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Performance of microbiological, serological, molecular, and modified seminal plasma methods in the diagnosis of *Brucella abortus* in semen and serum of bovine bulls☆

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ABSTRACT

Brucellosis remains as a major infectious disease of domestic animals and is considered a re-emerging zoonosis in several countries. *B. abortus* infections in bulls are related to reproductive tract infections, although infected animals show transient serological titers or nonreactor status. Thus, diagnosis of bovine brucellosis based exclusively on serological tests probably underestimates *B. abortus* infections in bulls. In this scenario, three hundred thirty-five serum samples from reproductively mature bovine bulls were subjected simultaneously to standard serodiagnosis using the rose Bengal test (RBT), 2-mercaptoethanol (2-ME), complement fixation (CFT), and fluorescence polarization assay (FPA). Furthermore, conventional semen plasma agglutination (SPA) and modified 2-ME, FC and, FPA were carried out in all bulls replaing serum by seminal plasma. Semen from all bulls was also analyzed for sperm viability, microbiological culture in Farrell media, and polymerase chain reaction (PCR). Only eight (2.38%) semen samples were considered improper for reproduction services (necrospermia and azoospermia), although none of these animals was positive in any of the diagnosis methods used. Five bulls (1.49%) were simultaneously positive in conventional RBT, 2-ME, SPA, modified 2-ME, microbiological culture in Farrell media, and in PCR for *B. abortus* strain 19. Two (1.67%) bulls were positive in PCR for *B. abortus* field strains and negative in all other tests, although semen was considered viable to reproduction service. The identification of *B. abortus* B19 strain in serum and semen of bulls occurred probably due to improper vaccination of males or infection by B19 strain shedding by vaccinated females that could to contaminated environment of farms. In addition, detection of *B. abortus* field strains only using PCR in bulls without sperm viability abnormalities indicate the need for including molecular methods to improve diagnosis of the disease in bovine bulls.

1. Introduction

Brucellosis remains a public health concern and is considered a re-emerging zoonosis in several countries [1]. *Brucella* infections represent the greatest economic threat to livestock worldwide, particularly in developing countries because of reproductive problems, reduced milk yield, and restrictions to animal movement

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and trade imposed by international regulatory organizations [2].

Brucella abortus is a well-recognized intracellular gram-negative bacteria considered to be the major cause of brucellosis in domestic cattle. In livestock, *B. abortus* infection is commonly acquired by direct exposure to fluids and tissues from infected fetuses or vaginal discharges after abortion. Alternatively, ingestion of water or pasture contaminated with fetal fluids and tissues, inhalation, sexual contact, and artificial insemination with contaminated semen may be considered in the transmission [3].

In bovine bulls, after intermittent bacteremia, pathogen infects reproductive organs and accessory glands of mature animals [4]. The pathogenicity of *B. abortus* to bovine males is related to seminal vesiculitis, orchitis, and epididymitis [3,5]. Nevertheless, infected bulls could show transient antibody titers or nonreactor status when subjected to conventional serological tests [4]. Thus, diagnosis of bovine brucellosis based exclusively on serological tests probably underestimates *B. abortus* infections in bulls [6].

Despite comprehensive studies involving serodiagnosis of cattle brucellosis [7], minor attention has been reserved to the diagnosis of the disease in bovine bulls using different methods, particularly regarding semen shedding of *B. abortus* [8]. In the current study, 335 serum samples from reproductive mature bovine bulls without apparent signs of reproductive tract inflammation were subjected simultaneously to serodiagnosis using rose Bengal test (RBT), 2-mercaptoethanol (2-ME), complement fixation (CFT), and fluorescence polarization assay (FPA). Semen of the same bulls was also analyzed for sperm viability and submitted to microbiological culture in Farrell media, semen plasma agglutination test (SPA), and polymerase chain reaction (PCR). Furthermore, 2-ME, FC and FPA tests were carried out replacing serum by semen of all bulls (modified tests).

2. Material and methods

2.1. Animals and samples

Serum and semen of 335 bovine bulls used both for natural mating and as semen donors were sampled from 2013 to 2015. These bulls were reproductively mature (older than 36 months) of different breeds or crossbreeds, without any apparent signs of orchitis or inflammation of accessory reproductive glands. Animals came from three states of the central region of Brazil where bovine breeding is common. Semen samples were aseptically collected by electroejaculation. The material was immediately analyzed for sperm viability [9], and classified as viable or non-viable for reproduction services. An aliquot of the semen samples was frozen (−20 °C) for further microbiological culture. Molecular diagnosis of *Brucella* species was carried out directly from semen, as well as colonies isolated through microbiological culture.

2.2. Microbiological diagnosis

All semen samples were subjected to microbiological culture using Farrell media (Oxoid™). Plates were maintained under micro-aerobic conditions (10% CO_2) at 37 °C, and observed every 24 h, for up to 14 days. Colonies suspected of *B. abortus* were subjected to Gram and Koster's stains. Conventional phenotypic (biochemical) characterization of *B. abortus* was based on CO_2 requirements, catalase, oxidase, urease, citrate, urease, thionin, fuchsin, indol, and nitrate reduction tests [10]. In addition, phenotypic differentiation of *B. abortus* field strains and *B. abortus* B19 vaccine strain was carried out by growing the isolates in thionin (2 μg/mL), penicillin (5 UI/mL), and rifampicin (50 μg/mL) [3,10].

2.3. Serological diagnosis

Serological diagnosis was performed using RBT, 2ME, CFT, and FPA. RBT, 2-ME, and CFT were performed using previously described antigens and procedures [6,11]. CFT was considered positive when at least 50% hemolysis occurred at serum dilution ≥20 ICFTU (international complement fixation test units)/mL [11]. The fluorescence polarization assay (FPA), was carried out with the *Brucella abortus* antibody test kit (Diachemix, USA™), composed of control serum-positive and serum-negative, and 25 times concentrated buffer lipopolysaccharide antigen conjugated fluorescein. Readings were carried out in a polarization analyzer Fluorescent Sentry 100 model (Diachemix, USA™). Results were expressed in milipolarization units (mP). FPA is based on the rotational difference between the soluble antigen molecule (fluorochrome-labeled) and the same molecule attached to the antibody [12]. To determine the cutoff point (CP), two-graph-receiver operation technique characteristic (TG-ROC) were used [13].

2.4. Seminal plasma technique

All semen samples were submitted to SPA. After treatment with 1% sodium azide (30 μL of 1% sodium azide/mL of semen), the samples were subjected to centrifugation. Seminal plasma was withdrawn and submitted to the conventional SPA [14]. In addition, modified 2-ME, CFT and FPA were performed replacing the same amount of serum by seminal plasma.

2.5. Molecular diagnosis of semen

PCR was carried out as described by Richtzenhain et al. [15] based on the 233-bp expected sizes of amplicons for the diagnosis of the genus *Brucella* direct from semen samples.

Genomic DNA was extracted from semen using enzymatic treatment (proteinase K) and boiling. The steps and cycle conditions (40 cycles) for the PCR assay were: initial DNA denaturation at 94 °C for 5 min, DNA denaturation at 94 °C for 60 s, primer annealing at 60 °C for 60 s, DNA extension at 72 °C for 60 s, and final extension at 72 °C for 10 min. PCR products were visualized after electrophoresis in 2% agarose gel stained by ethidium bromide. A molecular weight marker (100-bp ladder Gibco-BRL) was used as size standard. Species of *Brucella* were detected by PCR according to the description by Lopez-Goñi et al. [16] modified according to Lopez-Goñi et al. [17].

2.6. Molecular speciation from Brucella colonies

Genomic DNA was extracted from colonies isolated thought microbiological cultures using boiling procedure. Briefly, 1 μL of the DNA sample, 12.5 μL of enzyme Kapa 2G Fast Multiplex PCR Kit (Kapa Biosystems™), and 10 pmoles of each primer in a final volume of 25 μL were used. The following cycles conditions were used: initial denaturation at 95 °C for 3 min, followed by template denaturation at 95 °C for 15 s, primer annealing at 64 °C for 75 s. Later, a 30-s primer extension at 72 °C (30 cycles), and final extension phase of 10 min at 72 °C were performed. Reactions were carried out in a Multigene (Labnet International, Inc.™) thermocycler. PCR products were analyzed in a 1.5% agarose gel [15,17].

3. Results and discussion

The major results of the current study revealed the occurrence of five bulls (1.49%) were simultaneously positive in conventional RBT, 2-ME, SPA, modified 2-ME, microbiological culture in Farrell media, and in PCR for *B. abortus* strain 19. In addition, two (1.67%)