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RAPD-PCR TYPING OF *Yersinia enterocolitica* (ENTEROBACTERIACEAE) O:3 SEROTYPE STRAINS ISOLATED FROM PIGS AND HUMANS

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ABSTRACT

Sixteen strains of *Yersinia enterocolitica* serotype O:3, isolated from apparently healthy pigs collected in Rio de Janeiro, and four human strains of serotypes O:4, O:5, O:6 and O:13 were analyzed by RAPD-PCR. The strains were grouped into five genotypic profiles according to the amplification patterns obtained with three random primers. Fifteen of the 16 pig strains had identical amplification patterns, which was named genotypic profile 1. The one different profile was named genotypic profile 2. Genotypic profile 1 was also exhibited by the O:6 human serotype strain. The O:4 and O:13 human serotype strains showed similar amplification profiles with two primers. However, the third primer induced a distinct profile in each strain. Therefore, these two strains were placed into genotypic profile 3 and 4, respectively. Each primer produced a completely different amplification profile in the O:5 human serotype strain; therefore, it was named genotypic profile 5. The presence or absence of plasmids in the strains studied did not affect the amplification results. These results show that genetic variations can exist within a serotype, and strains of different serotypes can exhibit the same amplification profile when compared using other primers.

INTRODUCTION

Yersinia enterocolitica is a gram-negative bacterium, belonging to the family Enterobacteriaceae. It is widespread throughout the environment and among healthy and sick animals of several species. Humans can be carriers. Furthermore, pigs can carry the same serotypes found in man. These animals carry the bacteria in their throat and excrete them in their feces (Bottone, 1977). Several isolations of *Y. enterocolitica* have been obtained in Brazil, from healthy and sick humans and animals (dogs and pigs), water and some kinds of foodstuff (milk, dairy products, meat and derivatives, vegetables, etc.) mainly in the States of São Paulo, Rio de Janeiro and Rio Grande do Sul (Nunes and Ricciardi, 1986; Warnken *et al*., 1987; Ceccarelli *et al*., 1990; Tassinari *et al*., 1994).

Y. enterocolitica is a very heterogeneous species and is divided into six biotypes, eight phage-types and more than 60 serotypes (Bottone, 1977). Moreover, it has been observed that traditional techniques of bio/sera/phage-typing are not sufficiently discriminatory. Strains of one serotype have been subdivided, while strains of different serotypes have been regrouped through analysis of restriction fragment length in pulsed field gel electrophoresis (Najdenski *et al.*, 1994; Saken *et al*., 1994).

Since its discovery, the PCR technique has been increasingly used for its simplicity, reproducibility and rapidity. The random amplification of polymorphic DNA (RAPD-PCR) technique, a variation of PCR, is based on the random amplification of polymorphic DNA fragments and uses a single primer directed at a random DNA sequence. In low stringency conditions, the primer anneals to homologous or very similar segments of DNA (Welsh and McClelland, 1990; William *et al*., 1990).

RAPD has been used with success by Rasmussen *et al*. (1994) to divide isolates of *Y. enterocolitica* into the different pathogenic groups and by Odinot *et al*. (1995) to discriminate between different biotypes of *Y. enterocolitica*. In the present work we used three random primers to characterize, by RAPD-PCR, *Y. enterocolitica* strains isolated from healthy pigs in the State of Rio de Janeiro, Brazil.

MATERIAL AND METHODS

Bacteria and culture conditions

Sixteen O:3 *Y. enterocolitica* serotype strains were analyzed. The samples were obtained from the culture collection of the Department of Bacteriology of the Oswaldo Cruz Institute (IOC/FIOCRUZ). They were obtained from healthy pigs (tongue, tonsils, mesenteric lymph node, rectal swab and cecum content). Additionally, four strains isolated from human feces (one of each serotype: O:4, O:5, O:6 and O:13) were included (Table I). The strains were plated on blood agar base (Difco) and subcultured in brain heart infusion broth (Difco) for 18 to 24 h at 28ºC.

Plasmid preparation

Plasmid DNA was extracted by the technique of Birnboim and Doly (1979), subjected to electrophoresis in 0.6% agarose gels, stained with ethidium bromide (10 mg/ml) and observed under ultraviolet light (UV).

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Table I - Phenotypic characteristics of *Yersinia enterocolitica* strains and their genotypic profiles distinguished by RAPD-PCR.

Strain	Origin	Serotype	Plasmid (kb)	Genotypic profile
Ye 1	Pig	O:3	-	1
Ye 2	Pig	O:3	-	1
Ye 5	Pig	O:3	-	1
Ye 5a	Pig	O:3	8.6	1
Ye f28	Pig	O:3	-	1
Ye 6	Pig	O:3	-	1
Ye 10	Pig	O:3	-	1
Ye 14	Pig	O:3	-	1
Ye 16	Pig	O:3	-	1
Ye 17	Pig	O:3	-	1
Ye 25c	Pig	O:3	70	1
Ye 25l	Pig	O:3	70	1
Ye 35	Pig	O:3	70	1
Ye 37	Pig	O:3	70	1
Ye 40	Pig	O:3	70	1
Ye 41	Pig	O:3	70	2
Ye 43p	Human	O:6	-	1
Ye 102p	Human	O:5	-	5
Ye 124p	Human	O:4	-	3
Ye 184p	Human	O:13	-	4

Total DNA preparation

Total DNA from the 20 strains was extracted as described by Maniatis *et al.* (1982). The DNA was quantified, after electrophoresis in a 1% agarose gel, by comparison with known amounts of *Hin*dIII-digested bacteriophage *lambda* DNA.

Selection of primers, establishment of thermal cycle and stringency conditions

Preliminary assays with strain Ye 184p (pig serotype O:3) were carried out in two different concentrations of Mg^{2+} (3 mM and 4 mM) and two thermal cycles: thermal cycle No. 1 was programmed for five cycles, composed of one initial step of denaturation for 5 min at 94°C, one step of low-stringency temperature of annealing for 5 min at 36°C and one step of synthesis or amplification for 5 min at 72°C. This was followed by 25 cycles of high-stringency temperature of annealing, consisting of 1 min at 94°C, 2 min at 50°C and 3 min at 72°C, finishing with a step of amplification for 7 min at 72°C. Thermal cycle No. 2 was programmed for 30 cycles composed of one initial step of denaturation for 1 min at 94°C, one step of annealing for 1 min at 36°C and one of synthesis for 2 min at 72°C followed by a final step of amplification for 7 min at 72°C. Assays were made with 24 primers of different sizes (9, 15 and 20 mer), to test their ability to produce discriminatory RAPD profiles in the *Y. enterocolitica* strains.

Amplification

Thermal cycle No. 1 reactions were prepared in a total volume of 25 µl per tube, containing 20 ng DNA, 1 U Taq DNA polymerase (CENBIOT/RS), 10 mM Tris-HCl, 3 mM $MgCl_2$, 50 mM KCl, 100 mg gelatin, 200 mM of each dNTP and 20 pmol of primer. Amplification products were submitted to electrophoresis in 1.5% agarose gel, Tris-borate buffer and a constant voltage of 100 V, followed by staining with ethidium bromide and visualization in a UV transilluminator. A negative control was included in each PCR run with no target DNA. Reproducibility of the amplification results was evaluated in parallel experiments by the repetition of the PCR reactions with all strains and each primer at least three times.

RESULTS

Selection of primers

The following primers produced a neat, bright and reproducible band and were selected for genotyping by random amplification: primer 785 = 5'-CCGCAGCCAA-3', primer 786 = 5'-GCGATCCCCA-3' and primer 795 = 5' GAGACGCACA-3'.

Amplification profiles

Of the 16 serotype O:3 *Y. enterocolitica* strains isolated from pigs, 15 presented the same profile, characterized by amplification of the same number of bands, specific for each of the three primers. This amplification profile was named genotypic profile 1. Amplification with primer 785 resulted in an array of seven well-defined and reproducible bands with 600 to 2400 base pairs (bp) (Figure 1C, lanes 3, 5, 8 and 9). Primer 795 produced 11 bands with 150 to 2500 bp (Figure 1B, lanes 3, 5, 8 and 9). Amplification with primer 786 resulted in seven bands with 300 to 1700 bp (Figure 1A, lanes 3, 5, 8 and 9). The human serotype O:6 strain exhibited a profile identical to the one observed in the 15 pig strains. Therefore, this strain was included in genotypic profile 1 (Figure 1A, B and C, lane 6). There were only two visible bands in Figure 1B (lane 6), out of the seven bands actually produced by the human strain O:6 serotype with primer 795. The other bands, visible on the other gels, were too faint to be noticed in the photo.

One pig serotype O:3 strain of *Y. enterocolitica* (Ye 41) displayed a different amplification pattern. The reaction with primer 785 resulted in the amplification of two bands of 1000 and 1200 bp. With primer 795 one band of 300 bp was produced, and primer 786 amplified two bands of 1000 and 1600 bp. This amplification pattern was named genotypic profile 2 (Figure 1A, B and C: lane 2, with primers 786, 795 and 785, respectively).

Strains Ye 184p (O:13) and Ye 124p (O:4) showed similar profiles, characterized by the amplification of nine bands with 300 to 2400 bp with primer 795 (Figure 1B, lanes 4 and 10). These two strains also produced identical eight band profiles with primer 786 (Figure 1A, lane 4

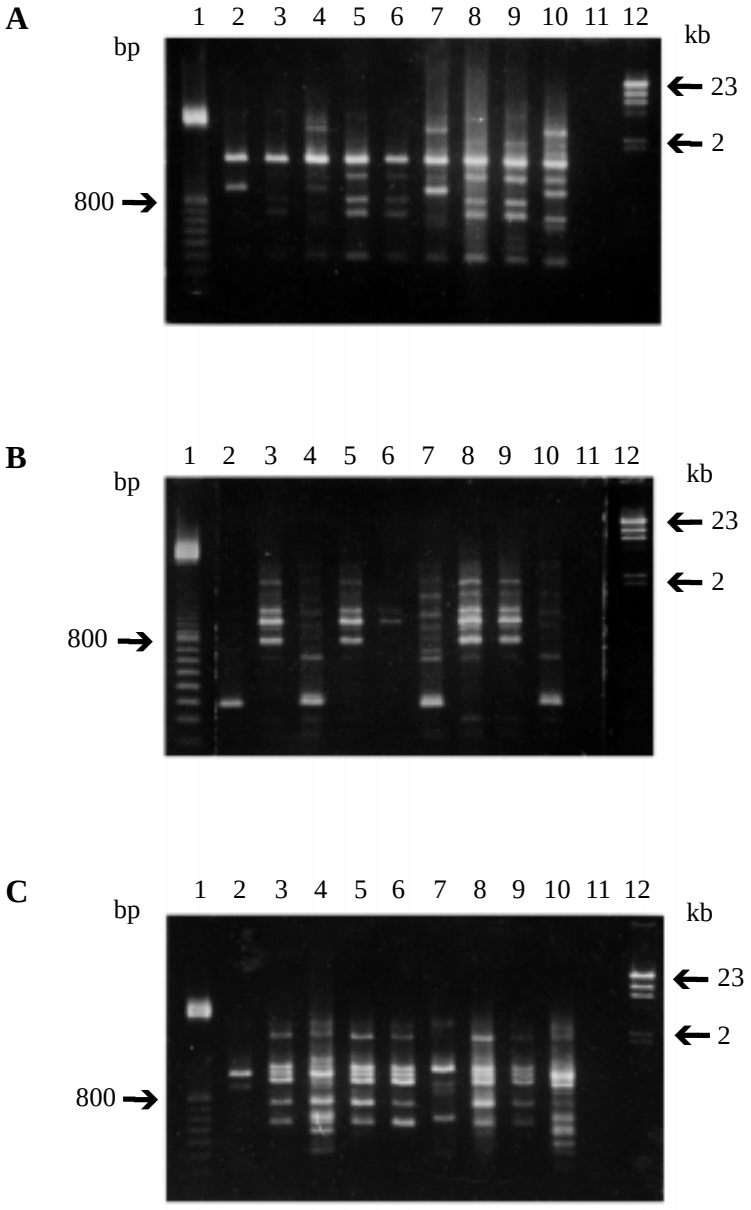

Figure 1A to 1C - RAPD-PCR profiles of amplification from DNA of serotype O:3 *Yersinia enterocolitica* pig strains. Lanes 2: Ye 41; 3: Ye 2; 5: Ye 1; 8: Ye 5, and 9: Ye 5A. Idem with serotypes O:13, O:6, O:5 and O:4 *Y. enterocolitica* human strains. Lanes 4: Ye184p; 6: Ye 43p; 7: Ye 102p, and 10: Ye 124p. Lanes 1: 100-bp DNA ladder; 11: negative control, and 12: λ *Hin*dIII. **1A.** Amplification with primer 786, **1B.** amplification with primer 795 and **1C.** amplification with primer 785.

and 10). However, each showed a distinct profile with primer 785, characterized by fewer bands in strain Ye 184p (O:13) than in Ye 124p (O:4). In spite of this, these two strains shared some common bands (Figure 1C, lanes 4 and 10). Two other profiles could be defined by combining the amplification profiles obtained for these two strains with the three primers: strain Ye 184p (O:13), placed in genotypic profile 4, and Ye 124p (O:4), in genotypic profile 3.

Serotype O:5 strain produced an amplification pattern completely different from those observed in the other strains. Each primer produced a distinct amplification pattern. This pattern was named genotypic profile 5 (Figure 1A, B and C, lane 7, primers 786, 795 and 785, respectively).

DISCUSSION

Y. enterocolitica is an emergent pathogen that is presently spreading throughout the entire world (Ichinohe *et al*., 1991; Iiuna *et al*., 1992; Miller, 1992). This species is proving to be very adaptable and seems to be changing, giving origin to strains with new characteristics, not detectable by traditional techniques of bio/sera/phage-typing.

We used three random primers to characterize, through RAPD-PCR, 16 *Y. enterocolitica* strains isolated from healthy pigs and four human strains collected in Rio de Janeiro. By the combination of the different amplification patterns observed, the 20 *Y. enterocolitica* strains analyzed were divided into five different genetic groups. The 16 serotype O:3 strains were subdivided into two different genotypes. The majority fit into genotypic profile 1. Only one strain (Ye 41) presented a different pattern (genotypic profile 2). This strain also displayed different behavior in relation to the 15 other strains. Surprisingly, this strain harbored several pathogenetic markers that were not found in the other 15 (Leal *et al*., 1997). On the other hand, one serotype O:6 human strain also fit the genotypic profile 1, confirming previous observations that human strains are also found in pigs. Each of the three other human strains produced a different amplification pattern and they were placed into three different genotypic profiles.

Amplification results by RAPD-PCR in the 20 human and pig *Y. enterocolitica* strains analyzed in this present work showed different intensities of amplified bands, suggesting that some DNA fragments had been produced at a higher rate during amplification. Alternatively, these fragments could be the result of multiple copies of identical DNA sequences along the genome, or the amplification of different sequences of DNA, producing fragments of the same size (Sayada *et al*., 1994). Variation in segment size of DNA amplified through RAPD, between strains of the same group, could be the result of deletions or insertions, modifying the size of the DNA segments (Welsh and McClelland, 1990). However, this does not explain why a strain from one serogroup produced an identical profile as that from a different serogroup.

Pathogenic *Y. enterocolitica* strains harbor a 70-kilobase (kb) plasmid named pYV, which is essential for virulence. However, this plasmid is unstable *in vitro* and can be lost through handling or storage in the laboratory. Of the 16 serotype O:3 *Y. enterocolitica* analyzed, six harbored pYV, and one strain harbored a small cryptic plasmid of about 8.6 kb. The others had no plasmids. Plasmid presence could affect the comparison of the amplification results of strains with and without plasmids, as plasmid DNA could serve as a template and generate additional bands in the profiles obtained through RAPD. In this case, comparison of strains with and without plasmids would not be possible. It appears, from our results, that plasmid content did not interfere with amplification results. Indeed, the presence or absence of plasmids did not seem to interfere with typing results with the primers studied, as the serotype O:3 strains with and without plasmids exhibited the same amplification patterns. Likewise, the presence of plasmids in strain Ye 41 did not cause a difference in its amplification profile. Indeed, the other O:3 serotype *Y. enterocolitica* strains with and without plasmids showed the same profile (genotypic profile 1). Despite the fact that strain Ye 5a has a small plasmid, about 8.6 kb, it also fits into genotypic profile 1 (Table I). We concluded that strains of the same serotype (O:3) and origin (pigs) can have genetic variations. New groups can be made illustrating the discriminatory power of RAPD. On the other hand, finding one O:6 human strain with a similar profile to that of O:3 serotype strains isolated from pigs confirms that RAPD is an efficient tool for regrouping *Y. enterocolitica* strains, and it could be a useful technique in epidemiological studies. It could also be used for tracking and following *Y. enterocolitica* dissemination and for evaluating modifications that could occur, with the emergence of strains possessing new characteristics.

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RESUMO

Foram utilizados três “primers” aleatórios para caracterizar pela técnica RAPD-PCR 16 cepas de *Yersinia enterocolitica* do sorotipo O:3, isoladas de suínos sadios do Rio de Janeiro. Pelos resultados dos padrões de amplificação, as 16 cepas dos suínos e as 4 cepas humanas usadas como referência (sorotipos O:4, O:5, O:6 e O:13) foram agrupadas em 5 perfis genotípicos. Quinze cepas de suínos apresentaram um padrão de amplificação idêntico (perfil genotípico 1) e somente uma apresentou um perfil de amplificação diferente (perfil genotípico 2). O mesmo padrão de amplificação do perfil genotípico 1 foi também observado em uma cepa humana do sorotipo O:6. As cepas humanas dos sorotipos O:4 e O:13 exibiram perfis de amplificação semelhantes com 2 “primers”, porém com o terceiro “primer” cada uma apresentou um perfil próprio. Essas duas cepas foram enquadradas, cada uma, em um tipo de perfil (perfis genotípicos 3 e 4, respectivamente). A cepa humana do sorotipo O:5 apresentou um perfil de amplificação com cada “primer” completamente diferente dos observados nas outras cepas (perfil genotípico 5). A presença ou ausência de plasmídios nas cepas estudadas não interferiu nos resultados das amplificações. Esses resultados mostram que dentro de um mesmo sorotipo podem existir modificações genéticas e que cepas de sorotipos diferentes apresentam o mesmo perfil de amplificação com alguns “primers”, comprovando que o RAPD-PCR é uma ferramenta eficaz para reagrupamento de cepas e poderá ser útil em estudos epidemiológicos para rastreamento de uma cepa e assim acompanhar a disseminação de *Y. enterocolitica*.

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