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Development of a new flow-through immunoassay to detect human IgM class antibodies to Brucella melitensis *16M S-lipopolysaccharide and comparison with IgM-ELISA*

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DESARROLLO DE UN NUEVO INMUNOENSAYO TIPO FLOW-TROUGH *PARA LA DETECCIÓN DE ANTICUERPOS IgM HUMANOS CONTRA S-LIPOPOLISACÁRIDO DE* BRUCELLA MELITENSIS *16M Y COMPARACIÓN CON ELISA PARA IgM*

RESUMEN

La brucelosis, una zoonosis de distribución mundial, es causada por varias especies del género *Brucella*. La infección causa síntomas inespecíficos, y su diagnóstico diferencial en los primeros periodos de la enfermedad es muy importante. El lipopolisacárido de la pared celular es un antígeno dominante, y las inmunoglobulinas específicas anti-LPS de la clase IgM son los primeros anticuerpos cuya concentración aumenta durante una infección aguda. Los ensayos que se usan con mayor frecuencia para detectar anticuerpos IgM anti-LPS son: aglutinación del suero en presencia de ditiotreitol (SAT-DTT) y ELISA-IgM. Sin embargo, ambos métodos tienen la desventaja para ser llevados a cabo de que requieren personal entrenado y laboratorios bien equipados. En este trabajo se describe un nuevo ensayo rápido para IgM anti-LPS de *Brucella*, que puede ser útil aun en laboratorios de bajos recursos. La base de este ensayo es la reacción de la IgM sobre una membrana de nitrocelulosa donde previamente se han adsorbido anticuerpos de conejo anti- IgM humana. Un conjugado monodisperso y estable de LPS ligado a partículas de látex azules se utiliza para visualizar la reacción. Con este ensayo, se puede obtener en aproximadamente 10 minutos el resultado de una muestra.

PALABRAS CLAVE: Inmunoensayo/ *Inmunoblotting/ Brucella.*

ABSTRACT

Brucellosis is a zoonosis of world-wide distribution, caused by several species of the genus Brucella. *The infection causes non-specific symptoms, and differential diagnosis is very important in the early stages of the disease. Lipopolysaccharide (LPS) of the cell wall is a dominant antigen, and specific anti-LPS immunoglobulins of the IgM class are the first antibodies whose concentration increases during an acute infection. The tests most often used to detect specific anti-LPS IgM antibodies are a serum agglutination test in the presence of dithiothreitol (SAT-DTT), and an IgM-based enzyme-linked immunosorbent assay (IgM-ELISA). However, both these methods have the disadvantage that they require skilled personnel and well-equipped laboratories to perform them. Here we describe a new rapid test for anti-*Brucella *LPS IgM, which can be useful even in circumstances where few laboratory facilities are available. The basis of this test was the binding of IgM onto a nitrocellulose membrane previously coated with rabbit anti-human IgM antibodies as the solid phase. A monodispersed, stable conjugate of LPS attached to deeply blue-dyed latex particles was used to visualise the reaction. With this assay, results for a specimen were obtained in about ten minutes.*

KEY WORDS: *Serologic test /Immunoblotting /*Brucella.

INTRODUCTION

Brucellosis, a zoonosis of world-wide importance, is caused by one of the following four species of Gram- negative intracellular coccobacilli: *Brucella melitensis, Brucella abortus, Brucella suis* and *Brucella canis* (1). Two other species, *Brucella ovis* and *Brucella neotomae*, have not been described as infectious for humans (2).

It is a serious public health problem and causes significant economic losses in domestic livestock. The main domestic animals that are affected are cattle, sheeps, goats and pigs. Brucellosis is readily transmissible to humans, causing undulant fever which may progress to a more chronic form with

serious complications affecting the musculo-skeletal, cardiovascular, and central nervous systems. *Brucellae* proliferate within host macrophages, and virulence is associated with the ability to multiply intracellularly, avoiding the fusion of the phagosome with the lysosome (3). As in other Gram-negative bacteria, the bacterial cell wall contains a dominant antigen, called lipopolysaccharide (LPS), composed of three distinct domains: lipid A, core oligosaccharide and O-antigen (4). Cultures of *Brucella* can grow with different characteristics, classically designated "smooth" (S) and "rough" (R). The R-variants lack the O- chain which is responsible for the higher virulence of the S-variants. The relative amounts of epitopes A and M on the O-chain of S-LPS vary for the different strains of *Brucella* (5). Antibodies to this S-LPS antigen are the first to appear once infection by smooth *Brucella* has occurred (6), and their detection is crucial for diagnosis. The most important antibodies to be detected are specific anti-LPS immunoglobulins of the IgM class because they are the antibodies whose concentration first increases after infection. Brucellosis is usually diagnosed by laboratory tests such as bacterial culture, and serological tests, including tests for serum agglutination (SAT), anti-human immunoglobulin test (Coombs test), complement fixation test (CF) and enzyme-linked immunosorbent assay (ELISA) (6). An important test which is often used to detect specific anti-LPS IgM, is serum agglutination in the presence of dithiotreitol (SAT-DTT) (2).

The epidemiological characteristics of brucellosis, described above, emphasize the need for rapid diagnostic tests that can be performed in situations where laboratory facilities and/or trained personnel are not available. Some examples of flow-through immunoassays described in the literature are also examples of rapid immunodiagnostic tests. Most of them are employed in the veterinary field (8-10), and they use an enzymatic reaction as the detection system, although dyed colloidal particles have been used as well (7).

The aim of the present study was the development of a new flow-through immunoassay (FTI) to detect human IgM-class antibodies specifically directed against S-LPS of *Brucella melitensis*. Dyed latex particles were used as the detection system. The results of the assay we describe here were compared with those obtained from a commercially-available ELISA kit.

MATERIALS AND METHODS

Sera: 251 specimens were obtained from healthy blood donors (Centro de Referencia para Transfusión Sanguínea-CRTS, Granada, Spain), and 93 from patients who had previously suffered brucellosis. The sera came from existing collections and were selected to include a wide range of reactivities from negative to strongly positive for IgM. All the specimens were stored frozen at –20 º C until tested.

S-LPS antigen: S-LPS antigen was prepared from a culture of *Brucella melitensis 16 M* by the phenol/water method (11).

Latex particles: 0.220 ± 0.012 µm diameter deep blue-dyed latex particles, 2.6% solids (Polysciences, PA, EE.UU.) were used as supplied.

Latex reagent preparation: This conjugate was prepared by passive adsorption of the S-LPS antigen onto latex particles by incubating together 750 µg of LPS (750 µl at a concentration of 1 mg/ml) diluted in phosphate buffered saline (PBS) pH 7.2, ionic strength 0.15 M, and 270 µl of latex at room temperature for 2 hours. After that, the mixture was centrifuged to eliminate unadsorbed LPS and final resuspension of the particles was carried out in PBS containing 10 mg/ml bovine serum albumin (BSA, A-7030, Sigma Chemical Co., EE.UU.) (12).

Membrane: FT 120 nitrocellulose membrane (Schleicher & Schuell, Germany) was used, onto which rabbit polyclonal antibodies specifically directed against human IgM (Dako, Denmark) were adsorbed, at a concentration of 1 mg/ml (13). The unoccupied sites of the membrane were blocked with an inert protein (BSA). Before its use, the membrane was cut into pieces of about 0.8 cm^2.

Enzyme-linked immunosorbent assay for detecting IgM (IgM-ELISA): IgM-ELISA tests were performed using an anti-*Brucella* IgM commercial assay kit in accordance with the manufacturer's instructions (Vircell S.L., Spain). The test provides 96-well flat-bottomed polystyrene plates pre-coated with an appropriate amount of *Brucella melitensis 16 M* SLPS antigen; IgG adsorbent (rabbit polyclonal anti-human IgG antibodies), positive, negative and cut-off control sera and anti-human IgM-peroxidase conjugate. Cut-off control sera were obtained from donors and selected so as to allow discrimination between healthy and *Brucella*-infected people.

Flow–through immunoassays (FTI): The test described here consists of two components: a) pieces of FT 120 nitrocellulose membrane onto which rabbit polyclonal antibodies specifically directed against human IgM had been adsorbed as previously described; y b) a monodispersed and stable preparation of deeply blue-dyed latex particles covered with SLPS on the surface, employed to visualize the reaction. Latex particles were prepared as described above. To perform the immunoassays, 50 µl of 1:5 dilutions of each serum were added to the membrane pieces. After incubation for 3 minutes, the membranes were washed by addition of PBS-Tween 0.05% to eliminate non-specifically bound proteins. Then, latex reagent (15 µl) was added and the membrane washed again as before. This stage of the assay does not need additional incubation time, since the membrane

Figure 1. *Some typical results obtained with the FTI-test. A,B-negative; C,D-positive; E,F- strongly positive sera.*

is wet, so capillary attraction forces migration of liquid while the excess is absorbed at the back of the membrane. The first step of the assay captures the IgM- immunoglobulins exclusively, obviating the need for addition of anti-IgG antibodies to avoid the competitive interference of IgG; the washing step eliminates the IgG together with the rest of the serum components. A positive result was observed as a blue line on the surface of the membrane. If no specific IgM was present in the specimen, the test zone remained not stained.

RESULTS

Results are summarised in table I. Some typical results obtained with the FTI test are shown in figure 1. Table I records IgM-ELISA results as the value *q*, where *q* is the ratio of $OD_{specimen}$ to $OD_{cut\text{-}off.}$ Sera can be classified in several groups, taking into account IgM-ELISA results and patient histories, as follows:

1. Sera from healthy blood donors, which were negative in IgM- ELISA ($q < 0.9$).

2. Sera from patients with a previous *Brucella* infection, which were negative in IgM-ELISA ($q < 0.9$).

3. Sera from patients with a previous *Brucella* infection, with a borderline titre in IgM-ELISA ($0.9 < q < 1.1$).

4. Sera from patients with a previous Brucella infection, now positive in IgM- ELISA ($q > 1.1$).

FTI results were classified as positive or negative. All of the sera coming from healthy blood donors were negative in the FTI test.

Taking the IgM-ELISA test as the reference assay, diagnostic sensitivity and specificity of the FTI assay were estimated as 80 and 98%, respectively, when borderline sera in IgM-ELISA were considered positive. When these borderline sera were considered negative, the sensitivity of the FTI assay was calculated to be 95%.

DISCUSSION

In the present work a new FTI assay to detect specific antibodies of the IgM class to *Brucella* is described and compared with a commercial IgM-ELISA test. Although the same S-LPS antigen preparation was used in both immunoassays, their formats were different. In the IgM-ELISA test the antigen was provided already adsorbed onto 96-microwell polystyrene plates. In the FTI test, however, capture antibodies against human IgM were adsorbed directly onto nitrocellulose membranes. The FTI here described is a potentially useful method for detecting IgM in the presence of IgG. Since the capture immunoreagent was specifically directed against IgM, the step for removing IgG antibodies was avoided, as IgG molecules were easily removed by means of the washing steps. This is an important comparative advantage over the IgM-ELISA method, where it was necessary to perform an additional step to eliminate IgG. The FTI assay did not require expensive equipment or highly-trained personnel. The cost of FTI is lower compared to ELISA. Good correlation was observed between the results obtained with the IgM-ELISA test and those obtained with the FTI (Table I). The FTI's specificity was good, and its sensitivity was acceptable for a rapid test. The FTI test is simpler than the SAT-DTT or IgM- ELISA tests, which need skilled personnel to perform them. Recently, a dipstick assay has been described (6) for diagnosing the early stages of human brucello-

Table I
Results of correlation between the commercial IgM-ELISA test and the FTI for the different groups of sera. The number of specimens tested by IgM-ELISA in each group are defined by the q value

	IgM-ELISA	*FTI*	
		Positive	*Negative*
Healthy blood donors, negative	251	0	251
Patients with a past infection, negative	69	4	65
Patients with a previous infection, with borderline titre	6	2	4
Patients with a previous infection, positive	18	17	1

sis by detecting anti-*Brucella* IgM. Although the dipstick assay is performed in only one step, it takes 3 hours to obtain the result from a specimen. The FTI test here described is even faster to carry out.

Brucellosis is an important zoonosis, and the present FTI test is potentially applicable to diagnosis of the disease in other species. To do this, the anti-human IgM antibodies we used must be replaced by anti-IgM antibodies specifically directed against the IgM of the species selected. New potential applications for this FTI system are being pursued by developing new reagents in further studies.

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