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Full Length Research Paper

Characterization of *Brucella* species in Mexico by Bruce-Ladder polymerase chain reaction (PCR)

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The typing of 153 *Brucella* strains isolated from different regions of Mexico was performed by multiplex PCR Bruce-Ladder. Results showed that microbiological typing and multiplex Bruce-ladder amplification were identical for all *Brucella* isolates tested. Because of its speed, Bruce-Ladder PCR is a useful method in the typing of *Brucella* species isolated from animal and humans.

Key words: *Brucella*, brucellosis, typing, Mexico, Bruce-ladder polymerase chain reaction (PCR).

INTRODUCTION

Brucella species are facultative intracellular pathogens that establish and maintain chronic infections in a wide variety of mammalian hosts (Moreno and Moriyón, 2002). Ten *Brucella* species are currently recognized for the genus *Brucella* (Whatmore et al., 2009). Brucellosis occurs worldwide, including the Mediterranean countries of Europe, northern and eastern Africa, India, Central Asia, Mexico, Central and South America are still no brucellosis free (Pappas et al., 2006). In Mexico, 93% of human brucellosis cases are due to *B. melitensis* acquired from goats, and a further 5% are of bovine origin (Luna-Martínez and Mejía-Terán, 2002; López-Merino et al., 1992). The program for animal brucellosis control in Mexico is based on vaccination with *B. abortus* S19 and *B. abortus* RB51, and *B. melitensis* Rev 1: this program is limited to cattle, sheep and goats and includes standard preventive measures, such as quarantine and inspection of slaughterhouses (Luna-Martínez and Mejía-Terán, 2002).

The tests to perform the typing of different species of genus *Brucella* are time-consuming and require skilled technicians; moreover, handling of the microorganism represents a high risk for laboratory personnel. In the last 5 years, several procedures using DNA-based techniques have been developed for typing the different *Brucella* species (Whatmore et al., 2007). One of these approaches is the multiplex PCR assay named Bruce-ladder that is able to identify and differentiate all *Brucella* species including *B. abortus* S19 and RB51 and *B. melitensis* Rev1 (García-Yoldi et al., 2006). Multiplex Bruce-Ladder was tested with *Brucella* strains from different countries, including some *B. melitensis* from Mexico, but no *B. abortus* isolates were tested (López-Goñi et al., 2008). This study described the typing of 153 *Brucella* strains isolated in the last five years from different regions and sources of Mexico by PCR multiplex Bruce-Ladder; results were compared with microbiological standard tests.

MATERIALS AND METHODS

Strains and growth conditions

Brucella strains (153 isolates) (Table 1) originating from Mexico was isolated from humans, animals, manure from goats, and dairy products. Also included in this study were 3 vaccine strains (*B. abortus* S19 and RB51, and *B. melitensis* Rev1). *Brucella* type strains used: *B. melitensis* bv.1 ATTC 23456 (16M), *B. melitensis* bv.*2* ATTC 23457, *B. melitensis* bv.3 ATTC 23458, *B. abortus* bv. 1 ATTC 23448 (544), *B. abortus* bv. 2 ATTC 23449, *B. abortus* bv. 3

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Table 1. Source of 153 *Brucella* Mexican strains tested by Bruce-ladder PCR.

Species*	Source	Number of strains tested
B. abortus	Cattle	31
	Manure from goat	3
	Human	8
	Cheese	3
	Goat	6
B. melitensis	Cattle	3
	Human	96
	Goat	2
B. suis	Cattle	1

*All isolated strains were typed as biovar 1.

ATTC 23450, *B. abortus* bv. 4 ATTC 23451, *B. abortus* bv. 5 ATTC 23452, *B. suis* bv. 1 ATCC 23444 (1330), *B. suis* bv. 2 ATCC 23445, *B. suis* bv. 3 ATCC 23446, *B. suis* bv. 4 ATCC 23447, *B. suis* bv. 5, *B. canis* 23365 (RM6/66), *B. ovis* 23840 (23/290) and *B. neotomae* 23456 (5K33). *Yersinia enterocolitica* 0:9, *Escherichia coli* 0:157 H7, *Salmonella typhi*, *Shigella* sp., *Staphylococcus aureus* 25923, *Ochrobactrum anthropi* 24566, *Ochrobactrum anthropi* 33786, *Rhizobium etli* CE3, *Rhizobium leguminosarum* USA 2370, *Sinorrhizobium meliloti* 1021, *Agrobacterium radiobacterium* IAM 13570, and *Agrobacterium rhizogenes* IAM 13570 were included in this work for specificity analysis.

Brucella strains were grown in soybean trypticase (Difco) broth at 37°C for 48 h. *Rhizobium* and *Ochrobactrum* strains were grown in Peptone-yeast medium at 28°C for 24 h. Enterobacteria and *S. aureus* were grown on Luria Bertani agar plates at 37°C for 28 h.

Microbiological test

Microbiological test includes hydrogen sulfide and urease production, growth in the presence of basic fuchsin and thionine dyes, susceptibility to phage lysis (Tbilisi, Weybridge and Berkeley) and agglutination with monospecific A and M anti-sera (Alton et al., 1988).

Deoxyribonucleic acid (DNA) extraction

Genomic DNA was extracted using the CTAB method previously described by Wilson (1987). Briefly, cells were suspended in 500 µl of TE buffer, 4 µl of proteinase K (10 mg/ml) and 15 µl of 10% SDS and incubated for 1.5 h at 37°C. Proteins were precipitated by the addition of 100 µl of 5 M NaCl and 80 µl of CTAB/NaCl solution, the mixture was incubated at 65°C for 10 min, and an equal volume of chloroform/isoamyl alcohol (24:1) was added. After centrifugation at 14,000x*g* for 10 min, DNA in the aqueous layer was precipitated with 500 µl of isopropanol, washed with 500 µl of 70% (v/v) ethanol in water and resuspended in molecular biology grade water. DNA concentration was measured eliminate by measuring the absorbance at 260 nm.

Polymerase chain reaction (PCR) assay

The 8 pairs of oligonucleotides primers and conditions for Bruce-ladder used were as previously described (García-Yoldi et al., 2006). Briefly, the multiplex PCR was performed with a reaction mixture containing 15 ng of extracted DNA of each strain, 1x PCR buffer, 3 mM $MgCl_2$, 1.5 U of DNA polymerase, 6.25 pmol of each primer and 400 µM each deoxynucleoside triphosphate. Conditions for PCR assay were: initial denaturation at 95°C for 7 min, followed by 25 cycles of template denaturation at 95°C for 35 s, primer annealing at 64°C for 45 s and primer extension at 72°C for 180 s, followed by a final extension at 72°C for 6 min. The amplified products were resolved by electrophoresis using a 1.5% agarose gel and followed by staining with ethidum bromide.

RESULTS

The amplification profile for *Brucella* reference strains obtained were as expected (data not shown). We also confirmed that Bruce-Ladder PCR could be performed in less than 24 h, and it was reproducible following the conditions previously described (Garcia-Yoldi et al., 2006).

The expected amplification pattern for each *Brucella* species was very easy to observe in the agarose gel. In Figure 1, a representative agarose gel reveals the PCR profile obtained for isolates of *B. abortus*, *B. melitensis*, and *B. suis* obtained from different sources and geographical regions of Mexico. Microbiological typing and multiplex Bruce-ladder profile matched in 51 *B. abortus*, 101 *B. melitensis*, and 1 *B. suis* tested in this work. Our results showed that no strain was phylogenetically or serologically related to *Brucella* amplified with Bruce-Ladder PCR (data not shown). Additionally, using Bruce-Ladder PCR, no vaccine strains were found in the isolates surveyed.

DISCUSSION

During the last 5 years more than 20,000 human cases have been recorded in Mexico (according to the Dirección General de Epidemiología, Secretaría de Salud, Mexico, http://www.dgepi.salud.gov.mx). Animal brucellosis is widely spread in different regions of the country. Despite the implementation of the national brucellosis eradication program more than forty years ago, some regions still have a high prevalence in bovine and goat herds. In contrast, the isolation of *Brucella* from humans and animals is not a common practice; moreover, just a few laboratories in Mexico are able to accurately identify *Brucella* spp. (Luna-Martinez and Mejía-Terán, 2002). The diagnosis of brucellosis is carry out by serological test, both in human and animals.

We evaluated the multiplex Bruce-ladder PCR as a tool for the typing of *Brucella* isolates from Mexico obtained in the last 5 years. This PCR assay was designed by Garcia-Yoldi et al. (2006) for the typing of different *Brucella* species, including the vaccine strains, based on the amplification of different genes that display a differential amplification pattern.

Multiplex PCR Bruce-ladder was previously evaluated in different laboratories including strains isolated from

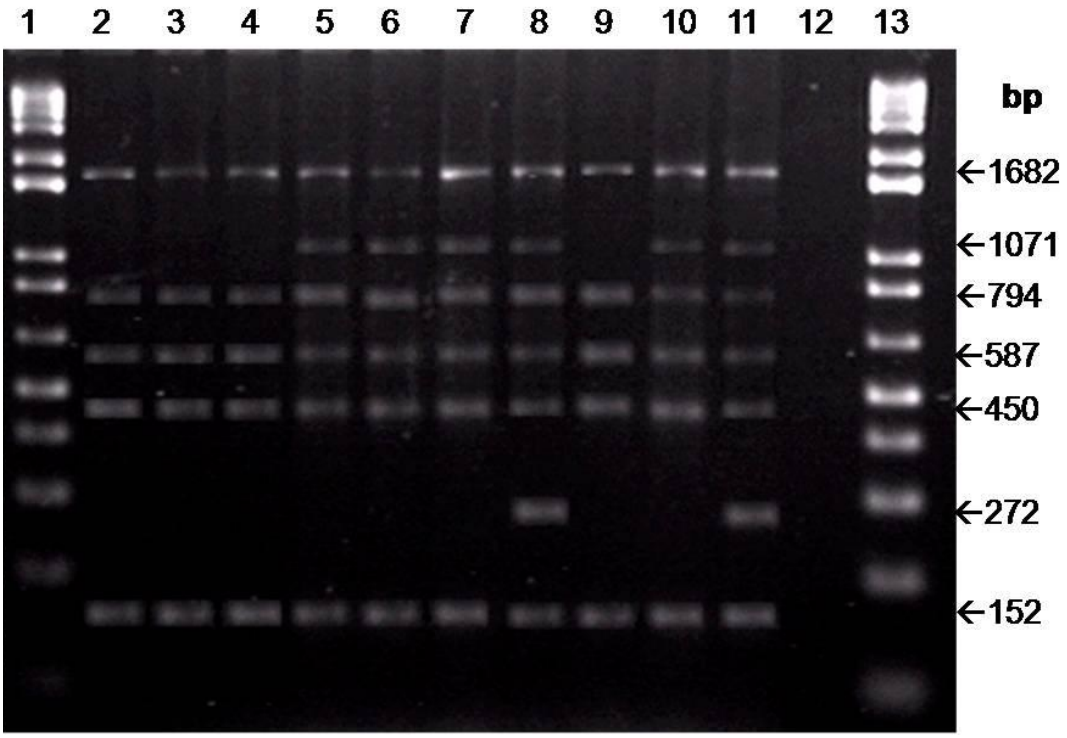

Figure 1. Multiplex PCR assay for typing of *Brucella* mexican isolates. Lanes 1 and 13, 1 Kb plus DNA ladder (Invitrogen); lanes 2-4, isolates of *B. abortus*; lanes 5-7, isolates of *B. melitensis*; lane 8, isolate of *B. suis*. Lane 9-11, positive control (DNA from *B. abortus* 544, *B. melitensis* 16M and *B. suis* 1330); lane 12, negative control (*Ochrobactrum anthropi*).

European countries, from Latin America, North America, Africa, and Asia. However, few isolates of *B. melitensis* and none of *B. abortus* from Mexico were included in the validation of this PCR assay (López-Goñi et al., 2008). In Mexico, *B. melitensis* is mainly isolated from humans and goats while *B. abortus* is mainly isolated from cattle. Since, milk is sold retail in a raw form (unpasteurized) and unbottled, the consumer is at risk for contracting brucellosis. Moreover, unsold raw milk is processed to make cheese, cream, and cultured dairy products (Losada et al., 2000). In areas with endemic brucellosis, this milk comes from infected goats and cows. Thus, the ingestion of unpasteurized dairy products is the most common way to acquire *Brucella* in Mexico (Luna-Martínez and Mejía-Terán, 2002).

Our findings showed one isolate of *B. suis* from a cow, six isolates of *B. abortus* from goats, and three strains of *B. melitensis* from cows. These findings showed that the transmission of *Brucella* may be due to the proximity between different hosts sharing the same stable or to uncontrolled movement of infected animals. In this sense, typing of *Brucella* species in endemic regions is crucial to identifying the risk factors involved in disease transmission. Bruce-Ladder PCR offers the first approach in the identification of *Brucella* species in a single tube that could be performed in any basic laboratory. Recently, a selection of 16 variable-number-tandem-repeats has been proposed for fingerprinting *Brucella* isolates (Whatmore et al., 2006; Le Flèche et al., 2006). This multiple-locus variable-number tandem-repeats analysis provides information for epidemiological trace-back investigation of brucellosis; however, this technique is more complicated than Bruce-ladder PCR.

In summary, we reported the typing of 153 *Brucella* isolates from clinical human cases and infected animals obtained from different regions of Mexico by Multiplex PCR Bruce-Ladder. Bruce-ladder PCR was faster than the microbiological standard tests. Additionally, this PCR assay is easy to perform and could be performed in any laboratory with a minimum of investment.

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