USE OF 16S-23S rDNA INTERGENIC SPACER TO DIFFERENTIATE *Enterococcus* ISOLATES FROM HUMAN AND NON-HUMAN SOURCES

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ABSTRACT

Many surface waters are contaminated by faecal pollution, which results in increased health risks to persons exposed to the water and degradation of recreational and drinking water quality. In urban systems, faecal pollution originates from an array of human and non-human sources. Non point sources of pollution which contribute to the presence of faecal bacteria in surface waters, have proven very difficult to accurately identify. Detection of *Enterococcus* spp. by standard microbiological methods provides no information as to the host source origin. However, identifying the major contributing sources of contamination is the critical component for an accurate assessment and successful control measures. In the present study the 16S-23S rDNA intergenic spacer region (ITS-PCR) was used as a tool to identify the origin of faecal pollution in surface water. Additionally, the RFLP ITS-PCR was used together with the Shannon-Weaver index (**H'**) in order to verify the diversity among isolates from human and non-human sources. A total of 109 *Enterococcus* spp. isolates from human and non-human sources were analyzed. Clustering analysis of the ITS-PCR fingerprints revealed distinct clusters of strains by host origin, however some overlapping with some of the isolates were observed. The strains in human host samples had lower diversity than the ones represented in contaminated surface waters. In conclusion, 16S-23S rDNA intergenic spacer region of *Enterococcus* spp. isolates have the potential to identify the source of faecal contamination.

Key words: *Enterococcus*, ITS-PCR, bacterial diversity, fecal pollution tracking

RESUMO

Uso da região intergênica 16S-23S do rDNA para diferenciação de isolados de *Enterococcus* de origem humana e não-humana

Muitos corpos d´água estão contaminados por poluição fecal, resultando em riscos de saúde às pessoas expostas à esta água e na degradação da qualidade da água para banho e potável. A poluição fecal em sistemas urbanos é proveniente de várias fontes não pontuais de origem humana e não-humana. A detecção de *Enterococcus* spp. através de métodos microbiológicos clássicos não fornece informação à respeito da origem desta poluição fecal. Entretanto, identificar a origem da contaminação é uma etapa crítica para uma avaliação precisa e ações de controle bem sucedidas. No presente estudo, o espaço intergênico 16S-23S do rDNA foi utilizado como ferramenta para identificação da origem da poluição fecal em amostras de água superficial. Adicionalmente, a RFLP PCR-ITS foi utilizada, juntamente com o índice de *Shannon-weaver* (**H'**), para verificar a diversidade entre os isolados de fontes humanas e não-humanas. Foram analisados 109 isolados de *Enterococcus* spp. de origem humana e não-humana. A análise de agrupamento dos perfis da PCR-ITS revelou a formação de grupos de acordo com a origem dos isolados, todavia, foi observada sobreposição de alguns isolados. Os isolados de origem humana apresentaram menor diversidade que os isolados de origem não-humana. Em conclusão, o espaço intergênico 16S-23S do rDNA de isolados de *Enterococcus* spp. possui potencial para ser utilizado na identificação da origem da contaminação fecal.

Palavras-chave: *Enterococcus,* ITS-PCR, diversidade bacteriana, diferenciação da poluição fecal.

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INTRODUCTION

Differentiation of sources of faecal contamination of surface waters is an important problem, especially for waters receiving mixed agricultural and human waste. In most contaminated waters, the presence of fecal indicator organisms can be demonstrated, but the nature of the source of the pollution is unknown. The contamination of natural waters with untreated faecal material may result in an increased risk of transmission of diseases to humans, who use those waters (SINTON, 1993a, p. 101). Sinton (1993b) said that the risk to humans is greater from human waste than from animal waste, therefore the knowledge of the source of pollution is an important factor in determining the degree of risk. This is especially relevant when recognizing that human feces can carry various human enteric pathogens, such as *Salmonella* spp., *Shigella* spp., *E. coli* (enterotoxigenic, enterohemorrhagic, enteroinvasive and enteropathogenic), *Giardia lamblia* and hepatitis A virus. Accordantly to Moe (1996), agricultural animals can also serve as vector for important pathogens including *Cryptosporidium parvum* and *E. coli* O157:H7.

Several attempts to develop methods to determine sources of faecal pollution have been made, and to date most have not proven useful. These include the ratio of faecal coliforms to faecal streptococci (SINTON, 1993b, p. 120), source-specific bacteriophages (TARTERA, 1989, p. 2700), use of yellow-pigmented enterococci (BAHIRATHAN, 1998, p. 1070) and patterns of antibiotic resistance in faecal coliform (PILLAI, 1997, p. 1672). Modern methods have also been evaluated to determine whether they can be used to differentiate sources of fecal contamination, such as ribotyping (CARSON, 2001; PARVEEN, 1999), pulsed-field gel electrophoresis (PARVEEN, 2001, p. 379) and PCR analysis of the 16S-23S rDNA intergenic spacer region (ITS) (BUCHAN, 2001, p. 313). More recently, Poeta (2005) studied the antibiotic resistance genes and virulence factors in faecal enterococci of wild animal and observed significant differences between an antibiotic-free ecosystem (such as that of wild animals) and faecal enterococci of humans or food animals, in which higher percentage of antibiotic resistance were detected. This could be another methodology to differentiate the origin of faecal pollution.

Despite efforts to minimize faecal input into waterways, this kind of pollution continues to be a problem due to an inability to reliably identify nonpoint sources, which may include inefficient sewage treatment plants, agricultural runoff, or wildlife. Knowing the source of the contamination is crucial to effective resource management and, ultimately, solution of the problem. In this context, the objective of the present study was to find a candidate source-specific *Enterococcus* spp. fingerprint as a potential genotypic marker to differentiate human and non-human pollution. The 16S-23S rDNA intergenic spacer region (ITS-PCR) was evaluated as a tool to identify the origin of faecal pollution in water. Additionally, we used the RFLP ITS-PCR together with *Shannon-Weaver* index (**H'**) to verify the diversity between isolates from human and non-human sources.

MATERIALS AND METHODS

Bacterial Isolates

A total of 109 strains were studied, including 40 *Enterococcs faecalis*, 40 *Enterococcus faecium,* 15 *Enterococcus mundtii* and 14 *Enterococcus hirae.* The 54 human isolates were obtained from feces from Hospital São Vicente de Paulo (Passo Fundo, RS, Brazil). The 55 water isolates were obtained from Arroio Feijó, which is an important tributary of the Gravataí river, a major constituent of Guaíba Lake estuary (Porto Alegre, RS, Brazil). Strains were tested for their phenotypic characteristics with conventional biochemical tests as recommended by Facklam and Collins (1989). Briefly, the following tests were made: Gram stain, pyrrolodonil arylamidase, catalase, growth in broth containing 6,5% NaCl, growth in broth at 45°C, deamination of arginine, motility, production of yellow pigment, pyruvate and carbohydrate fermentation (manitol, arabinose and raffinose). Further phenotypic identification of the isolates until species level was done using the API Rapid ID 32 Strep system (BioMerieux, Marcy L'Etoile, France).

DNA extraction

The genomic DNA of isolates was obtained via enzyme lysis of bacterial cells cultivated in 10 mL BHI broth (Difco) for 18 h at 37°C, under agitation at 100 rpm. The extraction of chromosomal DNA was carried out as described in Givers (2001). The amount of DNA was quantified and assessed for impurities via spectrophotometry using a Uv-Vis Metrolab spectrophotometer.

ITS-PCR

DNA fragments of the 16S-23S rDNA intergenic region were amplified using two primers:

5'-CCCGAAGTCGGTGARGTAAC-3' and
5'-CCTTCATCGGCTCCTAGTGC-3'.

The primers (Invitrogen Life Technologies) were designed based on the alignment of 16S and 23S *Enterococcus* rDNA sequences as accessed in the GenBank, with the following accession numbers: AJ295311, AJ420806, AJ295303, Y18358, AJ295304, AJ420801, AJ420800, X79341, AF477496, AJ295306. The amplification reactions were carried out in a thermal cycler (Mastercycler personal, Eppendorf) with: 1 × PCR buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTP, 1 µM of each primer, 1 U **Taq** DNA polymerase (Invitrogen), and 200-400 ng bacterial DNA to a final volume of 35 µL. The amplification conditions were as follows: an initial denaturation at 94°C (2 min), 30 cycles at 94°C (1 min), at 51°C (1 min), at 72°C (1 min), and a final elongation step at 72°C (10 min). The PCR products were electrophoresed in non-denaturating 8% polyacrylamide gel and stained with ethidium bromide. The fragment size was used to produce a binary code matrix, with 0 representing the absence and 1 the presence of a given fragment. The strain *E. faecalis* ATCC 29212 was used as positive control.

RFLP ITS-PCR

An 10 µL aliquot of the amplicon solution was cleaved with 1 U of the endonuclease restriction enzyme **Sau**3AI (Amersham Biosciences) for 2 h at 37°C following the manufacturer's instructions. After incubation, the DNA was electrophoresed in polyacrylamide non-denaturating gel 8% and stained with ethidium bromide. The fragment size data obtained were again used to produce a binary code matrix, with 0 representing the absence and 1 the presence of a given band. The strain *E. faecalis* ATCC 29212 was used as positive control in all restriction reaction. Sixty isolates were randomly chosen to test the reproducibility of the band patterns.

Data analysis

The similarity matrices were constructed using the Dice coefficient and dendrograms were constructed using the UPGMA (Unweighted Pair Group Method with Arithmatic Mean) method. The data analyses were carried out using the SPSS software (version 11.0). The Shannon-Weaver similarity index was calculated using the formula $H = C/N(N \times \log N - \Sigma n_i \times \log n)$, in which **n** is the number of isolates with the same **Sau**3AI profile, **N** the total number of isolates, and **C** a constant (2.3).

RESULTS

ITS-PCR

The apparent genetic diversity of the 16S-23S rDNA intergenic spacer was used to characterize the genome of different *Enterococcus* species from human and non-human sources. Ten fragments with sizes ranging between 330 to 650 bp were obtained (Figure 1). Thirteen ITS-PCR profiles were found among the different species from human samples, and eight ITS-PCR profiles from non-human samples. All the isolates presented two or three major fragments that were analyzed in the present study. The fragments of 650 bp and 410 bp were exclusively observed among human isolates. The high molecular size bands, running near the top of the gel, occurred in an inconsistent manner, and hence, they were not particularly useful in *Enterococcus* genus characterization.

Clustering analysis of the ITS-PCR fingerprints revealed distinct clusters of strains by host origin. At least three large ones were observed with a similarity index of 70% or more (Figure 2). The Groups I and III were formed by the majority of human origin isolates. Group I had 50% of human origin isolates and Group III had 33.3%. Group II was formed mainly by isolates from non-human sources, 67.3% of the non-human isolates were assigned to these group. Other smaller clusters were also observed, formed mainly by isolates from one source.

RFLP ITS-PCR

Enterococcus spp. isolates from human source of faecal pollution were characterized by RFLP ITS-PCR and compared with *Enterococcus* spp. isolates from non-human source. The cleavage of the intergenic spacer using **Sau**3AI was carried out to asses the variability within the amplified nucleotide sequence and to provide additional information about diversity of the isolates from different origins. The cleavage generated 26 fragments with sizes ranging from 85 to 515 bp. The isolates generated 4 to 9 cleavage fragments, with an average of 6 bands per isolate (Figure 3). The diversity obtained using Shannon-Weaver

index (**H'**) was 3.0 for non-human and 2.6, for human samples.

DISCUSSION

Faecal pollution in urban systems is expected to originate from an array of human and non-human sources. Detection of *Enterococcus* spp. by standard microbiological methods provides no information about the host source. However, identifying the major contributing sources of contamination is a critical component for accurate assessment and successful control measures and to a better understanding of the potential health risk. By identifying the presence of human faecal pollution, and therefore the possible presence of human enteric pathogens, allows further resolution of the source of this risk. Source identification approaches have included methods based on the phenotypic and genotypic characteristics of the indicator organism, including evaluating percentages of faecal streptococci (SINTON, 1993b, p. 120), antibiotic resistance (HARWOOD, 2000; BLANCH, 2005) and DNA fingerprinting of faecal indicator bacteria (GUAN, 2002, p. 2694).

The aim of the present study was to determine how environmental strains could be compared and differentiated from human host source strains. The results of RFLP ITS-PCR and **Shannon-Weaver** diversity index showed that strains from human host samples had lower diversity than the ones from contaminated surface waters. This was an expected result and it can be explained because *Enterococcus* spp. are preferable maintained in human host. Parveen (1999) found, by ribotyping, that human *E. coli* isolates showed much less diversity than non human isolates. McLellan (2004) demonstrated that a single animal generally harbours one predominant strain. The genetic diversity of the contaminated surface water may result from environmental factors, such as the disposal of sewage, the presence of domestic animals and livestock and erosion of river banks. Overall, the high diversity in the surface water population demonstrates that diverse contamination sources have been introduced into the environment and that nonpoint pollution sources are subject to significant natural variability due to many environmental processes.

The use of genetic traits for faecal pollution source tracking is based on the hypothesis that there is a host-specific structure within *E. coli* population (MCLELLAN, 2004, p. 4663). Many studies that have described the genotypic characteristics of *E. coli* population have focused on subsets of pathogenic strains or have employed multilocus enzyme electrophoresis to assess clonality within these populations (SOUZA, 1999, p. 3380). The results of studies using rep-PCR and ribotyping analysis of *E. coli* for source tracking have been compared. They showed that the association between *E. coli* isolates and their source-host occurred in a consistent manner in different geographical regions (PARVEE, 1999; MCLELLAN, 2004). Blanch (2005) tried to determine the origin of faecal pollution in surface water, using different methods, like genotypes of F-specific RNA bacteriophages, bacteriophages infecting *Bacteroides fragilis*, phenotypes of faecal coliforms and enterococci, and sterols. The results of this study indicate that no single parameter alone is able to discriminate the sources, human or non-human, of faecal pollution, but that a 'basket' of 4 or 5 parameters, which includes one of the present faecal indicators, will do so. A new tool is necessary to the number of parameters in the 'basket'.

ITS-PCR was used to discriminate human and non-human *Enterococcus* spp. isolates. Clustering analysis showed that some of the isolates grouped according to their host origins and that this analysis was useful in separating isolates into human and non-human source groups. Similarly, Hagedorn (1999) were able to classify faecal streptococci isolates into groups by using antibiotic resistance patterns and some overlapping occurred between the human and non-human clusters. So, this study indicates that ITS-PCR profiles of *Enterococcus* spp. can be used as another parameter to differentiate human and non-human faecal pollution. Additionally, the RFLP ITS-PCR may be used to asses the variability between isolates from different sources. However, more statistical analyses would be needed to test this potential tool.

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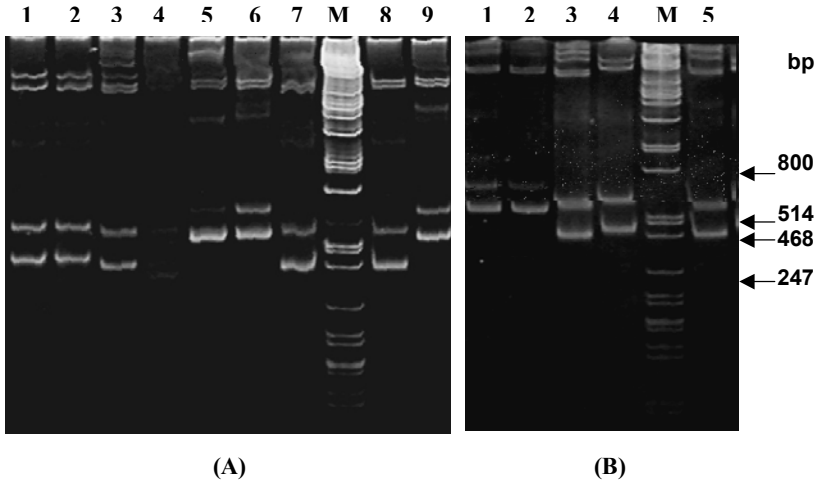

Fig. 1. ITS-PCR profiles observed among *Enterococcus* spp. from different sources, in non-denaturating polyaclylamide gel 8%. **(A)** Non-human isolates: 1 - *E. faecalis* ATCC 29212; 2 - *E. faecalis* A1; 3 - *E. faecalis* A2; 4 - *E. faecalis* A3; 5 - *E. faecalis* A4; 6 - *E. faecalis* A5; 7 - *E. faecalis* A6; M, molecular marker λ(*Pst*I); 8 - *E. faecalis* A7; 9 - *E. faecalis* A8. **(B)** Human isolates: 1 - *E. faecalis* H22; 2 - *E. faecalis* H24; 3 - *E. faecalis* H26; 4 - *E. faecalis* H29; M, molecular marker λ(*Pst*I); 5 - *E. faecalis* H33. The letter "A" indicates non-human isolates and the letter "H" indicates human isolates.

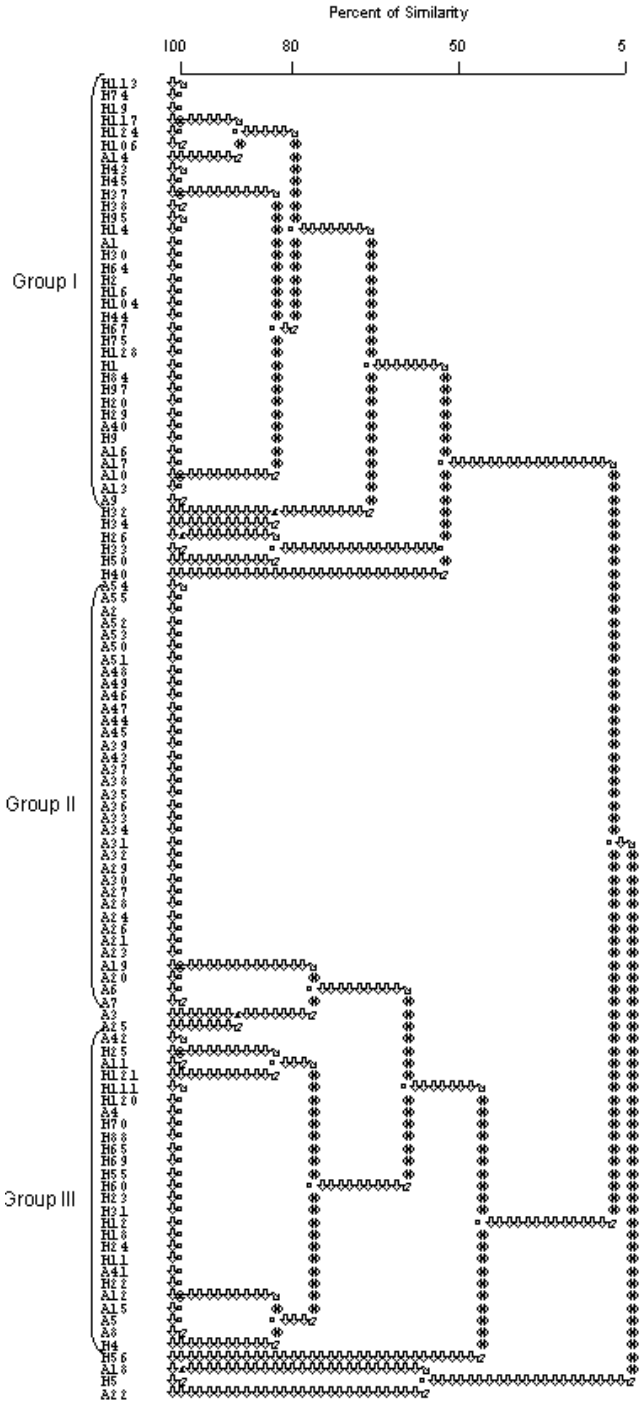

Fig. 2. Dendrogram constructed from the ITS-PCR by UPGMA method and using Dice coefficient. The letter "A" indicates non-human isolates and the letter "H" indicates human isolates. The Groups I, II and III were constructed with 70% of similarity.

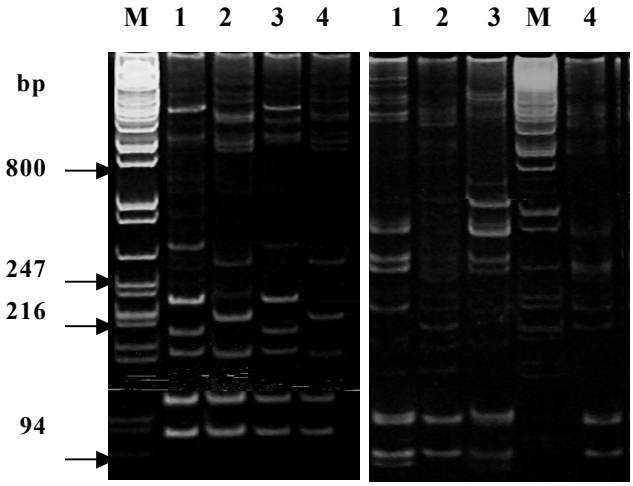

Fig. 3. RFLP ITS-PCR profiles observed among *Enterococcus* spp. from different sources in non-denaturating polyaclylamide gel 8%. **(A)** Non-human isolates: M, molecular marker λ(*Pst*I); 1 - *E. faecalis* A1 **Sau**3AI; 2 - *E. faecalis* A2 **Sau**3AI; 3 - *E. faecalis* A16 *Sau*3AI; 4 - *E. faecium* A33 **Sau**3AI. **(B)** Human isolates: 1 - *E. mundtii* H2 **Sau**3AI; 2 - *E. mundtii* H4 *Sau*3AI; 3 - *E. mundtii* H5 **Sau**3AI; M, molecular marker λ(*Pst*I); 4 - *E. mundtii* H16 **Sau**3AI. The letter "A" indicates non-human isolates and the letter "H" indicates human isolates.