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# Performance of skin tests with allergens from *B. melitensis* B115 and rough *B. abortus* mutants for diagnosing swine brucellosis

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ABSTRACT

Swine brucellosis by *Brucella suis* biovar 2 is an emerging disease whose control is based on serological testing and culling. However, current serological tests detect antibodies to the O-polysaccharide (O/PS) moiety of *Brucella* smooth lipopolysaccharide (S-LPS), and thus lack specificity when infections by *Yersinia enterocolitica* O:9 and other gram-negative bacteria carrying cross-reacting O/PS occur. The skin test with the protein-rich brucellin extract obtained from rough *B. melitensis* B115 is assumed to be specific for discriminating these false positive serological reactions (FPSR). However, B115 strain, although unable to synthesize S-LPS, accumulates O/PS internally, which could cause diagnostic problems. Since the brucellin skin test has been seldom used in pigs and FPSR are common in these animals, we assessed its performance using cytosoluble protein extracts obtained from *B. abortus* rough mutants in *manBcore* or *per* genes (critical for O/PS biosynthesis) and *B. melitensis* B115. The diagnostic sensitivity and specificity were determined in *B. suis* biovar 2 culture positive and brucellosis free sows, and apparent prevalence in sows of unknown individual bacteriological and serological status belonging to *B. suis* biovar 2 naturally infected herds. Moreover, the specificity in discriminating brucellosis from FPSR was assessed in brucellosis free boars showing FPSR. The skin test with *B. abortus* Δ*manBcore* and *B. melitensis* B115 allergens performed similarly, and the former one resulted in 100% specificity when testing animals showing FPSR in indirect ELISA, Rose Bengal and complement fixation serological tests. We conclude that O/PS-free genetically defined mutants represent an appropriate alternative to obtain *Brucella* protein extracts for diagnosing swine brucellosis.



## 1. Introduction

Swine brucellosis is caused by *Brucella suis* biovars 1, 2 and 3. Biovars 1 and 3 are endemic in America and Asia and are zoonotic, causing reproductive problems in pigs and severe infections in humans. In contrast, biovar 2 is restricted to Europe, where it infects domestic pigs and wildlife. Although *B. suis* biovar 2 has been isolated rarely from humans and seems to affect only immune-compromised patients (EFSA, 2009), it represents a common infection in pigs reared in outdoor breeding systems, likely as a spill-over from brucellosis of wild boar and hares,

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which are considered the wild reservoir of this infection (Abdoel et al., 2008; Cvetnic et al., 2009; Garin Bastuji et al., 2000; Muñoz et al., 2010). Thus, surveillance of brucellosis in pigs is important and, moreover, control and eradication has to be implemented in the affected farms. Surveillance and eradication programs are currently based on the serological monitoring of pigs and the subsequent culling of those seropositive or, more frequently, the stamping out of the affected farm. However, none of serological tests used currently for diagnosing brucellosis in pigs provides satisfactory specificity and sensitivity (EFSA, 2009; Ferris et al., 1995; Rogers et al., 1989). In addition to intrinsic problems or defects in validation that limit the diagnostic performance of these tests (McGiven et al., 2012; Muñoz et al., 2012), many pig farms in brucellosis-free countries are currently affected by false positive serological reactions (FPSR). FPSR result from infections by *Y. enterocolitica* O:9 and other gram-negative bacteria that share common epitopes with the *Brucella* O-polysaccharide (O/PS), the smooth lipopolysaccharide (S-LPS) moiety antigenically relevant in brucellosis serological tests (EFSA, 2009; McGiven et al., 2012). Because of the FPSR problem, seropositive pigs have to be unequivocally confirmed as infected through isolation of *B. suis* (Olsen et al., 2012). However, bacteriological diagnosis lacks sensitivity and is slow, cumbersome and expensive, being impractical for individual diagnosis in large animal populations. In contrast to S-LPS based tests, immunoassays using *Brucella* cytosoluble proteins are not affected by FPSR and, as brucellosis induces both cellular and antibody mediated responses to these antigens, the brucellin skin test is considered the test of choice in FPSR contexts. However, studies using this assay in swine are scanty (EFSA, 2009). Moreover, the only skin test allergen available (known as brucellergene OCB, brucellin INRA or brucellin) is a hypertonic cytosoluble extract obtained from the rough (R) *B. melitensis* B115 strain. Owing to its R phenotype (*i.e.* the lack of S-LPS in the cell surface), this strain is assumed to be free of O/PS and, in fact, a single brucellin inoculation does not elicit antibodies detectable in S-LPS based serological tests (EFSA, 2009). However, it is known that *B. melitensis* B115 synthesizes an O/PS molecule linked to the inner leaflet of the cytosolic membrane (Cloeckaert et al., 1992), in part because of the frame-shift mutation affecting the *wzm* gene required for O/PS export (Adone et al., 2011). We have previously shown that *B. melitensis* Δ*wzm* mutants are similar to B115 in that they accumulate cytosoluble O/PS and elicit anti-S-LPS antibodies (González et al., 2008). Accordingly, repeated inoculations with B115 brucellin might elicit antibodies interfering in S-LPS based diagnostic tests. Since current knowledge on the molecular basis of *Brucella* LPS biosynthesis (Monreal et al., 2003; González et al., 2008) allows proposing genetically defined mutants devoid of O/PS as an appropriate source of protein allergens, in this work we have assessed the performance of cytosoluble extracts of *B. abortus* mutants in *manBcore* and *per* genes (both critical for O/PS synthesis) for diagnosing *B. suis* biovar 2 infection in pigs, in comparison with the classical *B. melitensis* B115 brucellin skin test and several S-LPS based serological tests.

## 2. Materials and methods

### 2.1. Bacterial strains and growth conditions

The *B. melitensis* B115 is a naturally R strain isolated in Malta from goat's milk, which has been used classically for brucellin extraction (Jones et al., 1973). A partial characterization of this strain has been conducted (Adone et al., 2011), but their genetic defects remain to be fully clarified. *B. abortus* Δ*manBcore* and *B. abortus* Δ*per* are R mutants obtained by transposon mutagenesis from the smooth *B. abortus* 2308 reference strain (Monreal et al., 2003). *B. abortus* Δ*manBcore* carries a mini-Tn5 inserted in *manBcore* (ORF BAB2_0856), encoding putatively the phosphomannomutase required for mannose biosynthesis. Since mannose is both a *Brucella* LPS core component (Kubler-Kielb and Vinogradov, 2013), and the biosynthetic precursor of N-formyl-perosamine (the main component of *Brucella* O/PS), *Brucella* Δ*manBcore* mutants carry a deeply truncated LPS, are unable to synthesize any form of O/PS precursors, and show higher attenuation than other R mutants (González et al., 2008). The *B. abortus* Δ*per* mutant carries also a mini-Tn5 inserted in *per* (ORF BAB1_0544), the putative perosamine synthase gene. All strains were grown in 1.7% casitone (Pronadisa, Madrid, Spain), 0.3% soy tone (E. Merck, Darmstadt, Germany), 0.5% yeast extract (Merck, Darmstadt, Germany), 0.25% $K_2HPO_4$, 2% glucose, 0.5% NaCl, and 0.01% A-butyl acetate (1:3) (Sigma Chemical CO., St Louis, MO, USA), in a 15 litre Biostat fermentor (B. Braun Melsungen AG, Leinfelden, Germany) for 48–72 h at 36 °C, as described elsewhere (Velasco et al., 1997). Bacteria were inactivated with phenol (0.5%, 37 °C, 48 h), washed twice with sterile saline (0.85% NaCl), and harvested by centrifugation (9000 rpm, 12 min).

### 2.2. Allergen extraction and characterization

A cytosoluble extract was obtained from each strain using methods described previously (Blasco et al., 1994; Velasco et al., 1997). Briefly, bacteria in a thick suspension in 0.1 M phosphate-buffered saline (pH 7.2) containing DNA-ase and RNA-ase (50 μg per ml of suspension), were disintegrated in a 40 K French Pressure cell (SLM Instruments Inc. Urbana, IL, USA) at 40,000 psi. Envelopes and cell debris were centrifuged (40,000 × *g*, 2 h, 4 °C) and the supernatant incubated at 4 °C overnight. Then, this cytosoluble fraction was ultra-centrifuged (100,000 × *g*, 6 h, 4 °C), and the supernatant dialysed against deionised water, and freeze dried. The total protein contents of the cytosoluble fractions were calculated colorimetrically (Markwell et al., 1978) using bovine serum albumin as standard. These fractions were further characterized using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by periodate silver staining (Tsai and Frasch, 1982), Western blotting (Burnette, 1981), and immunoelectrophoresis (Grabar et al., 1955) with sera from *B. suis* biovar 2 infected and brucellosis free pigs (see below).

Moreover, the biological activity of *B. abortus* Δ*manBcore* allergen was assessed *in vivo* using the guinea-pig