Brazilian Journal of Microbiology (2011) 42: 1420-1426
ISSN 1517-8382

ERIC-PCR GENOTYPIC CHARACTERIZATION OF *HAEMOPHILUS PARASUIS* ISOLATED FROM BRAZILIAN SWINE

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Submitted: August 09, 2010; Returned to authors for corrections: February 11, 2011; Approved: June 06, 2011.

ABSTRACT

Haemophilus parasuis infection, known as Glässer's disease, is characterized by fibrinous polyserositis, arthritis and meningitis in piglets. Although traditional diagnosis is based on herd history, clinical signs, bacterial isolation and serotyping, the molecular-based methods are alternatives for species-specific tests and epidemiologic study. The aim of this study was to characterize *H. parasuis* strains isolated from different states of Brazil by serotyping, PCR and ERIC-PCR. Serotyping revealed serovar 4 as the most prevalent (24 %), followed by serovars 14 (14 %), 5 (12 %), 13 (8 %) and 2 (2 %), whereas 40 % of the strains were considered as non-typeable. From 50 strains tested 43 (86%) were positive to Group 1 *vta*A gene that have been related to virulent strains of *H.parasuis*. ERIC-PCR was able to type isolates tested among 23 different patterns, including non-typeable strains. ERIC-PCR patterns were very heterogeneous and presented high similarity between strains of the same animal or farm origin. The results indicated ERIC-PCR as a valuable tool for typing *H. parasuis* isolates collected in Brazil.

Key words: *Haemophilus parasuis*; Glässer disease; Genotyping; ERIC-PCR.

INTRODUCTION

Haemophilus parasuis is the etiological agent of Glässer's disease which is characterized by fibrinous polyserositis, arthritis and meningitis that can affect pigs from 2 weeks to 4 months old (11). It is a non-motile, pleomorphic, Gram-negative bacillus of the *Pasturellacea* family, nicotinamide adenine dinucleotide (NAD) dependent bacterium (1) which colonizes the upper respiratory tract of pigs before weaning (20). Therefore, samples of pleura, peritoneum, joints and meninges are preferable for clinical diagnosis (17), since *H. parasuis* can be isolated from the upper respiratory tract of healthy pigs.

The strains of *H. parasuis* were classified by serotyping in 15 serovars and demonstrated differences in their virulence, classifying strains from highly virulent to non-virulent (7).

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However, no clear correlation between serovar and virulence has been defined, since strains belonging to the same serotype can exhibit different degrees of virulence. Nevertheless, serotyping has been useful for vaccine production, but it is not discriminative enough for epidemiologic studies, since 15 to 41% of the isolates are non-typeable through the Kielstein and Rapp-Gabrielson (KRG) serotyping method (15, 22). For this reason, molecular methods have been developed to improve diagnosis and epidemiological characterization of *H. parasuis*.

Santos *et al*. (23) first described high incidence of serovars 1, 4, 5 and 12, which were considered from moderate to high virulence. Since then, few studies have been developed with *H. parasuis* in Brazil, but the epidemiology of the infection is still unknown. Therefore, the aim of this study was to characterize *Haemophilus parasuis* isolates collected in different States in Brazil, by serotyping, PCR and ERIC-PCR.

MATERIAL AND METHODS

Bacterial strains

Fifty isolates were evaluated in this study. The strains were isolated from lungs of 23 pigs with clinical signs of arthritis, meningitis, peritonitis and/or pericarditis, in 21 herds from four different States (Mato Grosso, Minas Gerais, Paraná and São Paulo) in Brazil. All isolates were maintained at -80^{o}C until analysis.

Serotyping

The strains were grown on Brain Heart Infusion (BHI - Difco-BBL, Detroit, MI /USA) supplemented with NAD (0.01 %) and fetal calf serum (5 %) from 24 to 48 h at 37^{o}C, under microaerophilic conditions (5 % of CO_2). Serotyping was carried out on 50 isolates by immunodiffusion test using autoclaved antigens (7) at the Microvet Laboratory of Veterinary Microbiology, Minas Gerais, Brazil.

DNA preparation and PCR

Purified DNA was recovered with Boom *et al*. (3) protocol of DNA extraction and stored at –20ºC. The 50 strains were identified by PCR based on species-specific amplification of the 16S rRNA gene as described by Oliveira *et al*. (12) and by amplification of Groups 1 and 3 *vta*A genes described by Olvera *et al.* (19). The PCR directed to 16S rRNA gene was performed in a 20µl reaction mixture containing 1 µl of extracted DNA, 0.3 mM of each primer, 50 mM KCl, 10 mM Tris-HCl, 1.5 mM MgCl2, 5 µl of PCR buffer, 0.24 mM of each deoxynucleoside triphosphate, and 0.5 U of Taq DNA polymerase (LGC Biotecnologia, São Paulo, Brazil). The PCR was carried out for 30 cycles consisting of denaturation for 30 seconds at 94 ^{o}C, annealing for 30 seconds at 59 ^{o}C, and extension for 2 minutes at 72 C. The PCR to detect Groups 1 and 3 *vta*A genes was performed in a 25µl reaction mixture containing 2.5 µl of extracted DNA, 800 mM of primer YADAF1 e PADHR1, 400 mM dos primers YADAF3 e PADHR3, 50 mM KCl, 10 mM Tris-HCl, 2 mM MgCl2, 5 µl of PCR buffer, 0.4 mM of each deoxynucleoside triphosphate, and 1 U of Taq DNA polymerase (LGC Biotecnologia, São Paulo, Brazil). Cycling conditions were 5 min at 94^{o}C, followed by 25 cycles of 45s at 94 ^{o}C, 45s at 64 ^{o}C and 1 min at 72 ^{o}C, then a final incubation at 72^{o}C for 7 min.

The amplified products were separated by electrophoresis in 1.5 % or 2.5% agarose gel stained with ethidium bromide. The 100bp DNA ladder (LGC Biotecnologia, São Paulo, Brazil) was used for molecular weight determinations.

Enterobacterial Repetitive Intergenic Consensus Sequences (ERIC-PCR)

ERIC-PCR was performed as described previously by Versalovic *et al*. (25), using 5 µl from extracted DNA, 1.5 mM of $MgCl_2$, 10 pmoles from primers ERIC1 (ATGTAA GCTCCTGGGGATTCAC) and ERIC2 (AAGTAAGTGACTG GGGTGAGCG), 1.0 U of *Taq* DNA polymerase (LGC Biotecnologia, São Paulo, Brazil), 1 X of PCR buffer and water to complete the total volume of 50 µl. PCR reaction was carried out with 35 cycles consisting of denaturation for 4 min at 94^{o}C, annealing for 60 sec at 50^{o}C, and extension of 2.5 min

at 72°C, increased with a final extension of 20 min at 72°C as described by Raffie *et al*. (21). Amplification products were detected through electrophoresis at 35 V for 20 h in 2 % Agarose 1000 (Invitrogen Corporation, Carlsbad, CA/USA) gel stained with ethidium bromide.

Data analysis

ERIC-PCR fingerprints analysis and similarity matrix calculations were carried out using Simple Matching coefficient and cluster analysis of fingerprints was performed using the unweighted-pair group method (UPGMA) by means of the NTSYS software to generate dendrogram. A 90% cut-off was used to analyze the genotypes generated by ERIC-PCR technique according to Oliveira *et al.* (13). Discriminatory index was calculated as described by Hunter and Gaston (6).

RESULTS

Serotyping and PCR

Serovar 4 was the most prevalent (24 %), followed by serovars 14 (14 %), 5 (12 %), 13 (8 %) and 2 (2 %), whereas 40 % of the strains were considered non-typeable. Strains from different serovars were identified on specimens from the same animal and same farm, as showed by Table 1. All tested strains were positive to species-specific amplification of the 16S rRNA gene as described by Oliveira *et al*. (12) and to Group 3 *vta*A gene described by Olvera *et al.* (19). From 50 strains tested 43 (86%) were positive to Group 1 *vta*A gene.

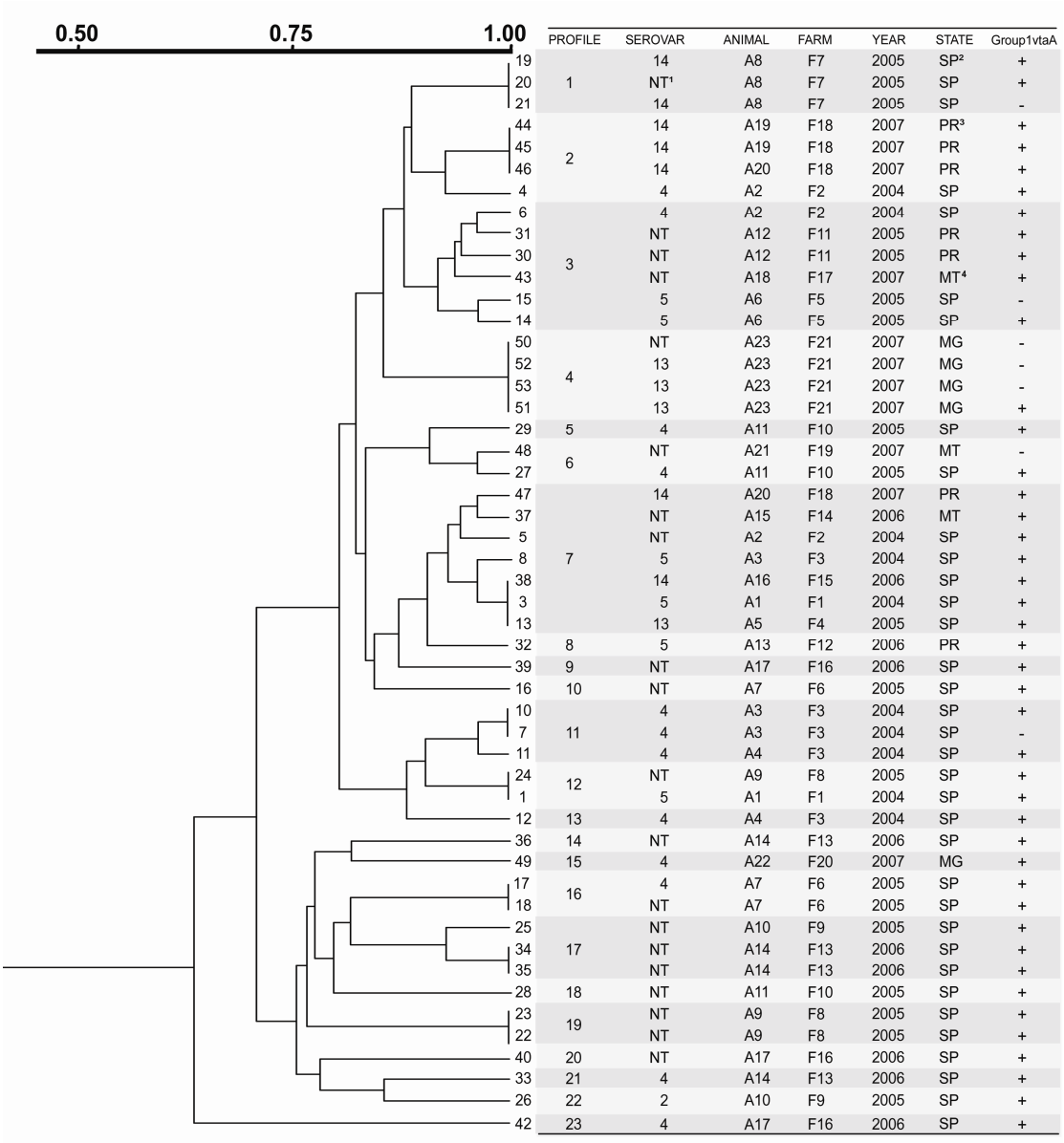
Table 1. Serovar distribution of *H. parasuis* isolates according with animal, farm of origin, State and year of isolation.

Animal	Farm	Serovar	State	Year
A1	F1	5, 5	SP[2]	2004
A2	F2	4, NT[1], 4	SP	2004
A3	F3	4,5,4	SP	2005
A4	F4	4,4	SP	2004
A5	F4	13	SP	2005
A6	F5	5,5	SP	2005
A7	F6	NT, NT, 4	SP	2005
A8	F7	14,UT,14	SP	2005
A9	F8	NT, NT, NT	SP	2005
A10	F9	NT, 2	SP	2005
A11	F10	4,4,NT	SP	2005
A12	F11	NT, NT	PR[3]	2005
A13	F12	5	PR	2006
A14	F13	4, NT, NT, NT	SP	2006
A15	F14	NT	MT[4]	2006
A16	F15	14	SP	2006
A17	F16	4, NT, NT	SP	2006
A18	F17	NT	MT	2007
A19	F18	14,14	PR	2007
A20	F18	14, 14	PR	2007
A21	F19	NT	MT	2007
A22	F20	4	MG[5]	2007
A23	F21	NT, 13, 13, 13	MG	2007

[1] non-typeable strains, [2] São Paulo, [3] Paraná, [4] Mato Grosso and [5]Minas Gerais

ERIC-PCR analysis

All 50 strains were examined and identified with the ERIC-PCR technique. Analysis of ERIC profiles utilizing a 90% cut-off revealed 23 distinct genotypes. ERIC patterns for *H. parasuis* isolates were highly heterogeneous, with DNA fragments ranging from 150 to 1300 bp in size. The ERIC-PCR dendrogram (Figure 1) distributed the 50 *H. parasuis* strains in two main groups with more than 70% of similarity. Group I comprised composed by 36 of tested strains from profiles 1 to 13. Inside these subtypes, strains from same animal and farm origin were clustered in an identical genotype, as well as strains from different animals, farms, state and even from different serotypes, were grouped in the same genetic profile. Group II comprised profiles 14 to 23 with *H. parasuis* isolates, ranging from 80% to 100% of similarity, 9 of which were non-typeable by serotyping.



	PROFILE	SEROVAR	ANIMAL	FARM	YEAR	STATE	Group1vtaA
19	1	14	A8	F7	2005	SP[2]	+
20		NT[1]	A8	F7	2005	SP	+
21		14	A8	F7	2005	SP	-
44	2	14	A19	F18	2007	PR[3]	+
45		14	A19	F18	2007	PR	+
46		14	A20	F18	2007	PR	+
4		4	A2	F2	2004	SP	+
6	3	4	A2	F2	2004	SP	+
31		NT	A12	F11	2005	PR	+
30		NT	A12	F11	2005	PR	+
43		NT	A18	F17	2007	MT[4]	+
15		5	A6	F5	2005	SP	-
14		5	A6	F5	2005	SP	+
50	4	NT	A23	F21	2007	MG	-
52		13	A23	F21	2007	MG	-
53		13	A23	F21	2007	MG	-
51		13	A23	F21	2007	MG	+
29	5	4	A11	F10	2005	SP	+
48	6	NT	A21	F19	2007	MT	-
27		4	A11	F10	2005	SP	+
47	7	14	A20	F18	2007	PR	+
37		NT	A15	F14	2006	MT	+
5		NT	A2	F2	2004	SP	+
8		5	A3	F3	2004	SP	+
38		14	A16	F15	2006	SP	+
3		5	A1	F1	2004	SP	+
13		13	A5	F4	2005	SP	+
32	8	5	A13	F12	2006	PR	+
39	9	NT	A17	F16	2006	SP	+
16	10	NT	A7	F6	2005	SP	+
10	11	4	A3	F3	2004	SP	+
7		4	A3	F3	2004	SP	-
11		4	A4	F3	2004	SP	+
24	12	NT	A9	F8	2005	SP	+
1		5	A1	F1	2004	SP	+
12	13	4	A4	F3	2004	SP	+
36	14	NT	A14	F13	2006	SP	+
49	15	4	A22	F20	2007	MG	+
17	16	4	A7	F6	2005	SP	+
18		NT	A7	F6	2005	SP	+
25	17	NT	A10	F9	2005	SP	+
34		NT	A14	F13	2006	SP	+
35		NT	A14	F13	2006	SP	+
28	18	NT	A11	F10	2005	SP	+
23	19	NT	A9	F8	2005	SP	+
22		NT	A9	F8	2005	SP	+
40	20	NT	A17	F16	2006	SP	+
33	21	4	A14	F13	2006	SP	+
26	22	2	A10	F9	2005	SP	+
42	23	4	A17	F16	2006	SP	+

Figure 1. ERIC-PCR dendrogram. Percentages of similarity between profiles were calculated by use of Dice's coefficient. The dendrogram was constructed by use of UPGMA (unweighted pair group method using arithmetic average).

Discriminatory power

The discriminatory index obtained for serotyping and ERIC-PCR were 0.75 and 0.95, respectively.

DISCUSSION

In this study, the application of the molecular typing method, ERIC-PCR, PCR and traditional serotyping method were used to characterize *Haemophilus parasuis* strains isolated from swine of different states in Brazil. Serotyping was able to characterize 60% of the isolates among five different serovars, including highly pathogenic strains (serovars 5, 13 and 14). The serovar 4 was the most prevalent (24%) among *H. parasuis* strains; however, more than 40% of the isolates tested were non-typeable by serotyping, which is compliant with Oliveira and Pijoan (15) findings that reported non-typable isolates as one of the most prevalent in United States herds. Macedo *et al.* (8) also reported high prevalence of serovar 4 (15.9%) and non-typeable (60.3%) strains on Brazilian territory. The occurrence of non-typeable strains is one limitation of serotyping as an epidemiological tool. For this reason, other techniques such as molecular methods are being developed to improve the study of the *H. parasuis* epidemiology.

Olvera *et al* (19) developed a PCR to characterize trimeric autotransporters genes (*vtaA*) associated to virulence, they describe that group 3 *vtaA* gene seems to be conserved among invasive and non-invasive strains and may be used to species identification, while group 1 *vtaA* gene showed better correlation with virulent strains. In the present study, all strains were isolated from lungs of swine with clinical signs of Glasser´s disease and the frequency of *vtaA*1 gene was high as could be expected (86%). Olvera *et al*, (19), also describe the occurrence of *vtaA*1 negative isolates from lungs as observed in seven strains examined here. In some cases were selected more than one isolate from the same animal, and was observed that six of seven *vtaA*1 negative strains were isolated from animals also carrying *vtaA*1positive *H. parasuis* strains (Figure 1- profiles 1, 3, 4 and 11).

Smart *et al*. (24) started the genotyping study of *H. parasuis* using REP (Restriction Endonuclease Pattern) technique to differentiate samples of the respiratory tract from isolates of the systemic infection. Other genotyping methods such as Multilocus Enzyme Electrophoresis (MEE), Restriction Fragment Length Polymorphism (RFLP), and more recently the fingerprinting and sequencing methods proved to be able to characterize different isolates of *H. parasuis* presenting highly heterogeneous patterns (2, 4, 5, 9, 13, 16, 18). However, most of these methods are long-lasting and expensive; therefore, an alternative technique to overcome these hindrances is ERIC-PCR.

Rafiee *et al*. (21) reported the use of ERIC-PCR to characterize isolates of *H. parasuis*. The method proved to be able to typify all isolates according to origin and serovar in a short period of time and at low cost. Oliveira *et al*. (14) and Olvera *et al*. (17) also validated ERIC-PCR as a useful technique and confirmed the high heterogeneity of *H. parasuis* isolates. However, they confirmed as well the low reproducibility of the technique which could hinder intra-laboratory comparisons. Macedo et al. (8) first used ERIC-PCR technique to characterize Brazilian *H. parasuis* strains and reported, not only that this genotyping method was more discriminatory than serotyping, but also a high genetic diversity among non-typeable strains and the isolates originated from respiratory tract. These results sustain the high genetic variability found in this study.

ERIC-PCR proved to be a valuable method for *H. parasuis* typing, since it discriminated all 50 strains tested, including the non-typeable isolates, among 23 genotypes. It was capable to distinguish isolates of the same and different serovars and, therefore, it is a useful epidemiological tool. Some profiles, as Genotype 4 and 12, comprises samples of the same serovar, animal and farm of origin, but one of them was characterized as non-typeable by serotyping. This could be explained by possible lost of cellular components of capsule naturally or after several reactivations at the laboratory,

resulting in the change of serovar type, a fact previously described for lung tissue specimens (10, 22).

Discriminatory power of ERIC-PCR was higher than serotyping, confirming the statement that serotyping is a poor epidemiological tool for the study of *H. parasuis,* when compared to genotyping methods, such as ERIC-PCR. Although ERIC-PCR proved to be an interesting technique to analyze bacterial samples, its low reproducibility still represents impairment to the future use of the method in intra-laboratory comparisons. Therefore, the optimization of genotyping methods to analyze *H. parasuis,* and the study of other pathogenic and virulence mechanisms of the bacterium are necessary to improve its epidemiological study and provide practical information to implement effective prevention and control programs at swine farms in Brazil.

ACKNOWLEDGEMENTS

This study was sponsored by FAPESP (process: 07/04893-9).

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