

**TABLE II. SPECIFICITY OF THE DIFFERENT ELISAS IN ADULT VACCINATED AND CALFHOOD VACCINATED BOVINES**

<table>
<thead>
<tr>
<th>Assay</th>
<th>Adult vaccinated</th>
<th>Calfhood vaccinated</th>
</tr>
</thead>
<tbody>
<tr>
<td>IELISA102 (70%)</td>
<td>95.67 ± 2.77%</td>
<td>92.12 ± 1.95%</td>
</tr>
<tr>
<td>IELISA103 (65%)</td>
<td>97.11 ± 2.28%</td>
<td>92.80 ± 1.87%</td>
</tr>
<tr>
<td>CELISA-1 (21%)</td>
<td>97.11 ± 2.28%</td>
<td>93.75 ± 1.75%</td>
</tr>
<tr>
<td>CELISA-2 (21%)</td>
<td>97.60 ± 2.08%</td>
<td>96.74 ± 1.28%</td>
</tr>
</tbody>
</table>

Adult vaccinated, positives defined: RB/CFT[-], N =208  
Calfhood vaccinated, positives defined: RB/CFT[-], N =736
TABLE III. KAPPA VALUES FOR THE DIFFERENT ELISAS

<table>
<thead>
<tr>
<th></th>
<th>ELISA102</th>
<th>ELISA103</th>
<th>CELISA1</th>
<th>CELISA2</th>
</tr>
</thead>
<tbody>
<tr>
<td>ELISA102</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>ELISA103</td>
<td>0.814 (0.768 - 0.860)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CELISA1</td>
<td>0.774 (0.723 - 0.825)</td>
<td>0.790 (0.742 - 0.839)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CELISA2</td>
<td>0.826 (0.780 - 0.873)</td>
<td>0.770 (0.717 - 0.822)</td>
<td>0.801 (0.751 - 0.850)</td>
<td>0.836 (0.790 - 0.883)</td>
</tr>
</tbody>
</table>

95% Confidence Limits in Brackets

4. DISCUSSION

Control and eradication of brucellosis requires at least four different coordinated measures: vaccination, diagnosis, removal of reactors and epidemiological surveillance. If one of these actions is absent or is partially accomplished, then the disease remains as a constant or periodically emergent nightmare. For the first of the requirements it has been clear for many years that *Brucella abortus* S-19 is an efficient vaccine against bovine brucellosis and for the future there are in process several live experimental vaccines (e.g. non pathogenic rough *Brucella abortus* RB51 and transposon *Brucella abortus* 2308 derived mutants). For the second of the conditions, we have demonstrated (together with our colleagues from other countries) the usefulness of several serological assays which allow us to distinguish vaccinated from infected bovines with high sensitivity and high specificity. The last two conditions are political and therefore out of the scope of this discussion.

From the different immunoenzymatic assays, the C-ELISA2, which uses LPS as antigen and a monoclonal antibody against the C/Y epitope as competing reagent, seems to be the most promising of the ELISAs. This is due to several clear cut facts. For instance, the C-ELISA2 has excellent sensitivity and specificity with good reproducibility and possesses a convenient cut-off value for diagnostic purposes. In addition, this test uses purified LPS as antigen which is relatively easy to prepare and standardise (for serological analysis). Finally it is not restricted for bovines and can be adapted for different species of animals such caprines, ovines, suines, dogs, horses and humans (in these species the test has been tested with positive experiences). One of the only restrictions of these assay is that is not suitable for testing samples in the field, since it requires a relatively sophisticated equipment (ELISA reader, computers), suitable laboratory conditions and skilled technicians. However this test could be recommended for screening a large number of serum samples on a laboratory basis. For testing samples in the field it is probably more realistic to use Rose Bengal test (which possesses high sensitivity) in combination with a RID assay (which is known to have high specificity). These two tests are simple, robust, long term tested and do not require sophisticated equipment and combined generate a powerful tool for sera testing in the field. Similar to the CELISA-2 both tests can be used for the diagnosis of *Brucella* infection in other species, including humans [8].

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REFERENCES