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MICROBIOLOGY

Phenotypic and genotypic characterization of *Brucella abortus* biovar 4 isolates from cattle in Brazil

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ABSTRACT: The aim of the present study was to characterize (phenotypically and genotypically) two strains of *Brucella abortus* identified as belonging to biovar 4 isolated from cattle in Brazil. The strains were isolated from cervical bursitis from cattle in the states of Pará and Rio Grande do Sul, respectively. In the phenotypic identification, the isolates were positive in CO_2 requirement, produced H_2S, were resistant to basic fuchsin (20 µg / mL) and sensitive to thionin (20 µg / mL and 40 µg / mL) and presented M surface antigen, but A surface antigen is absent. The isolates were positive in the PCR for the *bcsp31* gene (genus-specific) and in the AMOS-enhanced PCR, both isolates showed a band profile consistent with *B. abortus* biovar 1, 2 or 4. Moreover, both isolates also showed restriction patterns identical to the reference strain when tested by the *omp2b* PCR-RFLP. In genotyping using Multiple Locus Variable Number of Tandem Repeat (VNTR) Analysis - MLVA (MLVA16), the isolates showed differences in several *loci* (Bruce42, Bruce19, Bruce04, Bruce16 and Bruce30); by Multiple Locus Sequence Typing (MLST), they also exhibited differences in sequence type (ST), strain 16/02 ST1 (2-1-1-2-1-3-1-1-1) and strain 128/11 ST (22-1-1 -8-9-3-1-1-1). The extensive typing of *B. abortus* strains isolated from cattle in Brazil using different approaches confirmed the occurrence of rare *B. abortus* biovar 4 in the country.
Key words: Brucellosis, *Brucella abortus* biovar 4, genotyping, MLVA, MLST.

Caracterização fenotípica e genotípica de isolados de *Brucella abortus* biovar 4 em bovinos no Brasil

RESUMO: O objetivo do presente estudo foi caracterizar (fenotipicamente e genotipicamente) duas cepas de *Brucella abortus* identificadas como pertencentes ao biovar 4 isolada de bovinos no Brasil. As cepas foram isoladas de bursite cervical de bovinos dos estados do Pará e Rio Grande do Sul, respectivamente. Na identificação fenotípica, os isolados foram positivos na exigência de CO_2, produziram H_2S, foram resistentes à fucsina básica (20 µg / mL) e sensíveis à tionina (20 µg / mL e 40 µg / mL) e apresentaram antígeno de superfície M, mas o antígeno de superfície A foi ausente. Os isolados foram positivos na PCR para o gene *bcsp31* (gênero específico) e na PCR - AMOS, ambos os isolados apresentaram perfil de banda consistente com *B. abortus* biovar 1, 2 ou 4. Além disso, ambos os isolados também apresentaram padrões de restrição idêntica à cepa de referência quando testada pelo *omp2b* PCR-RFLP. Na genotipagem usando Multiple Locus Variable Number of Tandem Repeat (VNTR) - MLVA (MLVA16), os isolados apresentaram diferenças em vários *loci* (Bruce42, Bruce19, Bruce04, Bruce16 e Bruce30); no Multiple Locus Sequence Typing (MLST), os isolados também exibiram diferenças na sequência tipo (ST), amostra 16/02 ST1 (2-1-1-2-1-3-1-1-1) e amostra 128/11 ST (22-1-1-8-9-3-1-1-1). A extensa tipagem de cepas de *B. abortus* isoladas de bovinos no Brasil por diferentes abordagens confirmou a rara ocorrência de *B. abortus* biovar 4 no país.
Palavras-chave: Brucelose, *Brucella abortus* biovar 4, genotipagem, MLVA, MLST.

INTRODUCTION

Brucellosis is a worldwide zoonotic disease caused by bacteria of the genus *Brucella*, which infect a wide variety of wild and domestic animals, as well as humans (ALTON et al., 1988). In cattle, infection is mainly caused by *Brucella abortus* (CORBEL, 2006). Due to the impact of *B. abortus* infection on livestock and public health, the control and eradication of bovine brucellosis is an important goal of several countries where the disease is endemic, including Brazil, that since 2001 has implemented the Programa Nacional de Controle e Erradicação de Brucelose e Tuberculose - PNCEBT (National Program for the Control and Eradication of Animal Brucellosis and Tuberculosis) (FERREIRA NETO et al., 2016).

The diagnosis of *Brucella* spp. in brucellosis control and eradication programs is generally based on bacteriological and serological

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tests (ALTON et al., 1988). Although, important for the diagnosis of the disease, phenotypic typing methods generally have less discriminatory power compared with genotypic methods and; therefore, make it difficult to track outbreaks and control the spread of the disease (MINHARRO et al., 2013). In this context, the intraspecific characterization of *B. abortus* at biovar and molecular levels are fundamental for a better understanding of the disease epidemiology, for formulation of effective strategies of infection control and eradication and for solving outbreaks (DORNELES et al., 2014; OLIVEIRA et al., 2017).

By means of phenotypic techniques, it is possible to classify bacteria of the genus *Brucella* into biovars. The *Brucella* International Taxonomy Subcommittee recognizes seven *B. abortus* biovars,1 to 6 and 9 (HOLT, 1984; ALTON et al., 1988). In Brazil, the biovars of *B. abortus* most frequently found, in descending order, were 1, 3, 6 and 2, and, so far, only one single strain of *B. abortus* biovar 4 was identified in the country (MINHARRO et al., 2013). Molecular techniques have been developed for differentiation of *Brucella* strains and biovars (BAILY et al., 1992; BRICKER & HALLING, 1995; CLOECKAERT et al., 1995) that complement the conventional methods used to define the phenotypic profile. Among the molecular typing methodologies commonly used for *Brucella* spp., *Multiple Locus Variable Number of Tandem Repeat* (VNTR) *Analysis* (MLVA) and *Multiple Locus Sequence Typing* (MLST) are well-adapted techniques that have proved to be valuable tools in source tracking and in the intraspecific classification of *Brucella* spp. isolates (OLIVEIRA et al., 2017).

In this study, we performed a wide phenotypic and genotypic characterization of two strains of *B. abortus* biovar 4 first described in cattle from Brazil, in order to support PNCEBT by providing high resolution epidemiologic data on the rare *B. abortus* isolates among cattle in the country.

MATERIALS AND METHODS

Brucella strains

Two *B. abortus* strains, 16/02 and 128/11, are described in this study, being strain 16/02 previously reported by MINHARRO et al. (2013). The 16/02 strain was isolated from the stomach of an aborted fetus of European breed cow, in Rio Grande do Sul in 2002, and the strain 128/11 was isolated and characterized by the Laboratório Federal de Defesa Agropecuária (LFDA / MG) in 2011, from cervical ligament bursitis of a Nellore cattle slaughtered in Pará. The reference strains *B. abortus* biovar 4 292 = ATCC 23451, *B. abortus* biovar 1 544 = ATCC 23448^T, *B. abortus* biovar 1 2308, *B. abortus* biovar 1 S19, *B. abortus* biovar 1 RB51, *B. melitensis* biovar 1 16M = ATCC 23456^T, *B. ovis* Reo 198 and *B. suis* biovar 1 1330 = ATCC 23444 were used as controls in different tests.

Identification and biotyping

Phenotypic identification of the two isolates was performed according to international standards (ALTON et al., 1988), using the following procedures: (i) examination of colony morphology, Gram stain; (ii) metabolic tests based on catalase, oxidase, urease, nitrate reduction and citrate as carbon

Table 1 - Growth characteristics of *Brucella abortus* isolated from cervical ligament lesions and lymph nodes from cattle slaughtered in Rio Grande do Sul and Pará, Brazil, in 2002 and 2011.

Strain	CO_2 requirement	H_2S production	----------Growth on dyes----------			Agglutination in sera	
			Thionin (20 µg/mL)	Thionin (40 µg/mL)	Basic fuchsin (20 µg/mL)	Anti-A	Anti-M
16/02	+	+	-	-	+	-	+
128/11	+	+	-	-	+	-	+
B. abortus biovar 4 292 = ATCC[1] 23451	+	+	-	-	+	-	+
B. abortus biovar 1 544 = ATCC 23448^T	+	+	-	-	+	+	-

[1]ATCC = American type culture collection.

and energy source; (iii) requirement for supplementary carbon dioxide (CO_2) and the production of hydrogen sulfide (H_2S); (iv) sensitivity to thionin (20 and 40 mg / mL) and basic fuchsin (20 mg / mL) dyes in serum dextrose medium; and (v) agglutination with *Brucella* A and M monospecific antisera (Table 1).

Identification by PCR assays

In addition to identification by phenotypic routine tests (ALTON et al., 1988), the isolates were also tested by *bcsp31* PCR (BAILY et al., 1992), AMOS-enhanced PCR (BRICKER & HALLING, 1995) and *omp2b* PCR-RFLP (Polymerase Chain Reaction - Restriction Fragment Length Polymorphisms), with restriction by *Taq*I, to confirm them as *B. abortus* biovar 4 strains (CLOECKAERT et al., 1995; GARCIA-YOLDI et al., 2005).

DNA of the strains were obtained from colonies suspended in 100 µL TE buffer (Sigma-Aldrich, USA) (10 mM Tris-HCl, 1 mM EDTA, pH 8.0), inactivated at 65 °C for 1 hour in a water bath, and subjected to genomic DNA extraction by guanidine method according to PITCHER et al. (1989). DNA of reference strains were used as positive controls in each PCR assay. PCR reagents without DNA were also included as negative controls.

Visualization of the amplified products of all PCR reactions was performed in 1.0 % agarose gel in tris-borate-EDTA buffer (TBE) (Sigma-Aldrich, USA) (89 mM Tris Base, 89 mM boric acid, 2 mM EDTA, pH 8.0) stained with ethidium bromide (Sigma-Aldrich, USA) (0.5 mg/mL). Following electrophoresis, the gels were visualized under ultraviolet light and photographed (L-PIX EX, Loccus Biotechnology, Brazil). The molecular marker 100 bp DNA ladder (100 bp DNA Ladder, New England Biolabs, USA) was used in all electrophoresis.

MLST and MLVA genotyping

MLST was performed as previously described by WHATMORE et al. (2007). Nine distinct genomic fragments were PCR amplified (*loci: gap, aroA, glk, dnaK, gyrB, trpE, cobQ, omp25* and int-*hyp*). Products were separated by agarose gel electrophoresis to check for efficiency of amplification and to ensure that only a single product of the expected size was present. Then, they were purified using a PCR purification kit (Invitek, USA) and sequenced using Big Dye™ 3.1 (Applied Biosystems, USA) on an ABI-3500 automatic sequencer (Applied Biosystems, USA). Sequences were edited using Seqman Pro (Laser Gene, USA) and aligned and edited using BioEdit (HALL, 1999).

To evaluate the genetic relationships among the isolates from this study, *B. abortus* reference strains for each biovar and other *B. abortus* biovar 4 strains, we used the MLST profiles of twenty-three *B. abortus* strains deposited in PubMLST database (https://www.pubmlst.org) (13 biovar 4 strains) and MLST genotypes obtained from the genome of four strains (Ba col-B012, 68-3396P, 90-0775 and 01-4165) available on PATRIC (https://patricbrc.org/job) and NCBI plataform (https://www.ncbi.nlm.nih.gov) (Table 2).

The MLVA was carried out as described by AL DAHOUK et al. (2007) (MLVA16). The MLVA16 *loci* were divided into three panels: panel 1 (P1) or MLVA8 composed of eight minisatellites (Bruce06, Bruce08, Bruce11, Bruce12, Bruce42, Bruce43, Bruce45 and Bruce55); panel 2A (P2A) composed of three microsatellites (Bruce18, Bruce19 and Bruce21); and panel 2B (P2B) with five microsatellites (Bruce04, Bruce07, Bruce09, Bruce16 and Bruce30). The PCR conditions for MLVA16 were as previously described by AL DAHOUK et al. (2007).

The amplified products were submitted to electrophoresis in 2% or 3% agarose gel, for the mini and microsatellites, respectively, in Tris-borate-EDTA 1X (TBE) buffer, stained with 0.5 mg/mL ethidium bromide, visualized under UV light, and photographed (L-Pix EX, Loccus Biotecnologia, Brazil). DNA ladders 100 bp (100 bp DNA Ladder, New England Biolabs, USA) and 25 bp (25 bp DNA Step Ladder, Promega, USA) were used to estimate the tandem repeat unit length (MINHARRO et al., 2013).

Band size estimates were converted into number of repeat units for each *locus* (AL DAHOUK et al., 2007; DORNELES et al., 2014) and compared with the internal standard strains (*B. abortus* RB51 and *B. melitensis* 16M), using the software BioNumerics 7.6 (Applied Maths, Belgium). Clustering analysis was performed using the same software based on the category coefficient and the unweighted pair group method with arithmetic mean (UPGMA) algorithm (AL DAHOUK et al., 2007; DORNELES et al., 2014). The minimum spanning tree (MST) built was the one with the highest overall reliability score and was calculated using UPGMA associated with the priority rule and the bootstrap resampling (BioNumerics 7.6).

Besides the *B. abortus* biovar 4 strains assessed in the present study, all three MLVA16 (BCCN#95-31, BCCN R7#* and 292 ATCC 23451) genotypes of *B. abortus* biovar 4 available in the MLVAbank 2020 (http://mlva.i2bc.paris-saclay.fr/brucella), including the *B. abortus* biovar 4 strain 292, were used in clustering and MST analyses.

Table 2 - Information on *B. abortus* biovar 4 isolates available in databases (PubMLST, NCBI, PATRIC and MLVABank)*.

Method	Strain	Host	Species	Biovar	Country	Continent	Year[1]	Plataform
MLST	84/35	Human	*B. abortus*	4	Mexico	North America	1984	PubMLST
MLST	SPINK527	Unknown	*B. abortus*	4	United Kingdom	North America	1951	PubMLST
MLST	600/64	Bovine	*B. abortus*	4	United Kingdom	Europe	1964	PubMLST
MLST	707/65	Bovine	*B. abortus*	4	United Kingdom	Europe	1965	PubMLST
MLST	863/67	Bovine	*B. abortus*	4	United Kingdom	Europe	1967	PubMLST
MLST	84/26	Human	*B. abortus*	4	Mexico	North America	1984	PubMLST
MLST	67/93	Buffalo	*B. abortus*	4	Iraq	Asia	1967	PubMLST
MLST	79/14	Bovine	*B. abortus*	4	Chad	Africa	1979	PubMLST
MLST	351/78	Unknown	*B. abortus*	4	United Kingdom	Europe	1978	PubMLST
MLST	184/68	Unknown	*B. abortus*	4	United Kingdom	Europe	1968	PubMLST
MLST	24/68	Bovine	*B. abortus*	4	United Kingdom	Europe	1968	PubMLST
MLST	UK7/07	Human	*B. abortus*	4	United Kingdom	Europe	2007	PubMLST
MLST	Ba 01-4165	Bovine	*B. abortus*	4	France	Europe	Unknown	PubMLST
MLST	2308	Unknown	*B. abortus*	1	United States	North America	2008	PubMLST
MLST	86/8/59	Bovine	*B. abortus*	2	United Kingdom	Europe	1959	PubMLST
MLST	Tulya	Human	*B. abortus*	3	Uganda	Africa	1958	PubMLST
MLST	B3196	Bovine	*B. abortus*	5	United Kingdom	Europe	1959	PubMLST
MLST	870	Bovine	*B. abortus*	6	The Netherlands	Europe	1959	PubMLST
MLST	C68	Bovine	*B. abortus*	9	United Kingdom	Europe	1958	PubMLST
MLST	Ba col-B012	Bovine	*B. abortus*	4	Colombia	South America	1997	NCBI and PATRIC
MLST	68-3396P	Unknown	*B. abortus*	4	USA	North America	1968	NCBI and PATRIC
MLST	90-0775	Bovine	*B. abortus*	4	USA	North America	1990	NCBI and PATRIC
MLST	01/65	Bovine	*B. abortus*	4	France	Europe	Unknown	NCBI and PATRIC
MLVA	BCCN R7#*	Bovine	*B. abortus*	4	United Kingdom	Europe	Unknown	MLVABank
MLVA	BCCN#95-13	Bovine	*B. abortus*	4	Italy: Silicia	Europe	1995	MLVABank
MLST/MLVA	292 ATCC[2] 23451	Bovine	*B. abortus*	4	United Kingdom	Europe	1961	PubMLST/ MLVABank
MLST/MLVA	128/11	Bovine	*B. abortus*	4	Brazil: Pará	South America	2011	This study
MLST/MLVA	16/02	Bovine	*B. abortus*	4	Brazil: Rio Grande do Sul	South America	2002	This study

[1]Year of isolation.
[2]ATCC = American type culture collection.
*PubMLST database (https://www.pubmlst.org), NCBI plataform (National Center for Biotechnology Information) - (https://www.ncbi.nlm.nih.gov), PATRIC (Pathosystems Resource Integration Center) - (https://patricbrc.org/job) and MLVABank (http://mlva.i2bc.paris-saclay.fr/brucella).

RESULTS

The two field isolates studied showed a phenotype consistent with *Brucella* spp. and a biochemical and metabolic pattern identical to the reference strain of *B. abortus* biovar 4 292 (Table 1). Both isolates were Gram-negative, coccobacilli, non-mobile, non-fermentative, oxidase and catalase positive. The colonies also exhibited whitish color, smooth and shiny surface, and were small and non-hemolytic. The two isolates also showed specific characteristics of *B. abortus* biovar 4: CO_2 requirement, H_2S production and growth in the presence of basic fuchsin (20 µg/mL), but not in the presence of thionin (20 µg/mL and 40 µg/mL) (ALTON et al., 1988). Moreover, in agglutination tests, the isolates agglutinated with monospecific antiserum M, but not with monospecific antiserum A (Table 1).

Also, amplification of the *bcsp31* gene confirmed the isolates as *Brucella* spp. (BAILY et al., 1992) (Figure 1-A) and AMOS-enhanced PCR

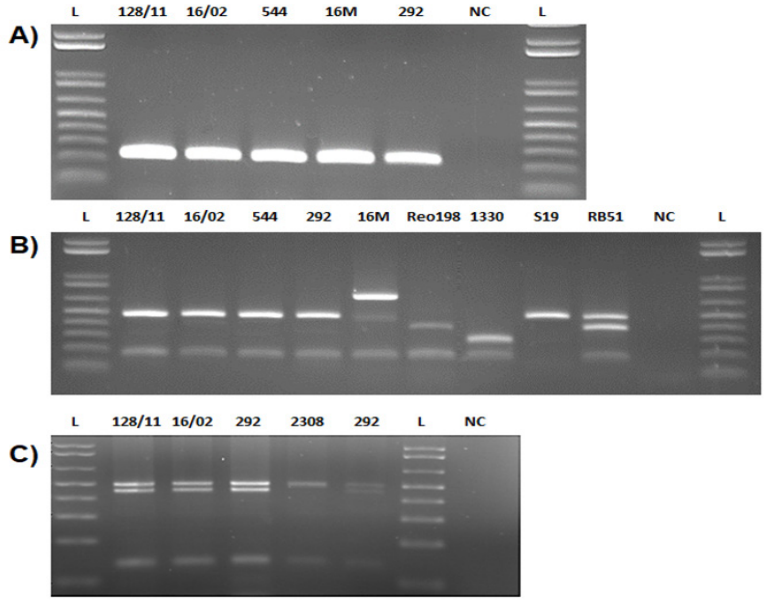

Figure 1 - (A) Agarose 1% gel showing PCR amplification for bcsp31 gene (*Brucella genus* specific) stained with ethidium bromide (0.5 mg / mL). Lanes L - 1Kb plus DNA Ladder molecular weight marker (Invitrogen, USA); lanes 2 e 3 - field isolates 128/11 e 16/02, respectively; lanes 4, 5 and 6 - positive controls B. abortus biovar 1 544, B. melitensis biovar 1 16M, *B. abortus* biovar 4 292, respectively; NC - negative control. (B). Agarose 1% gel showing amplification of ethidium bromide stained AMOS-enhanced-PCR (0.5 mg / mL). Lanes L - molecular weight marker 1Kb plus DNA Ladder (Invitrogen, USA); lanes 2 and 3 - field isolates 128/11, 16/02, respectively; lane 4 - *B. abortus* biovar 1,544; lane 5 - *B. abortus* biovar 4 292; lane 6 - *B. melitensis* biovar 1 16M; lane 7 - *B. ovis* Reo 198; lane 8 - *B. suis* biovar 1 1330; lane 9 - *B. abortus* biovar 1 S19; lane 10 - B. abortus biovar 1 RB51; NC - negative control.(C) Restriction patterns of the PCR-amplified omp2b gene digested with TaqI enzyme. Lanes L - 1Kb plus molecular weight marker (Invitrogen, USA), lane 2 - *B. abortus* strain 128/11, lane 3 - *B. abortus* strain 16/02, lane 4 = B. abortus biovar 4 292; 5 = *B. abortus* biovar 1 2308; laen 6 - *B. abortus* biovar 4,292 (ATCC 23451); NC - Negative control.

(Figure 1-B) results were compatible with *B. abortus* biovar 1, 2 or 4 for both strains (BRICKER & HALLING, 1995). In the PCR-RFLP for the *omp2b* gene, the field strains showed an identical restriction pattern to that of the reference strain *B. abortus* biovar 4 292 (CLOECKAERT et al., 1995; GARCIA-YOLDI et al., 2005) (Figure 1-C).

The MLST analysis showed different genotypes for both isolates (16/02 and 128/11), strain 16/02 depicted a ST1 (2-1-1-2-1-3-1-1-1) and strain 128/11 did not show a ST (22-1-1-8-9-3-1-1-1) with complete correspondence with any other ST previously described in the PubMLST database for *Brucella* spp. (accessed December 17, 2021) (Figure 2A-2). The STs of both strains were deposited in the PubMLST.

Likewise, the analysis of the MLVA *loci* revealed different genotypes among the field isolates (16/02 and 128/11). Patterns obtained in the sixteen VNTR *loci* are summarized in figure 2B-1. Genotyping based on MLVA8 and MLVA11 identified previously described genotypes in MLVAbank 2020 (access on May 22th 2020) for both strains, 16/02 (MLVA8 = 28, MLVA11 = 75) and 128/11 (MLVA8 = 32, MLVA11 = 182). The MLVA16 genotypes for the isolates 16/02 and 128/11 did not match any of those deposited in the MLVAbank 2020 (access on May 22th 2020). The differences reported in MLVA16 between field isolates compared with the *B. abortus* biovar 4 reference strain 292 occurred in all panels (P1, P2A and P2B), the strain 16/02 showed addition of one repeat unit in Bruce19 and Bruce30, addition of two repeat units in Bruce04 and deletion of one repeat unit in Bruce42 (Figure 2B-1). For the strain 128/11, the comparison with the reference strain 292 revealed addition of one repeat unit in Bruce16 and deletion of one repeat unit in Bruce19 and Bruce30 (Figure 2B-1). The MST created based on MLVA16 genotypes is shown in figure 2B-2.

DISCUSSION

Intraspecific characterization of *Brucella* spp. circulating strains is critical for elimination of outbreaks, tracking infection spread and periodic assessment of anti-brucellosis strategies (BRICKER & HALLING, 1994; DORNELES et al., 2014). Therefore, this study characterized phenotypically and genotypically two isolates of *B. abortus* biovar 4, rare in cattle from Brazil, as part of the actions to support PNCEBT, the program for the control and eradication of bovine brucellosis in place in the country.

The two strains of *B. abortus* isolated from cervical bursitis exhibited different biochemical and molecular tests than *B. abortus* biovar 1, the most common strain causing bovine brucellosis in Brazilian territory (MINHARRO et al., 2013; OLIVEIRA et al., 2017). All the tests used allowed us to state without doubt that the two isolates are in fact *B. abortus* biovar 4, being the first isolated strains of this biovar in Brazil (MINHARRO et al., 2013). Considering that both strains were isolated after the implementation of the PNCEBT, that brucellosis is endemic with medium to high prevalence in a large part of the Brazilian territory and that many states have not been able to significantly reduce the prevalence of the disease (FERREIRA-NETO et al., 2016), it is possible to suggest that *B. abortus* biovar 4 is still circulating in the Brazilian cattle herd; although, the strains were isolated in 2002 and 2011.

Despite the low frequency of this biovar worldwide compared with other more prevalent *B. abortus* biovars (1, 2, 3 and 6) (BRICKER & HALLING, 1994), biovar 4 strains were previously identified in some countries, such as Argentina, Chad, Chile, Colombia, Cuba, El Salvador, Ecuador, France, India, Iraq, United States, Mexico, Nicaragua, and Venezuela (LUCERO et al., 2008; HIGGINS et al., 2012; TORRES HIGUERA et al., 2019). The host mainly associated with the isolation of this biovar is cattle (LUCERO et al., 2008; MINHARRO et al., 2013; DARSHANA et al., 2016); however, *B. abortus* biovar 4 have also been isolated from elk (ETTER & DREW, 2006), bison (HIGGINS et al., 2012), Rocky Mountain bighorn sheep (KREEGER et al., 2004), dogs (FORBES, 1990) and, sheep and goats (DARSHANA et al., 2016).

Considering that classical epidemiological tools alone usually do not have sufficient resolution to allow a complete understanding of the dynamics of zoonotic infectious diseases with multiple hosts, such as brucellosis, genotyping data contribute indicating the direction of transmission between hosts and assist in the decision-making process for the management of wildlife populations (HIGGINS et al., 2012). Therefore, to increase reliability and complement the results of phenotypic and molecular tests, we genotyped the strains using MLST and MLVA techniques, which allowed the differentiation of *B. abortus* biovar 4 strains into genotypes and the drawing of some inferences on their epidemiological relationships.

The MLST analysis showed different genotypes for both isolates (16/02 and 128/11), the genotype of the strain 16/02 was ST1, which has a global distribution, being widely distributed in many

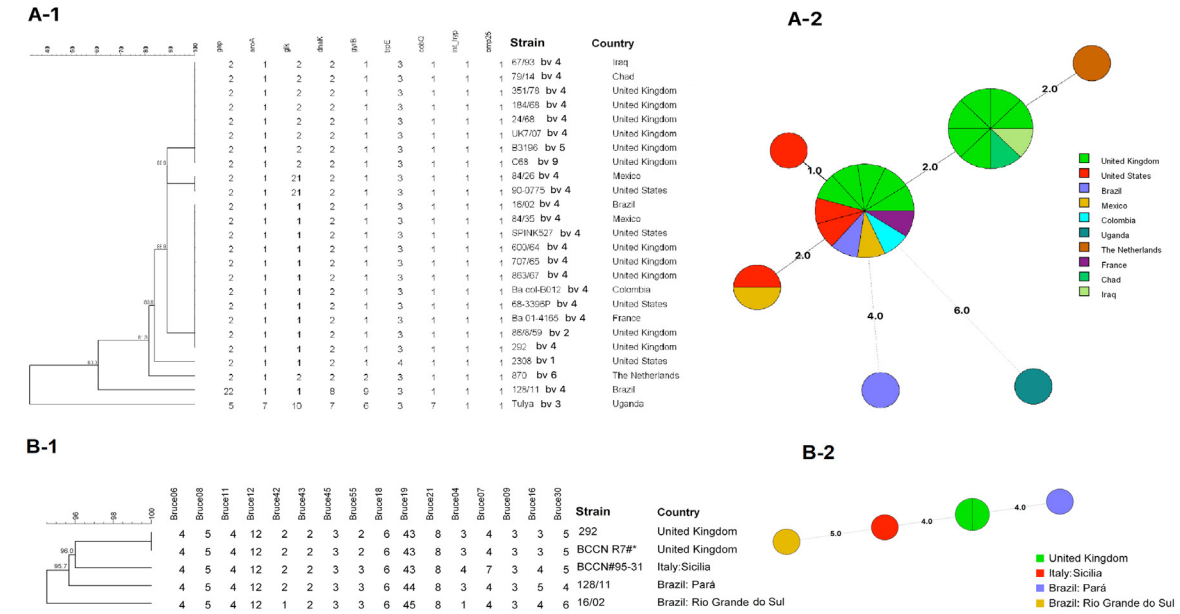

Figure 2 - Cluster analyzes of *Brucella abortus* biovar 4 by molecular typing methods MLST and MLVA with the aid of the Bionumerics software (version 7.6, Applied-Maths, Belgium). A-1) Dendrogram based on the MLST genotyping test showing relationships of *B. abortus* reference strains for each biovar and other *B. abortus* biovar 4 strains (thirteen available in PubMLST and four obtained from whole genome sequence in the NCBI) and the two isolates from this study (128/11 and 16/02). A-2) Minimal Spanning Tree (MST) analysis of *B. abortus* strains using MLST data. B-1) Dendrogram based on MLVA16 for all three *B. abortus* biovar 4 MLVA16 genotypes (BCCN # 95-31, BCCN R7 # * and 292 ATCC 23451) available at MLVAbank 2020 and the two isolates from this study (128/11 and 16/02). B-2) MST analysis of *B. abortus* biovar 4 isolates using MLVA16 data.

continents (WHATMORE et al., 2016; WHATMORE & FOSTER, 2021). Strain 128/11, conversely, exhibited a different and unique genotype, not described in the PubMLST database and far from any other profile already described for *B. abortus* biovar 4 (Figure 2A), suggesting the absence of an epidemiological link between the isolate (128/11) of the present study and others already described in other countries. In fact, despite the low number of strains analyzed, the allelic profile observed for the strain 128/11 indicated the existence of several polymorphisms at the individual *locus* and at the level of the combined alleles, compared with other STs already described for *B. abortus* biovar 4 (Figure 2A). Likewise, the comparison of ST between both Brazilian biovar 4 isolates also suggests a lack of epidemiological relationship between these two strains, due to the large genetic distance observed (Figure 2A).

Similarly, MLVA results showed a great genetic diversity among the studied strains using MLVA8, MLVA11 and MLVA16. The differences observed between the genotypes of the field strains were not limited to the differences at the most variable *loci* (Bruce19, Bruce04, Bruce16 and Bruce30), but they were also observed in more conserved *loci*, such as Bruce42 (Figure 2B-1). In fact, the MST (Figure 2B-2) analysis shows that the Brazilian isolates (16/02 and 128/11) are very distant genetically considering the MLVA16 markers, as each one is at one end of the tree. Taking into account all the *B. abortus* biovar 4 genotypes available at MLVABank, the strain 16/02 was closest to the isolate from Italy (BCCN#95-31), whereas the strain 128/11 to the strains isolated in the United Kingdom (BCCN R7#* and 292-ATCC 23451).

This large genetic distance in MLST and MLVA genotypic profile in the comparison between the two *B. abortus* biovar 4 strains isolated from Brazil, together with the absence of an epidemiological link between them, strongly suggest that both strains originated from a different ancestor. Indeed, considering the great geographic distance between the place of origin of the isolates, since Pará

and Rio Grande do Sul are far opposite states in the Brazilian territory (north and south, respectively, more than 3000 km apart, approximately 1864 miles), and also the difference in the productive profiles and historical origin of the cattle herds between these two states, it is very likely that the strains have different origins. Cattle herd from Rio Grande do Sul has a historical influence from neighboring countries, Uruguay and Argentina, which make up the herd mainly from European breeds (*Bos taurus* subsp. *taurus*), on the other hand Pará has a large part of the territory occupied by the Nellore breed (*Bos taurus* subsp. *indicus*), influenced by the proximity to states of high representativeness in the national livestock, such as Mato Grosso, Goiás and Mato Grosso do Sul (LÁU, 2006; CANOZZI et al., 2019).

In this context, it is tempting to speculate that the studied strain 16/02 isolated in the state of Rio Grande do Sul may be associated with animal import and transport, since to date there have been 17 isolates characterized as *B. abortus* biovar 4 in the world, from which 52.94% (9/17) belong to ST1, including the reference strain *B. abortus* biovar 4 292 from United Kingdom. Nevertheless, albeit the 16/02 strain depicted the ST1, considered to be widespread in many continents, other *B. abortus* biovar 1 and 2 also exhibited ST1 and have shown to be historically closely related genetically (GARGANI & LOPEZ-MERINO, 2006). Indeed, from 54 *B. abortus* isolates from Brazil deposited in the PubMLST 75.92% (41/54) showed ST1; although, not classified biovar 4. Based on these findings, it is not yet possible to identify distinct genetic lineages corresponding to these biovars (WHATMORE et al., 2016; WHATMORE & FOSTER, 2021).

Another possible origin of the strain 16/02; although, there is no epidemiological or molecular evidence, since very few strains of *B. abortus* biovar 4 were genotyped by MLST or MLVA16, could be *B. abortus* biovar 4 in neighboring countries, such as Argentina, where this biovar has already been found (LUCERO et al., 2008; MINHARRO et al., 2013). However, it is important to emphasize that these strains identified and classified as *B. abortus* biovar 4 were not genotyped, making it impossible to trace their origin.

Regarding the transmission chain associated with the 128/11 strain isolated in Pará, it is difficult to state a hypothesis for the origin of the strain based on MLST and MLVA results, due to the scarcity of available data. MLST data of the *B. abortus* biovar 4 strain Ba col-B012 isolated in Colombia (TORRES HIGUERA et al., 2019), which is at the northern Brazilian border, on the contrary, indicated the isolates are unrelated.

Considering the different results obtained between MLST and MLVA, it is important to highlight that these techniques characterized the isolates at different levels of resolution, being the MLST based on polymorphism observed in conserved portions of the genome, while MLVA is built by a set of VNTRs (non-coding regions). Thereby, they are complementary from an epidemiological point of view. Moreover, it is important to mention that the identification of *B. abortus* biovar 4 isolates is uncommon, despite the easy availability of serological data on bovine brucellosis in Brazil and worldwide. This is probably due to the largely limited data on its etiological agent, considering the complexity of handling, as it is a level 3 agent, leaving information on the prevalent species and biovars of *Brucella* obscure. Additionally, the complementation of these findings with genotypic analysis by MLST or MLVA is even rarer. In fact, only seventeen sequence types (thirteen available in PubMLST and four obtained from whole genome sequencing by NCBI) and three MLVA16 genotypes are available for *B. abortus* biovar 4 strains (Table 2). Although, HIGGINS et al. (2012) also genotyped *B. abortus* biovar 4 isolates, they used different VNTR *loci* (HOOF-Print1; HOOF-Print3; HOOF-Print4; HOOF-Print8; VNTR2; VNTR5A; VNTR5B; VNTR16; VNTR17; VNTR21) precluding any comparison among their and other studies.

The identification and characterization of *Brucella* species and biovars that affect animals and humans is of fundamental importance to understand the epidemiological situation of the brucellosis, allowing the improvement of control and eradication strategies.

CONCLUSION

The typing of *B. abortus* strains isolated from cattle in Brazil confirmed the occurrence of *B. abortus* biovar 4 in the country, providing support for surveillance of the pathogen within the program for the control and eradication of bovine brucellosis in the country.

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DECLARATION OF CONFLICT OF INTEREST

The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

AUTHOR'S CONTRIBUTION

RSA wrote the paper; EMSD and APL conceived and critically reviewed the manuscript for important intellectual content; SM, PGS and MC performed research; PMSF, CRP and AAFJ did the analysis and interpretation of data.

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