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 ($-20\,^{\circ}$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 $^{\circ}$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 $^{\circ}$C for 5 min, DNA denaturation at 94 $^{\circ}$C for 60 s, primer annealing at 60 $^{\circ}$C for 60 s, DNA extension at 72 $^{\circ}$C for 60 s, and final extension at 72 $^{\circ}$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 $^{\circ}$C for 3 min, followed by template denaturation at 95 $^{\circ}$C for 15 s, primer annealing at 64 $^{\circ}$C for 75 s. Later, a 30-s primer extension at 72 $^{\circ}$C (30 cycles), and final extension phase of 10 min at 72 $^{\circ}$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%)

bulls were positive in PCR for *B. abortus* field strains and negative in all other tests, although semen was considered viable to reproduction service; indicating the need for the association of methods in order to improve the diagnosis of brucellosis in bovine bulls.

Vaccination of heifers, periodic serological tests, and elimination of positive animals are the critical tools to control and eradicate bovine brucellosis worldwide [1]. In spite of the recently approved RB51 vaccine in the prevention of bovine brucellosis, the use of *B. abortus* strain 19 vaccine remains more widespread for bovine heifers in control and eradication programs for the disease worldwide [21]. Five animals sampled in the current study were positive in all conventional serological tests analyzed, and in modified 2-ME. The same animals were PCR-positive for *B. abortus* and showed isolation of *B. abortus* in Farrell media (Table 1). Particularly in Brazil, bovine brucellosis remains an endemic disease in several states and regions [22].

Based on microbiological culture, phenotypic characteristics and PCR analysis, these five isolates were identified as *B. abortus* strain 19. Positive serodiagnosis, SPA, and modified 2-ME in five bovine bulls from Brazil that shedding *B. abortus* strain 19 in the semen probably was caused by vaccination of male calves or through exposure to a strain 19-related abortion by an adult cow [23,24]. It is important to note that in Brazil, this vaccine is approved exclusively for heifers (3–8 months of age) [24]. In addition, the mechanism of *B. abortus* infection of bovines by B19 vaccine strain shedding in environment by females remains not entirely clear [25]. The presence of mature bulls seropositive for brucellosis in the sampled animals reinforce the recommendation to limit the use *B. abortus* strain 19 vaccine exclusively to heifers, since higher persistence of serum titers in conventional serological tests have been reported after vaccination of males with strain 19, compared with heifers [6].

Vaccination of bovine bulls with strain 19 may cause orchitis, seminal vesiculitis, and epididymitis, transitorily affecting spermatogenesis [1]. In addition, shedding of *B. abortus* strain 19 in the semen of bulls several months after vaccination was also described [6]. Five seropositive bulls sampled herein that eliminated *B. abortus* strain 19 in the semen were considered apt to reproduction services based only on andrologic examination (Table 1). These data highlight the risk of evaluating semen quality in bovine bulls based exclusively on parameters of seminal viability, without subjecting the animals to diagnosis of reproductive diseases, particularly brucellosis [4]. However, semen with *Brucella* deposited in the vagina does not appear to be high risk for infection, due to local immune response, as opposed to pathogen that is deposited in to the uterus by artificial insemination does frequently lead to infection [4,6].

Semen of only eight animals (2.38%) were considered nonviable for reproduction services (necrospermia or azoospermia). However, none of these animals showed reactions in serological tests, SPA, or positive results in Farrell culture and PCR (Table 1). Improper viability of semen of these eight animals negative for brucellosis may have been caused by other pathogens, as well as by traumatic, degenerative, and genetic disorders that may affect the quality of the ejaculate of bulls at reproductive age [4,9].

The pathogenic action of *B. abortus* in bulls is intimately associated with seminal vesiculitis, orchitis, and epididymitis [6]. In this context, one bovine bull was followed over 18 months isolating *B. abortus* from each of 80 ejaculations, with 500 viable organisms/mL of semen [18]. Another similar study, isolated *B. abortus* from 90 of 93 (97.0%) consecutive semen ejaculations from a bovine bull for five year period. In the same study, the pathogen was recovered from another bovine bull over 2 years and 6 months [19]. Both aforementioned studies detected macroscopic abnormalities in the semen [18,19]. Rough *B. abortus* biotype I was identified from the vas deferens and seminal vesicles in a bull, although no isolates were recovered from the semen of this animal on 12 attempts [20]. Nevertheless, despite *B. abortus* targeting the reproductive tract of bovine bulls and the potential elimination of pathogen in the semen, infected males may show transient antibody titers [14] or be nonreactors in conventional serological tests [4–6], difficulting serum diagnosis of brucellosis in bovine bulls.

Modified 2-ME, FCT and FPA were used in all semen samples of 335 bulls by replacing serum with seminal plasma. However, only modified 2-ME agreed with conventional RBT, 2-ME, SPA, PCR, and microbiological culture (Table 1), showing that modified 2-ME may be valuable in the diagnosis of bovine brucellosis in bulls.

Among the 335 semen samples, two (1.67%) were positive in PCR for field *B. abortus* isolates, and negative in all the other tests, although semen of these animals was considered viable for reproduction service (Table 1). Few studies have detected the DNA of *Brucella* species in the semen of seronegative bovine bulls [8].

Semen positive in PCR for *B. abortus* and seronegative in conventional serological tests may be explained by the lower threshold of PCR, which needs fewer microorganisms for positive detection. Overall, detection of *B. abortus* field strains only using PCR in bulls

Table 1

Andrologic examination, conventional serological methods (RBT, 2-ME, CFT, FPT), semen plasma agglutination (SPA), modified seminal plasma examination, polymerase chain reaction (PCR) and microbiological culture of semen of 335 bulls. Brazil, 2013–2015.

Diagnosis methods	Andrologic examination	Serological methods				Conventional SPA	Modified seminal plasma			Molecular procedures	Microbiological culture
Identification of bulls	viability of semen	RBT	2-ME	FCT	FPA	SPA	2-ME	FCT	FPA	PCR	Farrel media
13	viable	NR	NR	NR	NR	NR	NR	NR	NR	positive	negative
25	viable	NR	NR	NR	NR	NR	NR	NR	NR	positive	negative
43	necrospermia	NR	NR	NR	NR	NR	NR	NR	NR	negative	negative
52	necrospermia	NR	NR	NR	NR	NR	NR	NR	NR	negative	negative
84	azoospermia	NR	NR	NR	NR	NR	NR	NR	NR	negative	negative
98	azoospermia	NR	NR	NR	NR	NR	NR	NR	NR	negative	negative
110	azoospermia	NR	NR	NR	NR	NR	NR	NR	NR	negative	negative
136	necrospermia	NR	NR	NR	NR	NR	NR	NR	NR	negative	negative
157	necrospermia	NR	NR	NR	NR	NR	NR	NR	NR	negative	negative
161	necrospermia	NR	NR	NR	NR	NR	NR	NR	NR	negative	negative
331	viable	positive	positive	NR	NR	positive	positive	NR	NR	positive	positive
332	viable	positive	positive	NR	NR	positive	positive	NR	NR	positive	positive
333	viable	positive	positive	NR	NR	positive	positive	NR	NR	positive	positive
334	viable	positive	positive	NR	NR	positive	positive	NR	NR	positive	positive
335	viable	positive	positive	NR	NR	positive	positive	NR	NR	positive	positive

NR = nonreactive, RBT = rose bengal test, 2-ME = 2-mercaptoethanol, CFT = complement fixation test, FPA = fluorescence polarization assay, SPA = semen plasma agglutination.

without sperm viability abnormalities indicate the need for including molecular methods to improve diagnosis of the disease in bovine bulls.

Acknowledgments

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