Identification of *Brucella* sp. Isolated in Brazil from 1976 to 2013 by Bruce-Ladder PCR

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ABSTRACT

Background: *Brucella* sp. are the causative agents of brucellosis, an infectious disease that affects various species of animals and can be transmitted to humans through direct contact with infected animals, indirectly by the ingestion of raw-milk products, and during the handling of strains or infected material in the laboratory. Being a zoonosis, the detection of *Brucella* species in animals is essential for the prevention of the disease in humans and to perform a good program of control in infected herds. This study aimed at identifying *Brucella* field strains isolated from 1976 to 2013 in Brazil, using the modified Bruce-Ladder method, to evaluate the performance of this technique.

Materials, Methods & Results: Eighty-three strains of *Brucella* sp. were included in the study, i.e. 21 reference strains (nine *B. abortus*, one *B. canis*, four *B. melitensis*, two *B. ovis* and five *B. suis*) and 62 field strains (six *B. canis*, one *B. suis* and 55 *B. abortus*). For the identification of the genus and/or species of *Brucella*, biochemical and physiological tests, including MacConkey-agar growth, glucose fermentation, haemolysis, catalase, oxidase and urease tests, nitrate reduction, citrate utilization, H_2S production and CO_2 requirement, were performed. Genomic DNA was extracted from pure cultures through heat-lysis of bacterial cultures and the genus was confirmed by a genus-specific PCR (*bcsp31* target gene), before performing the modified Bruce-Ladder PCR for the confirmation of the *Brucella* species. No problems of specificity were observed with the Bruce-Ladder PCR. However, the 1,682 bp fragment was not systematically amplified, even after several modifications such as the concentration of mix components, annealing temperatures and time. Therefore, an individual PCR using primers specific to this fragment was needed for complete identification of some strains. Also, only one kind of Polymerase gave the best results. All *Brucella* reference strains and negative controls gave the expected results. All field strains previously identified as *B. abortus*, *B. canis* and *B. suis* by biochemical and physiological tests were confirmed by the modified Bruce-Ladder PCR. All isolated *Brucella abortus* presented a Bruce-Ladder PCR profile expected for field strains, excluding the vaccine strains.

Discussion: The modified Bruce-Ladder PCR identified properly all *Brucella* species (reference and field strains) and proved to be a reliable technique, thus facilitating the identification of the species in the laboratory, reducing the manipulation of these bacteria and the associated danger. Albeit the difficulties of amplification of one fragment for some strains, when using the multiplex technique, this method is fast and without risks after inactivation of the strains. Most studies on animal brucellosis in Brazil were only based on serological tests without identification of the pathogen; while the knowledge of the particular species and/or biovars that occur in Brazil, as well as their distribution, is important to monitor the spread of *Brucella* among sensitive species and among farms. Our results showed also that *B. abortus* is still the predominant species isolated in cattle in Brazil. The knowledge of the species that occur in Brazil can help to identify the source of infection and the measures of control to be applied, while it is also very important to trace the dispersion of strains among farms.

Keywords: brucellosis, *Brucella* identification, *Brucella* isolation, Bruce-Ladder PCR.

INTRODUCTION

Currently, there are 10 recognized species of Brucella, based on host preferences, phenotypic differences and pathogenesis: *B. abortus* (cattle), *B. canis* (dogs), *B. melitensis* (ovine and caprine), *B. neotomae* (desert rats), *B. ovis* (sheep), *B. suis* (pig, reindeer, and hares), *B. microti* (small rodents from Eastern Europe), *B. pinnipedialis* (pinnipeds), *B. ceti* (cetaceans), and *B. inopinata* (associated with human infection) [2,4,11]. Among these species, six are pathogenic for humans: *B. abortus*, *B. canis*, *B. inopinata*, *B. melitensis*, *B. pinnipedialis*, and *B. suis* [1,12,13].

Classically, *Brucella* isolates are divided into species thanks to a typing procedure that includes a number of tests related to their physiology, phenotype, phage sensitivity, and antigenic properties. Some species are divided into biovars (*B. abortus*, *B. melitensis*, *B. suis*) [1,2]. Such analyses are subject to different interpretations and expose the technicians to a risk of contamination, and therefore should be performed by experienced personnel in reference laboratories [1,10,14].

In 2006 a multiplex PCR test was developed, named Bruce-Ladder, and was able to distinguish *Brucella* species, including vaccine strains in the same test [5]. However, some strains of *B. canis* showed the same profile as *B. suis* therefore identifying a limitation of the technique [5,7]. In 2011, some modifications were proposed to the Bruce-Ladder technique, enabling this assay to distinguish all known species of *Brucella* [6].

This study aimed at identifying *Brucella* field strains isolated from 1976 to 2013 in Brazil, using the Bruce-Ladder modified method, in order to evaluate the performance of this technique.

MATERIALS AND METHODS

The experiment was carried out with 83 strains of *Brucella* sp., including 21 reference strains (nine *B. abortus*, one *B. canis*, four *B. melitensis*, two *B. ovis* and five *B. suis*) and 62 field strains from DeMIP collection (six *B. canis*, one *B. suis* and 55 *B. abortus*) (Tables 1 and 2). For the identification of the genus and/or species of *Brucella*, the biochemical and physiological tests, MacConkey-agar growth, glucose fermentation, haemolysis, catalase, oxidase and urease tests, nitrate reduction, citrate utilization, H_2S production and CO_2 requirement were performed [1]. A total of 15 field strains were sent to the EU/OIE/FAO Brucellosis Reference Laboratory, ANSES, France, to determine the biovar, thanks to additional tests, anti-A and -M agglutination as well as dye-sensitivity in particular [1]. A genus-specific PCR targeting the *bcsp31* gene was also used to confirm all strains [3]. No DNA in the reaction and DNA from *Staphylococcus aureus* (β-hemolytic field strain) were used as negative controls.

Genomic DNA was extracted from pure cultures through heat-lysis of bacterial cultures, as described elsewhere, with a previous inactivation of the strain by boiling for 1 h [9]. For the identification of *Brucella* species, the Bruce-Ladder multiplex PCR conditions described initially [5] was performed, but with primers[1] and Polymerase[2] suggested elsewhere [7]. No DNA and DNA from *Ochrobactrum anthropi* (reference strain 3301) were used as negative controls. The PCR products were analyzed by 1.5% agarose gel electrophoresis, and fragment sizes were estimated by comparison with a 100-bp DNA ladder[3] with the Kodak 1D program, version 3.5.2.

RESULTS

All *Brucella* reference strains and negative controls gave the expected results. All field strains previously identified to the species level by biochemical and physiological tests were confirmed by the genus-specific PCR and by the modified Bruce-Ladder PCR (Figure 1). None of the field strains of *B. abortus* presented the profile expected for vaccine strains B19 and RB51.
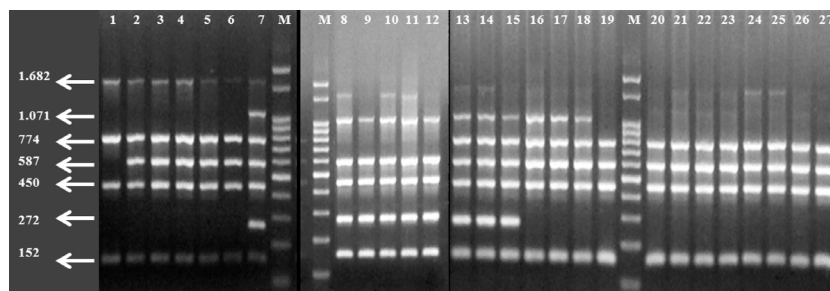
Figure 1. Examples of profiles of reference and field *Brucella* strains obtained after Bruce-Ladder PCR and gel electrophoresis. Lanes: 1 to 4 = *B. abortus* 1 (B19, 544, 119-3, 99). 5 = *B. abortus* 3 (Tulya). 6 = *B. abortus* 4 (292). 7 = *B. suis* 1 (1330). 8 to 12 = *B. canis* (RM6160, 35/03, 41/03, 09/95, 09/98). 13 = *B. suis* 1 (SEA). 14 = *B. suis* 2 (Thomsen). 15 = *B. suis* 3 (686). 16 = *B. melitensis* 3 (Ether). 17 = *B. melitensis* 2 (63/9). 18 = *B. melitensis* 1 (16M). 19 to 27 = *Brucella abortus* field strains (41, A1, 43, 44, 45, A6, 47, 55). M = ladder 100 bp. Reference strains: Lanes 1- 8, 14 -18. Field strains: All other lanes.

Table 1. *Brucella* reference strains tested in Bruce-Ladder PCR.

Species	Biovar	Strains	Origin[a]
Brucella abortus	1	544, 1119-3, B19, 99	IPVDF and ANSES
	2	86/08/59	ANSES
	3	Tulya	ANSES
	4	292	ANSES
	5	B3196	ANSES
	6	870	ANSES
	9	C68	ANSES
Brucella canis	NA[b]	RM6/66	ANSES
Brucella ovis	NA	REO198, 63/690	IPVDF and ANSES
Brucella melitensis	1	16M, REV1	IPVDF and ANSES
	2	63/9	ANSES
	3	Ether	ANSES
Brucella suis	1	1330	IPVDF
	2	Thomsen	ANSES
	3	686	ANSES
	4	40	ANSES
	5	5513	ANSES

[a]IPVDF: Instituto de Pesquisas Veterinárias Desidério Finamor. ANSES: French Agency for Food, Environmental and Occupational Health and Safety (EU/OIE/FAO Brucellosis Reference Laboratory). [b]NA: Not applicable.

Table 2. Field strains isolated in Brazil from 1976 to 2013 and their origin, tested in Bruce-Ladder PCR.

Species	Biovar	Strains	Origin[a]
B. abortus	1	B875	ND
	1	56, 96, 477, 577, 02/06, 13a/02, 13b/02, 13/03, 14/02, 14/03, 15/03	RS
	2	33MG, 34MG	MG
	3	17a/02	RS
	ND[b]	02, 06, 22, 13/02, 17b/02, 8p/04, 8g/04, 03/07, 09/07, am70, am75, NB57, NB94, NB135, VM07, VM10, VM82, VM88, VM551, 11/03/06, CB03, RPab/08, ab./08	RS
	ND	41, 43, 44, 45, 47, 55, A1, A4, A6	MG
	ND	03, 04, 05, 07, 08/12	MT
B. canis	NA[c]	09/95	RS
		09/98	PR
		35/03, 41/03	SP
		28/09/04, 21/10/98	ND
B. suis	1	SEA	ND

[a]RS: Rio Grande do Sul State. PR: Paraná State. SP: São Paulo State. MG: Minas Gerais State. MT: Mato Grosso State. [b]ND: not determined. [c]NA: not applicable.

DISCUSSION

All reference and field strains were properly differentiated at species level by PCR as described elsewhere [6]. The *Brucella* field strains isolated from bovines were confirmed as field *B. abortus* and not from vaccine origin. The only difficulty encountered with the realization of this methodology was the occasional absence of amplification of the 1,682 bp fragment. This fragment did not always appear on multiplex PCR reactions, and this variability persisted after several modifications such as different concentrations of primers, DNA, magnesium chloride, different annealing temperatures, different Polymerase (Platinum® Taq DNA polymerase[4] and GoTaq® DNA Polymerase[5]) enzymes, and times. Also, only one kind of Polymerase (Immolase[2]) gave the best results in all tests. Therefore an individual PCR using primers specific to this fragment (single PCR) was sometimes necessary to confirm its presence or absence.

These results demonstrate the added value of the modified Bruce-Ladder PCR that presents an excellent discriminating power for *Brucella* species, allowing to a rapid and correct identification of strains isolated from the field, and the possibility of differentiation among field and vaccine strains. This test represents a major improvement in the identification of *Brucella* sp., because it is a powerful test, safer for the technicians, and still allows a faster identification of the pathogen, while conventional methods for identification of *Brucella* sp. involve time, specific reagents unavailable commercially and a risk of human contamination [10,14].

CONCLUSION

In conclusion, all reference and field strains identified by biochemical and physiological analysis have been confirmed by the modified Bruce-Ladder PCR, and *B. abortus* is still the predominant species isolated in cattle in Brazil [8]. This fast and robust test is an important tool in routine laboratories for the microbiological diagnosis of brucellosis in domestic and wild animals, and can contribute to the control of the disease. In Brazil, few studies exist regarding the identification of *Brucella* species. Most studies on animal brucellosis in Brazil are based on serological tests and not on the identification of the pathogen. The knowledge of the species and /or biovars that occur in Brazil can help to identify the source of infection and the measures of control, and also it is very important to trace the dispersion of strains among animal species and among farms.

Declaration of interest. The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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