Journal of Microbiological Methods 119 (2015) 106–109

Contents lists available at ScienceDirect

Journal of Microbiological Methods

journal homepage: www.elsevier.com/locate/jmicmeth

Serological diagnosis of bovine brucellosis using *B. melitensis* strain B115

Marialaura Corrente [a,*], Costantina Desario [a], Antonio Parisi [b], Erika Grandolfo [a], Domenico Scaltrito [c], Gesualdo Vesco [d], Valeriana Colao [a], Domenico Buonavoglia [a]

[a] Department of Veterinary Medicine, University of Bari "Aldo Moro", Strada Provinciale per Casamassima km.3, 70010, Valenzano, BA, Italy
[b] Istituto Zooprofilattico della Puglia e Basilicata, Via Chiancolla, c.da San Pietro Piturno, 70017, Putignano, BA, Italy
[c] Istituto Zooprofilattico della Puglia e Basilicata, Via Manfredonia, 20, 71121, Foggia, Italy
[d] Istituto Zooprofilattico della Sicilia, Via Gino Marinuzzi, 3, 90129 Palermo, Italy

ARTICLE INFO

Article history:
Received 23 June 2015
Received in revised form 22 September 2015
Accepted 12 October 2015
Available online xxxx

Keywords:
Bovine brucellosis
False positive serological reactions
Brucella strain B115

ABSTRACT

Bovine brucellosis is diagnosed by official tests, such as Rose Bengal plate test (RBPT) and Complement Fixation test (CFT). Both tests detect antibodies directed against the lipolysaccharide (LPS) of *Brucella* cell wall. Despite their good sensitivity, those tests do not discriminate between true positive and false positive serological reactions (FPSR), the latter being generated by animals infected with other Gram negative microorganisms that share components of *Brucella* LPS. In this study, an antigenic extract from whole *Brucella melitensis* B115 strain was used to set up an ELISA assay for the serological diagnosis of bovine brucellosis. A total of 148 serum samples from five different groups of animals were tested: Group A: 28 samples from two calves experimentally infected with *Yersinia enterocolitica* O:9; Group B: 30 samples from bovines infected with *Brucella abortus*; Group C: 50 samples from brucellosis-free herds; Group D: 20 samples RBPT positive and CFT negative; Group E: 20 samples both RBPT and CFT positive. Group D and Group E serum samples were from brucellosis-free herds.
Positive reactions were detected only by RBPT and CFT in calves immunized with *Y. enterocolitica* O:9. Sera from Group B animals tested positive also in the ELISA assay, whereas sera from the remaining groups were all negative. The results obtained encourage the use of the ELISA assay to implement the serological diagnosis of brucellosis.

1. Introduction

Brucellosis is a worldwide disease affecting several animal species and humans. Among the numerous *Brucella* species, *Brucella abortus* and *Brucella melitensis* infect predominantly ruminant species (Michaux-Charachon et al., 2002).

Due to the economic losses in livestock farms and to the risk of transmission to humans, brucellosis eradication plans are active in European Countries. These plans require systematic diagnosis of brucellosis in ruminant herds and impose slaughtering of infected animals. Bacteriological analysis is the gold standard test to diagnose brucellosis but it is time-consuming and impractical for large-scale use. Furthermore, bacteriological analyses are performed on organs from slaughtered animals (Corbel et al., 1979). Serology is an alternative option to diagnose brucellosis (Bricker, 2002) and two tests (i.e., a rapid seroagglutination test, known as Rose Bengal plate test, RBPT, and a Complement Fixation test, CFT) are currently used to detect antibodies to the smooth-lipolysaccharide (S-LPS) of *Brucella* cell wall.

The S-LPS molecule comprises three parts: the lipid A, the core oligosaccharide and the distal O-polysaccharide chain (O-PS or O-antigen). The O-PS is a homopolymer of N-formyl-perosamine (Adone et al., 2011).

B. abortus, *B. melitensis*, *Brucella suis*, *Brucella neotomae*, *Brucella microti*, as well as the recently isolated *Brucella pinnipedialis* and *Brucella ceti* species, express a smooth phenotype and carry a complete S-LPS (Foster et al., 2007). Moreover, *B. abortus*, *B. melitensis*, and *B. suis* have common S-LPS epitopes; thus *B. abortus* can be used in serological tests, as recommended by the Office International des Epizooties (OIE) (Nielsen, 2002). Systematic use of serological assays and compliance to eradication plans has led to a drastic decrease in disease prevalence. However, some animals, called "single-reactors" (SR), test positive serologically although they are not infected with *Brucella* spp. (Godfroid et al., 2002). Indeed, false positive serological reactions (FPSR) are known to occur since other Gram-negative bacteria share LPS components with *Brucella* spp. LPS (Corbel and Cullen, 1970). In particular, *Yersinia enterocolitica* serotype O:9 shares an identical O-PS chain with *Brucella* spp. and this identity is responsible for FPSR when diagnosing brucellosis. Cross-reactions have been reported in humans, ruminants and pigs (Ahvonen and Sievers, 1969; Weynants et al., 1996; Jungersen et al., 2006). In particular, positivity to conventional

Abbreviations: RBPT, Rose Bengal plate test; CFT, Complement Fixation Test; LPS, lipolysaccharide; FPSR, false positive serological reactions; S-LPS, smooth-LPS; O-PS, O-polysaccharide; OIE, Office International des Epizooties; SR, single reactors; IZSs, Istituti Zooprofilattici; OMPs, outer membrane proteins; OD, optical density; R.O.C., receiver operating characteristic.

* Corresponding author.
E-mail address: marialaura.corrente@uniba.it (M. Corrente).

http://dx.doi.org/10.1016/j.mimet.2015.10.012
0167-7012/

brucellosis tests has been described in cattle and pigs experimentally infected with *Y. enterocolitica* O:9 (Garin-Bastuji et al., 1999; Thibodeau et al., 2001a).

According to the Italian Veterinary Regulations (Decreto Ministeriale 02/07/1992, n. 453; Decreto Ministeriale 27/08/1994, n. 651), an animal is considered infected with *Brucella* spp. when it is RBPT positive and CFT positive (antibody titer ≥20 ICFTU/ml) or when it is RBPT negative and CFT positive. In Italy, the "Istituti Zooprofilattici" (IZSs) are reference centers to diagnose brucellosis serologically. If a serum sample is RBPT positive and CFT negative, the animal is subjected to a second test. Meanwhile, even if the animal is a SR, the brucellosis-free status of the herd is suspended. If positivity is confirmed with the second test (either RBPT and/or CFT) the animal is slaughtered. Moreover, the brucellosis-free status can be re-established after slaughtering all the positive animals and when none of the remaining animals in the herd, tests positive to two consecutive serological controls (made at a 3-week interval) and to a final control (performed 3–6 months later). Thus, the brucellosis-free status can be re-established 5–7 months later.

Diagnostic tests using other *Brucella* antigens have been developed (Verstreate and Winter, 1984; Santos et al., 1984 ; Cloeckaert et al., 2002). Western blotting or competitive ELISAs, have been employed to analyze the antibody response against proteins of *Brucella* spp. (Letesson et al., 1997). Unfortunately, results obtained with those tests are not easily reproducible and conflicting results, in terms of sensitivity, hamper their use in serological diagnosis (Letesson et al., 1997; Corrente et al., 2004).

Rough (R) *Brucella* strains have been considered as potential antigens for diagnostic purposes since *B. abortus* RB51, *B. melitensis* B115, *Brucella ovis* and *Brucella canis* are typically R strains (Alton et al., 1988; Foster et al., 2007). R strains lack the O-PS chain and do not stimulate the production of cross-reactive antibodies (interfering with the diagnosis of brucellosis) as conventional tests detect antibodies to this antigen (Moriyon et al., 2004).

B. melitensis strain B115 is a R strain isolated in Malta from goat milk, which has been employed as antigen in Brucellin intradermal test (Jones et al., 1973) and it has been partially characterized (Adone et al., 2011). Strain B115 is defective of a gene that promotes translocation of O-PS to the outer membrane and, therefore, does not induce production of antibodies to this component. On the other hand, cytosoluble proteins of strain B115 elicit both humoral and cell-mediated response (Debbarh et al., 1995; Dieste-Perez et al., 2014). Thus, serum antibodies detected when *B. melitensis* strain B115 is used as an antigen, should be more specific than antibodies to S-LPS. In this note, we describe the development of an ELISA test employing an extract of whole *B. melitensis* strain B115, as antigen, and tested its specificity in serological diagnosis of brucellosis.

2. Materials and methods

2.1. Preparation of B. melitensis B115 extracts

B. melitensis attenuated strain B115 was kindly provided by Dr. Rosanna Adone (Istituto Superiore di Sanità), and it was used to prepare the bacterial extract. The bacteria were cultured in 1 l of *Brucella* broth (Becton Dickinson, France) at 37 °C in aerobic conditions under stirring.

When the culture reached an optical density (OD) of 2.080, the broth was centrifuged at 9000 rpm for 20 min. The pellet was resuspended with saline solution and centrifuged at 9000 rpm for 10 min. The pellet was then harvested, resuspended with 8 ml of saline solution and inactivated at 100 °C for 10 min. The bacterial suspension was sonicated, using an ultrasonic processor (Vibra-cell, Sonics and Materials, USA). After sonication, the suspension was centrifuged and subjected to ultracentrifugation (30,000 g for 20 min). The supernatant was dialyzed against distilled water and harvested. The sample was subjected to protein quantification using Biophotometer plus (Eppendorf, Germany).

2.2. Serum samples

A total of 148 serum samples from five different groups of animals were tested.

Group A: 28 sera were obtained from animals experimentally infected with *Y. enterocolitica* O:9. The sera were provided by the IZS–Sicilia and were collected weekly, from time 0 (before infection) until week 13 post infection (p.i.), from two *Brucella*-free calves which had been infected with 4 ml of a bacterial suspension containing 10^{12} *Y. enterocolitica* O:9 cells/ml.

Group B: 30 sera, both RBPT and CFT positive, were collected in *Brucella* infected herds. These seropositive animals were slaughtered and infection with *B. abortus* was confirmed by bacteriological analysis of lymph nodes (Alton et al., 1988).

Group C: 50 sera were collected from brucellosis-free herds (i.e., herds with no positive animals detected in the last 5 years).

Group D: 20 sera were from SR animals, RBPT positive and CFT negative.

Group E: 20 sera were both RBPT and CFT positive.

Group D and Group E serum samples were from brucellosis-free herds. In addition, *Y. enterocolitica* O:9 was isolated from rectal swabs of animals from Groups D and E after bacteriological cultures (according to the International Standard ISO 10273:2003).

The OIE international standard serum, supplied by the OIE Reference Laboratory for brucellosis at the Veterinary Laboratories Agency (VLA) of Weybridge, was used as positive control serum.

2.3. Serological tests

RBPT and CFT were performed according to international standard procedures (World Organization for Animal Health, OIE, 2015). ELISA was performed according to a previously described protocol with some modifications (Elia et al., 2010). In particular, polysorp microtiter plates (Nunc, Milan, Italy) were coated with serial dilutions of the bacterial extract (50–250 ng/well) and incubated overnight at +4 °C. Since preliminary testing established that the optimal concentration of the bacterial extract was 50 ng/well diluted in 100 μl of carbonated buffer (15 mM Na_2CO_3, 35 M $NaHCO_3$ pH 9.6) that concentration was used in all subsequent tests. The plates were coated with 100 μl of the bacterial extract and incubated overnight at +4 °C under gentle shaking. The plates were then washed three times with PBS containing 0.05% Tween 20 (PBS-T) and 100 μl of blocking solution (0.2% gelatin in carbonate buffer) were added to each well for 90 min at 37 °C. After repeated washes, 100 μl of serum, diluted 1:50 in PBS-T, were added in duplicate and the plates were incubated at 37 °C for 90 min. After washings, a rabbit anti-bovine antibody labeled with peroxidase (Sigma Aldrich, Milan, Italy) was diluted 1:1000 in PBS-T and added to the plates which were then incubated for 60 min at 37 °C. After final washings, an ABTS [2,2′-Azino-di-(3-ethylbenzothiazoline sulfonate)] solution (Sigma Aldrich, Milan, Italy) was added to each well and the plate was incubated at room temperature for 25 min. The OD was measured at 405 nm using an automated ELISA reader. The negative samples (Group C, n = 50) were used to determine the cut-off value of the ELISA test (i.e., the arithmetic mean of the OD of all negative samples plus 3 standard deviations).

2.4. Statistical analysis

Results were analyzed by chi square test with Yates correction. A p value ≤ 0.05 was considered statistically significant. For CFT, a value ≥ 20 ICFTU was considered positive while for ELISA, an OD value above the cut off was considered positive.

In addition, a R.O.C. (receiver operating characteristic) curve was generated using the R Software, version 2.8.1 (http://www.r-project.org/).