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ANTIMICROBIAL SUSCEPTIBILITY PROFILE OF *Brucella* spp. ISOLATED IN BRAZIL

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ABSTRACT

The intention of this work was to investigate the susceptibility profile of 27 *Brucella* strains isolated from animals in Brazil, using the E-test method with antimicrobials recommended for the treatment of human brucellosis, to monitor the activities of these antimicrobials and their potential efficacy for human brucellosis treatment. Efficiency of SE-AFLP in determining the genetic diversity of the species of *Brucella* and its correlation with their susceptibility profile was also evaluated. All 27 strains were susceptible to doxycycline. With the exception of one strain of *B. canis* and of *B. abortus*, all strains were susceptible to gentamicin and streptomycin. Of the wild *Brucella* strains tested, ten, nine and five showed reduced susceptibility to rifampicin, ceftriaxone and trimetoprim/sulfamethoxazole, respectively. One *B. abortus* and three *B. canis* strains showed multi-resistance profiles. The strain of *B. abortus* was resistant to streptomycin, rifampicin and ceftriaxone. Two strains of *B. canis* were resistant to rifampicin, ceftriaxone and trimetoprim/sulfamethoxazole, and one strain was resistant to rifampicin, ceftriaxone, streptomycin and gentamicin. Rifampicin, in combination with doxycycline, is one of the principal antibiotics prescribed to treat human brucellosis. The occurrence of strains resistant to rifampicin and other antimicrobials must be monitored before initiating this treatment, since the resistance of these strains could be one of the causes of the failure of some brucellosis treatment. No relationship was observed between SE-AFLP profiles and regional origin of the strains; neither between SE-AFLP profiles and antimicrobial profiles.

KEY WORDS: *Brucella;* brucellosis; E-test; resistance; rifampicin; SE-AFLP.

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RESUMO

Perfil de susceptibilidade antimicrobiana de cepas de *Brucella* sp. isoladas no Brasil

O objetivo deste trabalho foi investigar o perfil de susceptibilidade de 27 cepas de *Brucella* isoladas de animais no Brasil, utilizando-se o método E-test com os antimicrobianos recomendados para o tratamento da brucelose humana. Com este método, pretendeu-se monitorar a atividade destes antimicrobianos e seu potencial de eficacidade no tratamento desta enfermidade no homem. Também foi avaliada a eficiência da técnica SE-AFLP para discriminar as diferentes cepas de *Brucella* sp. e para analisar se os perfis gerados mostram alguma relação com os resultados de susceptibilidade. Todas as 27 cepas testadas foram sensíveis à doxiciclina, com exceção de uma cepa de *B. canis* e outra de *B. abortus*; as demais cepas foram sensíveis à gentamicina e à estreptomicina. Do total de cepas de campo testadas, respectivamente, dez, nove e cinco apresentaram susceptibilidade reduzida à rifampicina, ceftriaxona e trimetoprim/sulfametoxazol. Uma cepa de *B. abortus* e três de *B. canis* apresentaram perfil de multirresistência. A cepa de *B. abortus* mostrou-se resistente à estreptomicina, rifampicina e ceftriaxona. Duas cepas de *B. canis* foram resistentes à rifampicina, ceftriaxona e trimetoprim/sulfametoxazol e uma cepa foi resistente à rifampicina, ceftriaxona, streptomicina e gentamicina. Rifampicina e doxiciclina, associadas, são os principais antibióticos recomendados para o tratamento da brucelose humana. A ocorrência de cepas resistentes à rifampicina e outros antimicrobianos deve ser monitorada antes do início do tratamento, pois a resistência a esses antimicrobianos pode ser uma das causas do insucesso de alguns tratamentos de brucelose. Não foi observada nenhuma correlação entre os perfis SE- AFLP gerados e a origem das cepas, nem com os perfis de susceptibilidade destas cepas.

DESCRITORES: *Brucella;* brucelose; E-test; resistência; rifampicina; SE-AFLP.

INTRODUCTION

Brucellosis is an infectious disease transmitted by domestic or wild animals. Humans are normally infected by direct contact with infected animals, when working with culture strains in the laboratory or by the consumption of contaminated food (mainly non-pasteurized milk) (7, 33). The estimated number of human cases of the disease in the world is not precise, and it is thought that many cases are not declared or diagnosed (35). There are many reports of human brucellosis in Brazil. *Brucella abortus* and *Brucella suis* were the species isolated, and outbreak reports are scarce (10, 12, 23, 25, 28, 31). Only one *Brucella canis* human infection has been described in Brazil, though this infection occurs in dogs in many regions of Brazil (1, 11, 17, 22, 27, 31, 32). The *Brucella* genus is genetically homogeneous. Some techniques have been used with *Brucella* spp. and other bacteria to verify polymorphism among species or strains. Whatmore et al. analyzed several strains of *Brucella* by AFLP, and have obtained good discrimination between the majority of species tested (34). Single-Enzyme Amplified Fragment Length Polymorphism (SE-AFLP) is considered a promising technique, and has been used in molecular typing of several bacterial genera, but not yet with *Brucella* (13, 14, 30). This technique is based on the amplification of a subset of DNA fragments generated by digestion with rare-cutter restriction enzymes (e.g. *Hind*III).

Compared to the original AFLP method, which uses rare-cutter and frequently-cutter restriction enzymes, SE-AFLP generates a smaller number of fragments, and therefore produces less genetic information, but it can be more easily applied (6).

The recommended treatment for human brucellosis is the combination of two antimicrobials, doxycycline with streptomycin, rifampicin or gentamicin (36). To treat brucellosis in children, the combination of trimetoprim/sulfamethoxazole with an aminoglycoside (streptomycin, gentamycin) or rifampicin is recommended. Susceptibility tests are not normally recommended for *Brucella* infections. But, it would be important to verify the susceptibility profile of new isolates, since the majority of these antimicrobials are used to control many other infections, which increases the risk of selection of resistant strains.

The intention of this work was to investigate the susceptibility profile of *Brucella* strains isolated from animals in Brazil using the E-test method with the antimicrobials recommended for the treatment of human brucellosis, to monitor the activities of these antimicrobials and their potential efficacy for human brucellosis treatment, and to evaluate the efficiency of SE-AFLP in determining the genetic diversity of the species of *Brucella* and its correlation with their susceptibility profile.

MATERIALS AND METHODS

Strains

Wild strains were isolated from animal tissues and bovine milk from the southern region (Rio Grande do Sul, Santa Catarina and Paraná) and the south-eastern region (São Paulo and Minas Gerais) of Brazil. Twenty-seven strains of *Brucella* were tested: 19 *B. abortus*, seven *B. canis*, and one *B. suis* (Table 1). Reference strains *Escherichia coli* (ATCC 25922), *B. abortus* (biovar 1, strain 554), and *B. suis* (biovar 1, strain 1330) were used as controls. All strains were freeze-dried or frozen at −20 ºC. Before performing the E-test and DNA extraction, all strains were assessed for purity and absence of dissociation, as described elsewhere (2).

E-test

The E-test (AB Biodisk, Solna, Sweden) was performed according to the manufacturer's instructions for *Haemophilus influenzae*, with the exception that the 1.0 McFarland turbidity and foetal bovine serum were used. Bacteria were inoculated onto Mueller-Hinton agar plates (150 mm) with 10% foetal bovine serum and, after application of the E-test strips, plates were incubated at 35 °C in a 10% CO_2 atmosphere, for 48 h. The following antimicrobials were used: doxycycline, streptomycin, rifampicin, ceftriaxone, gentamicin and trimetoprim/sulfamethoxazole. The tests were performed in duplicate and were evaluated

according to the E-test manufacturer's instructions and the Clinical and Laboratory Standards Institute (CLSI) susceptibility criteria for *Brucella* spp. or *H. influenzae* (8). When there was no information displayed in the CLSI table for *Brucella* spp. or *Haemophillus* spp., the CLSI and E-test manufacturer's cut-offs for aerobic bacteria were used.

Table 1. *Brucella abortus, B. canis, B. suis* wild strains and their SE-ALFP profile

Species	Strains[a]	SE-AFLP profile[f]	SE-AFLP Classification
B. abortus			
	01/06, JA07, 477	A1 C1 G1 T1	A
	551g, 03/06	A1 C1 G1 T2	B
	Par08	A1 C1 G1 T5	C
	17b/02[b]	A1 C2 G1 T1	D
	88	A2 C1 G1 T1	E
	15/03	A2 C2 G1 T1	F
	551i, 577	A3 C1 G1 T1	G
	30MG[c]	A3 C8 G3 T4	H
	13b/02	A3 C8 G6 T4	I
	8p/04	A4 C3 G4 T8	J
	02/06	A4 C4 G3 T3	K
	14/02	A4 C4 G4 T4	L
	13a/02	A4 C5 G5 T5	M
	32MG[c]	A4 C8 G4 T4	N
	17a/02[b]	A5 C5 G5 T5	O
B. canis			
	Dalpel	A7 C7 G11 T10	P
	07/08, C95[d], C98	A8 C4 G8 T8	Q
	35/03[e]	A8 C7 G11 T10	R
	C97	A10 C10 G10 T10	S
	Pitpel	A11 C7 G7 T7	T
B. suis			
	SEA[b]	A11 C7 G11 T10	U

a: All strains were isolated in Rio Grande do Sul State except those indicated by letters. Region where those strains were isolated: **b** Santa Catarina; **c** Minas Gerais; **d** Paraná; **e** São Paulo. **f**: Profiles obtained after individual PCR with primers HI-A, HI-C, HI-G and HI-T. Each number indicates different profile to each primer used.

SE-AFLP

DNA extraction: Prior to DNA extraction, bacteria were suspended in 1 mL of TE buffer (10 mM Tris-HCL, 1 mM EDTA, pH 8.0) and inactivated at 80 ºC for 1 h. Following inactivation, a NucleoSpin Tissue XS kit (Macherey-Nagel) was used for DNA extraction.

Enzyme digestion and ligation of adapters SE-AFLP: For the digestion and ligation steps, the protocol described elsewhere was used, with modifications

(14). In a final volume of 10 µL, DNA (500 ng) was digested with 5U of *Hind*III, for 4 h at 37 ºC, and then for a final 15 min at 70 ºC. For a final volume of 20 µL of ligation reaction, 1U of T4 DNA ligase (Promega), ligation buffer, and 0.2 µg of each adapter (ADH-1 and ADH-2, Integrated DNA Technologies, Inc.) were added to the digested DNA and the mixture was incubated at 37 ºC for 4 h.

SE-AFLP: PCR was carried out as described elsewhere with modifications only in amplification conditions (13). The amplification was carried out with an initial cycle at 94 ºC for 5 min, followed by 13 cycles (94 ºC, 65 ºC, and 72 ºC for 1 min), reducing the temperature by 1 ºC in each cycle; 17 cycles of (94 ºC, 52 ºC, and 72 ºC for 1 min), and a final extension at 72 ºC for 30 min. Amplification products were separated by electrophoresis in 1.5% agarose gel, stained with ethidium bromide (0.5 µg mL^{-1}), and the image captured by a Kodak digital camera (DC120 Zoom Digital Picture Transfer Application, version 1.0.2).

Statistical and genetic diversity analysis

Statistical analyses were performed using the Statistical Analysis System version 9.2 (2005, SAS Institute Inc., Cary, NC, USA). Percentages of resistant strains were compared by the Chi-Squared test or Fisher's test. MIC values were analyzed using the procedure NPAR1WAY and comparison between *B. abortus* and *B. canis* strains was performed with the Wilcoxon test. SE-AFLP data analyses were performed using the Numerical Taxonomy and Multivariate Analysis System (NTSYS-pc) program, version 1.70 (Applied Biostatistics, 1992). An SE-AFLP profile was considered to be distinct if it had at least one fragment different. To evaluate the reproducibility of the technique, DNA from 21 strains of *Brucella* that showed different profiles was re-extracted and submitted to SE-AFLP.

RESULTS

The two reference strains were susceptible to all antimicrobials tested; *B. abortus* showed full resistance to trimetoprim/sulfamethoxazole. Tables 2, 3 and 4 summarize the results of the E-test with wild *Brucella* strains. Rifampicin and ceftriaxone showed the worst results. All wild strains of *B. abortus* were susceptible to doxycycline and gentamicin. One strain of *B. abortus* was resistant to streptomycin. This same strain was also resistant to rifampicin and ceftriaxone. All *B. canis* strains were susceptible to doxycycline, and one isolate out of the seven tested showed resistance to streptomycin and gentamicin. The same isolate also showed resistance to rifampicin and ceftriaxone. Minimum inhibitory concentrations (MIC) are shown in Tables 2 and 3; *B. canis* had the highest proportion of resistant strains to rifampicin and trimetoprim/sulfamethoxazole, and higher MIC values to rifampicin ($P<0.05$)(see also Table 4).

Table 2. MIC values of 19 *B. abortus* strains isolated in Brazil

Antimicrobials[a]	MIC (mg/L)				
	Wild strains			*B. abortus*	CLSI cut-offs
	MIC (min–max)	MIC_{50}	MIC_{90}	Biovar 1[b]	Susceptible
DC	0.032–1	0.5	1	0.5	≤1
SM	0.064–192	0.75	2	0.064	≤8
RI	0.08–32	0.75	32[c]	0.38	≤1[d]
TX	0.094–256	0.75	256[c]	0.25	≤2[d]
GM	0.016–4	1	1.5	0.38	≤4[e]
TS	0.003–32	0.125	0.25	32	≤2

a: DC Doxycycline; SM Streptomycin, RI Rifampicin, TX Ceftriaxone, GM Gentamicin, TS Trimethoprim/ Sulphametoxazole (only the trimethoprim portion of the 1/19 drug ratio is displayed). **b**: Reference strain 544. **c**: Less than 90% of strains were classified as susceptible. **d**: Not displayed in CLSI table for *Brucella* spp., used the CLSI cut-off for *Haemophilus* spp. **e**: Not displayed in CLSI table for *Brucella* spp. nor for *Haemophilus* spp., used CLSI and E-test manufacturer cut-off for aerobic bacteria.

Table 3. MIC (mg/L) values of seven *B. canis* strains isolated in Brazil

Antimicrobials[a]	Wild strains			CLSI cut-offs
	MIC (min–max)	MIC_{50}	MIC_{90}	Susceptible
DC	0.094–0.5	0.25	0.5	≤1
SM	0.75–192	1	192[b]	≤8
RI	1–32	1.5[b]	32[b]	≤1[c]
TX	0.5–256	4[b]	256[b]	≤2[c]
GM	0.19–8	0.25	8[b]	≤4[d]
TS	0.004–32	2	32[b]	≤2

a: DC Doxycycline; SM Streptomycin, RI Rifampicin, TX Ceftriaxone, GM Gentamicin, TS Trimethoprim/Sulphametoxazole (only the trimethoprim portion of the 1/19 drug ratio is displayed). **b**: Less than 50% or 90% of strains were classified as susceptible. **c**: Not displayed in CLSI table for *Brucella* spp., used the CLSI cut-off for *Haemophilus* spp. **d**: Not displayed in CLSI table to *Brucella* spp. nor to *Haemophilus* spp., used CLSI and E-test manufacturer cut-offsfor aerobic bacteria.

Resistance to trimethoprim/sulphametoxazole was observed within *B. canis*, *B. abortus* and the unique strain of *B. suis* tested (Table 4). Ceftriaxone showed reduced activity to nine *Brucella* strains. Among the 19 *B. abortus* and seven *B. canis* strains tested, seven and six, respectively, showed reduced susceptibility to one or more of the antimicrobials tested. One strain of *B. abortus* showed multi-resistance, based on its reduced susceptibility to at least three different classes of antimicrobials (rifampicin, ceftriaxone and streptomycin). In addition, three of the seven *B. canis* strains tested were also considered multi-resistant: all were resistant to rifampicin and ceftriaxone. These multi-resistant *B. canis* strains also showed resistance to one or more of the following antimicrobials: streptomycin (one strain), gentamicin (one strain), and trimetoprim/sulfamethoxazole (two strains).

The number of fragments generated by the SE-AFLP method for each strain and each primer ranged from 1 to 13, with sizes between 200 and 2,200 bp. In this study, we analyzed the fragments between 400 and 1,500 bp. Analyzing the

profiles generated by the four primers for each strain, 21 different grouped profiles were created and named from A to U (Table 1).

DISCUSSION

The MIC_{50} values obtained with *B. abortus* to ceftriaxone and rifampicin were similar to those obtained by other authors, but to doxycycline the MIC_{50} value was higher in this study despite all strains being susceptible to this antibiotic (4, 5). The results showed that doxycycline, the main antimicrobial used for brucellosis treatment, is efficient, as are streptomycin and gentamicin, inhibiting almost all tested strains (36). Rifampicin is generally a second-choice antimicrobial used to treat brucellosis in combination with doxycycline. The wild strains showed a pronounced reduced susceptibility to this antibiotic, and *B. canis* had a higher MIC_{50} value than *B. abortus* (Tables 2 and 3). This fact must be taken into account when using this antimicrobial (4).

Resistance to trimethoprim/sulphametoxazole was observed within *B. canis*, *B. abortus* and the unique wild strain of *B. suis* tested. This antimicrobial is also recommended for brucellosis treatment, mainly in children. Other authors have shown the low activity of this antimicrobial on the *Brucella* genus, so it is important that its susceptibility be monitored before performing treatment (9, 19, 36). Nine *Brucella* strains showed reduced susceptibility to ceftriaxone, which fortunately is not frequently used for human brucellosis treatment.

More than 50% of the strains showed reduced susceptibility to one or more of the antimicrobials tested. This reduced susceptibility may be associated with their use in the treatment of other infections, a situation that can contribute to the selection of resistant strains.

The determination of the antimicrobial susceptibility of *Brucella* spp. using the E-test is carried out for certain groups because of its practicality; requiring less manipulations of the bacteria compared to dilution tests (4, 5, 15, 16, 18, 21, 24). As studies about the correlation between *Brucella* antibiograms and treatment efficiency are scarce, it is difficult to assert the importance of the resistances observed (29). As suggested by other authors, it would be interesting to monitor the antimicrobial susceptibility of the isolates, since it has been observed that the treatment of brucellosis carries a relative risk of relapse (5, 21). Certainly it is difficult to claim whether this relapse is due to the difficulty of tissue penetration by the drug, the correct treatment period or the resistance of those strains (3, 26). Some investigators have suggested also that resistance variation among strains exists in different localities, maybe as a result of local selection pressure, and this could be linked to the failure of some brucellosis treatment (5, 20).

The 19 *B. abortus* strains tested by SE-AFLP were distributed in 15 profiles, and the seven *B. canis* strains tested were distributed in five profiles, demonstrating the high discrimination of this technique. No relationship was observed between

SE-AFLP profiles and the regional origin of strains, although there was a large difference in the number of strains from those regions for this to be significant (P>0.05). In the same way, no relationship was observed between SE-AFLP profiles and antimicrobials. For example, a same profile of susceptibility to all antimicrobials tested showed 10 different SE-AFLP profiles for some *B. abortus* strains (Table 4). Also these same profiles of susceptibility to all antimicrobials grouped strains from three regions showing no relationship to the origin of these strains. But, as mentioned above, the numbers of strains from the different regions were not equal; therefore it was difficult to analyze any significance.

Table 4. *Brucella* spp. origin, antimicrobial susceptibility and SE-AFLP classification

Region	Strains	Antimicrobials[a]						SE-AFLP[b]
		DC	SM	RI	TX	GM	TS	Profile classification
	B. abortus							
RS[c]	88	S[d]	R	R	I	S	S	E
RS	14/02	S	S	R	R	S	S	L
RS	01/06, 8p/04	S	S	R	R	S	S	A, J
MG	30MG	S	S	I	S	S	S	H
RS(9)[e], SC(2), MG(1)	JA07, 477, 551g, Par08, 17b/02, 15/03, 551i, 577, 02/06, 13a/02, 32MG, 17a/02	S	S	S	S	S	S	A(2)[f], B, C, D, F, G(2), K, M, N, O
RS	03/06	S	S	S	S	S	R	B
RS	13b/02	S	S	S	I	S	S	I
	B. canis							
RS	07/08	S	R	R	R	R	S	Q
RS, PR	C95, C97	S	S	R	R	S	R	Q, S
RS, SP	35/03, Pitpel	S	S	R	S	S	S	R, T
RS	Dalpel	S	S	S	S	S	S	P
RS	C98	S	S	S	R	S	R	Q
	B. suis							
SC	SEA	S	S	S	S	S	R	U

a: DC Doxycycline; SM Streptomycin, RI Rifampicin, TX Ceftriaxone, GM Gentamicin, TS Trimethoprim/Sulphametoxazole. **b**: Different letters indicate different profiles after SE-AFLP. **c**. Regions where strains were isolated: RS Rio Grande do Sul, SC Santa Catarina, MG Minas Gerais, PR Paraná, SP São Paulo. **d**: S: susceptible, R: resistant, I: intermediate. **e**: In brackets, number of strains from those regions. **f**: In brackets, number of strains with those profiles.

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