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ARTIGO ORIGINAL

Genotypic analysis of *Helicobacter pylori* by Multiple-Locus Variable-Number Tandem-Repeats method in southern Brazil

Análise genotípica do Helicobacter pylori pelo método Multiple-Locus Variable-Number Tandem-Repeats no sul do Brasil

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DESCRITORES
Diversidade genética; Helicobacter pylori; Variable Number of Tandem Repeats.

KEYWORDS
Genetic diversity; Helicobacter pylori; Variable Number of Tandem Repeats.

RESUMO

Justificativa e Objetivos: Apesar de ser utilizado como um método de genotipagem para diferentes microrganismos, poucos estudos relatam a utilização de Multiple-*Locus* Variable Number of Tandem Repeats Analysis (MLVA) para análise da diversidade clonal do *Helicobacter pylori*. O objetivo deste estudo foi determinar a variabilidade genética de cepas de *H. pylori* pelo MLVA no sul do Brasil. **Métodos:** 95 amostras de DNA de *H. pylori* foram obtidas a partir de biópsias gástricas de pacientes *H. pylori*-positivos provenientes de duas cidades do sul do Brasil e a diversidade genética das cepas foi avaliada pelo método MLVA utilizando eletroforese em gel de agarose. Para a seleção dos *loci* a serem analisados neste estudo, foi realizada uma análise *in silico* de 12 *loci* previamente descritos na literatura. **Resultados:** A partir da análise *in silico,* apenas quatro *loci* foram considerados viáveis para a análise genotípica das cepas, resultando em 90 cepas distribuídas em oito grupos diferentes e cinco cepas órfãs. **Conclusões:** Apesar de o método MLVA permitir fazer inferências acerca da diversidade genética de uma população, nossos resultados mostraram que os métodos de genotipagem do *H. pylori* devem ser criticamente avaliados antes de serem utilizados nessa região do Brasil.

ABSTRACT

Background and Objectives: Despite its use as a genotyping method for different microorganisms, few studies have reported the use of Multiple-Locus Variable Number of Tandem Repeats Analysis (MLVA) for the analysis of the clonal diversity of *Helicobacter pylori*. The aim of this study was to determine the genetic variability of *H. pylori* strains by MLVA in southern Brazil. **Methods:** 95 *H. pylori* DNA samples were obtained from the gastric biopsies of *H. pylori*-positive patients from two cities in southern Brazil and their clonal analysis was evaluated by MLVA method using electrophoresis in agarose gel. For selection of loci to be analyzed in this study, an in silico analysis of 12 loci previously described in the literature was performed. **Results:** From the in silico analysis, only four loci were viable for the genotypic analysis of these *H. pylori* strains, resulting in 90 strains distributed in eight different groups and five orphan strains. **Conclusion:** Despite MLVA method allow make inferences about the genotypic diversity of a population, our results showed that *H. pylori* genotyping methods should be critically evaluated prior to their use in this region of Brazil.

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INTRODUCTION

Genotyping is an important tool in epidemiology studies, enabling the identification of predominant clones and geospatial distribution.[1] *Helicobacter pylori* have been correlated to a variety of gastric disorders such as chronic gastritis, peptic ulcer and gastric cancer.[2,3] A comparison of the genome sequence of strains of *H. pylori* indicates that there is significant genetic variation, mainly due to the high rates of mutation and genetic recombination in this microorganism.[2,4] This genetic diversity allows for its adaptation to several unfavorable host conditions.[5,6] Furthermore, genotyping studies have demonstrated a relationship among microorganisms' and their host's genetics, permitting the inference of a potential co--evolution.[7] Although Multiple-*Locus* Variable-Number Tandem-Repeats Analysis (MLVA) is largely used for genotyping, including prokaryotes and eukaryotes, few studies have standardized MLVA for *H. pylori*.[8-10] The aim of this study was to evaluate 12 previously described *loci* for the genotyping of *H. pylori* using gastric biopsies of *H. pylori*-positive patients in southern Brazil.[9]

METHODS

95 gastric biopsy samples from *H. pylori*-positive patients from the metropolitan region of Pelotas and Rio Grande cities, situated in southern Brazil, were collected between May 2011 and April 2012 and stored in the sample bank of the Núcleo de Pesquisa em Microbiologia Médica (NUPEMM), Faculty of Medicine – Federal University of Rio Grande (FURG), Rio Grande do Sul, Brazil. This study was approved by the Ethics Committee in the Research of Health Area (FURG - 23116.001044/2011-16), and informed consent was obtained from all patients.

Extraction of total DNA was performed from the biopsies of the gastric antrum and body using DNAzol® Reagent (Invitrogen, USA) and 10 µg/µl of proteinase K (Promega, USA) as described by Fonseca et al. (2010).[11] The integrity of the extracted DNA was assessed by the amplification of a specific fragment of 110 base pairs (bp) from human β-globin using primers and methodology previously described by Saiki *et al*. (1985) while the presence of *H. pylori* in the biopsy was confirmed by the detection of *ureA* and *glmM* genes using PCR as previously described by Rota et al. (2001) and Espinoza et al. (2011), respectively.[12-14] On the basis of a previous study performed by Guo et al. (2011), we used the Vector NTI Suite 8® software to perform an *in silico* analysis of the 12 *loci* (VNTR 180, 263, 557, 607, 614, 1801, 2181, 2457, 2576, 5062, 5282 and 5581) in order to identify the annealing sites of the primers in the whole genomes of *H. pylori* strains 26695 (AE000511.1), J99 (AE001439.1) and HPAG1 (CP000241.1), which are available in the GenBank database, National Center of Biotechnology Information (NCBI), (http://www.ncbi.nlm.nih.gov/Genbank/index.html). The analysis of selected *loci* was performed by PCR with a final volume of 25µl containing 10 ng of DNA, 0.5 mM of each primer, 1 unit of Taq DNA polymerase, 200 mM of dNTPs, and 10 × PCR buffer (500 mM KCl, 100 mM Tris-HCl (pH 8.3) 25 mM $MgCl_2$) (LudwigBiotec®), using the primers and conditions described by Guo et al. (2011).[9]

The fragments amplified by PCR were analyzed by electrophoresis, in 3% agarose gel. After staining with ethidium bromide, was carried out the visualization and subsequent differentiation of the size of amplified fragments, in fluorescence under UV radiation emission. It was used 25bp (Invitrogen®), 50bp and 100bp DNA ladder (Ludwig Biotec®) for to define the size of the PCR products. The discriminatory power of the technique and of each *locus* was determined by calculating the diversity index Hunter-Gaston (HGDI).[15]

RESULTS

By the *in silico* analyses, the primers described for five *loci* amplification (VNTR 614, 607, 2457, 2576, and 5581) presented 100% similarity with more than one genomic region, which could generate nonspecific amplicons. Among the other seven VNTR (180, 263, 557, 1801, 2181, 5062 and 5282), the VNTR 2181 *locus* was amplified in only 61% of the samples. To further understand this result, a new *in silico* analysis using other strains of *H. pylori* (G27, F57, B8, HUP-B14 and Shi470), which also have sequenced genomes, was performed in addition to those already referenced in this study (data not shown). For HUP-B14 and Shi470 strains, we observed that the genomic regions in which the primer that was used to identify the VNTR 2181 *locus* annealing site was not present in the genome. This result may explain why this VNTR had amplified only 58 of the 95 samples analyzed. To determine the discriminatory power between *H. pylori* strains, samples that showed no amplification were identified as 0 to differentiate these samples from those that were amplified. Furthermore, although VNTR 1801, 5282 and 5062 showed total identification with the respective *loci*, they did not present a relationship among the number of alleles and amplicon size, and they were excluded from the genotypic analysis step. From the analyses performed with the four *loci*, the samples showed thirteen different patterns: 90 strains were distributed in eight different groups, and only five strains had an orphan pattern. Furthermore, strains obtained from Pelotas and Rio Grande shared similar genotypes.

Considering the variability found for four *loci*, the MLVA method showed a discriminatory power of 0.863 according to the HGDI.[15] The analyzed *loci* exhibited allelic diversity (AD) ranging from zero to 0.67 (Table 1). We found that the VNTR 557 *locus* had the lowest allelic diversity (AD = 0), while the VNTR 263 *locus* had the higher (AD = 0.67) (Table 1).

Table 1. Allelic diversity of each *locus*.

locus	Allelic diversity
VNTR 180	0.11
VNTR 263	0.67
VNTR 557	0
VNTR 2181	0.47

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DISCUSSION

The low diversity clonal observed in our study should be consider with caution because only four VNTR were used to perform the genotyping, which is an important limitation in the discriminatory power of the method. Interestingly, the main result of our study was the low useful VNTR, which included only 1/3 of those *loci* proposed by Guo et al. (2011).[9] This study is the first to use the MLVA method to investigate the genetic diversity of *H. pylori* in Latin American and the *H. pylori* genotyping by the MLVA method, based only on these four *loci*, using agarose gel electrophoresis, showed similar results to those originally found by Guo et al. (2011) about the discriminatory power of each *loci*.[9] Therefore, despite allow make inferences about the genotypic diversity of a population, the MLVA method has limitations. Although one could consider the genome plasticity of *H. pylori*, it is important to highlight that some primers and/or VNTR did not align with more frequent strains sequenced. In conclusion, our results showed that *H. pylori* genotyping methods should be critically evaluated prior to their use in our region.

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