

Improvement of Multiple-Locus VNTR Analysis Typing Scheme for *Helicobacter pylori*

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Authors' contributions

This work was carried out in collaboration between all authors. Author VMS designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Authors RVP and ASV managed the analyses of the study. Author VMS managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Aims: To improve a multiple-locus variable-number tandem-repeat (VNTR) analysis (MLVA) assay for *Helicobacter pylori* typing.

Materials and Methods: Polymorphic VNTRs were searched by Gene Expert. The distribution and polymorphism of each VNTR locus were analyzed in 18 *H. pylori* genomes from the NCBI genome database by BLAST and were compared with a collection of 15 clinical *H. pylori* strains. The MLVA assay was compared with MLST-typing for discriminating *H. pylori* isolates.

Results: Twelve VNTR loci were identified by bioinformatic screening of *H. pylori* genomes, and five of them were highly polymorphic. Therefore, an MLVA assay composed of five VNTR loci was developed with greatest discriminatory power.

Conclusion: MLVA typing is a faster and more standardized method for studying the genetic relatedness of *H. pylori* isolates. At preliminary stage it is sufficient to use only 3 VNTR loci for the differentiation of *H. pylori* strains.

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1. INTRODUCTION

Stomach infection with *Helicobacter pylori* (*H. pylori*) is one of the most common infectious disease of humans. *H. pylori* infection may cause gastric and duodenal ulcer disease, the development of gastric mucosal atrophy, gastric carcinoma. *H. pylori* infections cause very high morbidity and mortality and are of particular concern in developing countries, where *H. pylori* prevalence as high as 90% have been reported. The population of *H. pylori* shows a high genomic variability among isolates. Few molecular typing tools have been described to reflect genetic relatedness in *H. pylori* isolates. These include pulsed-field gel electrophoresis [1], random fragment length polymorphism [2], randomly amplified polymorphic DNA [3,4], amplified fragment length polymorphism [5,6], and PCR-based genotyping of repetitive sequences, namely, repetitive extragenic palindromes [7,8] and enterobacterial repetitive intergenic consensus elements [9]. The multi-locus sequencing-typing (MLST) method of the "housekeeping" genes was used as the basis for the use of *H. pylori* as a marker of human migration [10]. The practical use of these approaches is limited, both by the complexity of methodological nature, and by problems with reproducibility and evaluation of results. At the same time, one of the universal methods of molecular genotyping is the determination of the multiplicity of the variable tandem repeats of a locus (VNTR-analysis, variable number tandem repeats analysis) on the chromosome of the host. Its application is extremely important for the development of research in the field of taxonomy, evolutionary genetics and molecular epidemiology. The method has proven itself in the typing of various microorganisms, including plague, anthrax, tuberculosis, tularemia and many others. VNTR-analysis Not so far, two different methods of MLVA typing of *H. pylori* were described [11,12]. Aim of this study was to improve MLVA assay for *H. pylori* typing suggested earlier [13].

2. MATERIALS AND METHODS

DNA isolation, VNTR primer design and PCR conditions were described earlier [13], with one exception, annealing at 55°C for locus HpD*. Each PCR product (5 µL) was resolved by 5-8% polyacrylamide gel electrophoresis. Allelic sizes were estimated using a pBlue Script DNA /

MspI (MBI Fermentas, Vilnius, Lithuania) as a size marker. Gels were visualised using UV transillumination and the images captured using the ChemiDoc System (BioRad). The polymorphism index for individual or combined VNTR loci was calculated using the Hunter-Gaston diversity index (HGDI) [14]. All calculations were done by the free online tool Comparing Partitions (<https://www.comparingpartitions.info>). A dendrogram was created with the Mega4 software by using the unweighted-pair group method using average linkages (UPGMA) (<https://www.megasoftware.net/mega4/mega.htm>).

3. RESULTS AND DISCUSSION

As mentioned above, Chinese scientists have proposed their method for multi - locus VNTR-typing of *H. pylori* strains based on 12 VNTR-loci with repeat length from 12 to 138 bp and detection of fragments in agarose gel [11]. Later, when trying to use this method for typing Brazilian strains, it was shown that *in silico* analysis four loci out of 12 have a significant drawback: DNA of many *H. pylori* strains from the NCBI database has two or more positions for hybridization with the corresponding primers [15]. Another locus has insufficient incidence among Brazilian strains (60%), and three loci have no correlation between the number of repeats and the size of the amplified fragment. Thus, to characterize the population of Brazilian strains of *H. pylori* only 4 loci out of 12 were used, and one of them was monomorphic for this population. As a result, only 13 genotypes were found in 90 studied strains [15]. The method we offer allowed to reveal 48 strains from Russian population with 48 genotypes, which means 100% discrimination ability of the method [13]. However, in the study of *H. pylori* clinical isolates by HpF locus, sometimes we encountered two or more fragments in a single sample and defined this as the presence of a mixed culture with the exclusion of the sample from further consideration. With the increasing number of DNA sequences of *H. pylori* in the database NCBI, strains appeared with two or more positions for priming the primers to the HpF locus. Accordingly, it was decided to replace the HpF locus with a new one – HpG, devoid of this disadvantage. Detailed characteristics of this locus are given in Table 1 and 2. Also, to increase the accuracy of determining the size of

fragments at the HpD locus, new primers are proposed, reducing the size of amplified fragments by about 100 bp (Table 1).

To evaluate the changes made, we selected 15 out of 48 previously studied regional strains and experimentally determined their MLVA types (MTs) by five VNTR loci. Then, we determined *in silico* MTs of 18 strains from the NCBI database with known geographical origin, earlier detected by MLST typing [16]. Comparative dendrograms were generated using MLVA types of 15 regional strains *H. pylori* and 18 strains from the NCBI database. (Fig.1 and Fig. 2).

Four VNTR loci typing scheme (HpA, HpD, HpE and HpF) divided the greatest part of *H.pylori* strains into three large clusters (A, B, C) and allowed to detect 33 individual genotypes at 33 strains (Fig. 1).When comparing Figs. 1 and 2 it is obvious that the replacement of the HpF locus on HpG reduced number of clusters from 3 to 2, changed the location of some strains, but did not noticeable affect the discriminating ability of the method (32 individual genotypes at 33 strains) (Fig.2). The presented results indicate that the

proposed method allows not only to differentiate regional *H.pylori* strains, but *in silico* compare an experimental data with the NCBI database.

Due to the hypervariable locus HpD, at the preliminary stage of the study, it is possible to reduce the number of loci studied to three, which practically does not reduce the discrimination ability of the method for a given sample of strains (Fig. 3 and 4).

This variant allowed to detect 32 individual genotypes at 33 strains (strains RO90 and RO92 have the same genotype (Fig.3).

This variant allowed to detect 33 individual genotypes at 33 strains (Fig.4).

The first three variants (Figs. 1-3) allows for a more clear grouping of strains according to geographic features and the assumption that most regional strains belong to a subpopulation of, hpEurope because their greatest part was located in the cluster A with the greatest part of the hpEurope strains, which agrees with the previously obtained data [17].

Table 1. Oligonucleotide primers of VNTRs analyzed in this study

Primer name	Direction	Sequence (5'-3')
HpA	Forward	TGGGGAACAAAACGAAGTTAAAAGG
	Reverse	TCTTATTCGCCCATTTTCCAACG
HpD	Forward	CGTTTCTATCAACGCCCTATTTTC
	Reverse	AAAAGGCCGAAATACTGGGATAGCTT
HpE	Forward	ACCGCTCAAATCCCACCAACC
	Reverse	ATGATGCTATAATCACTAATCACT
HpF	Forward	GGTAATATTCATATTGCTTTTTGCGCG
	Reverse	AGATCGTTAAGATTTTGGACGCTTTC
HpG	Forward	CCAAAATAGCTTGGTTGAACAATCC
	Reverse	TGGGGGTGGGTATGTTATACATTTT
HpD*	Forward	TTGGATATTCAATCTATTTTGGTATAATAG
	Reverse	CTGGGATAGCTTATAGCGAGTTAGC

*newprimers for HpD

Table 2. HGDI and characters for individual VNTR locus

Locus	Repeat	Repeat Size (bp)	N*	HGDI
HpA	TTTTGATGA	9	9	0.836
HpD	AAATACAT	8	23	0.979
HpE	TAATCAC	7	13	0.866
HpF	AATTCTGTGTTT	12	6	0.727
HpG	GCCAAGTA	8	7	0.795

*number of different repeats

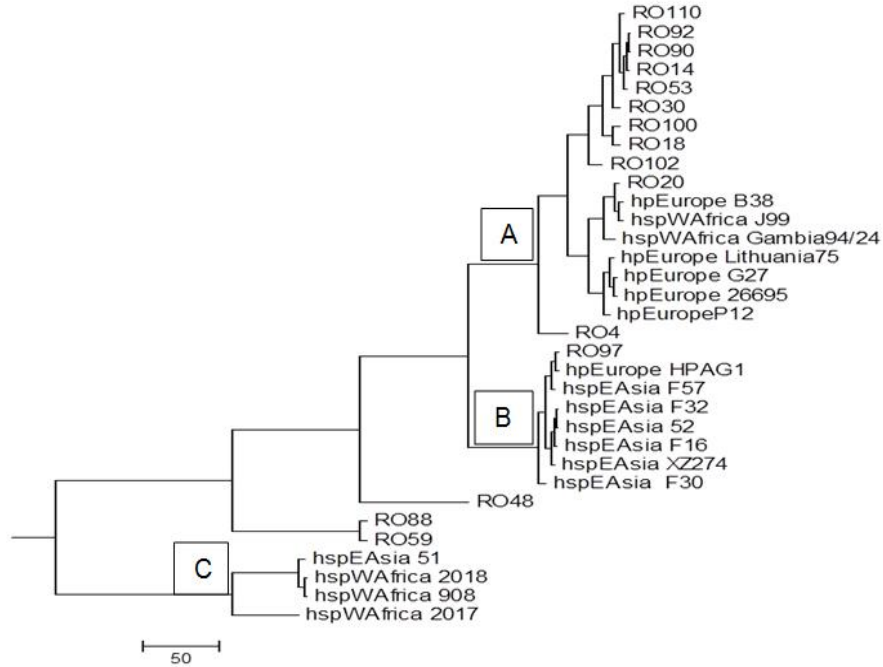


Fig. 1. Dendrogram of genetic relationships of *H. pylori* strains at the loci of HpA, HpD, HpE, HpF

RO – Regional strain

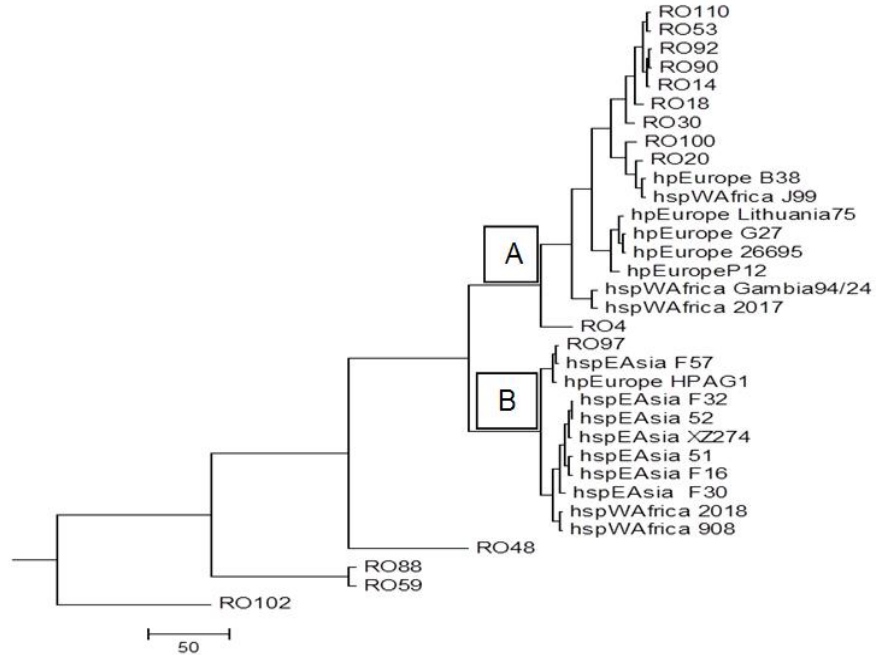


Fig. 2. Dendrogram of genetic relationships of *H. pylori* strains at the loci of HpA, HpD, HpE, HpG

RO – Regional strains

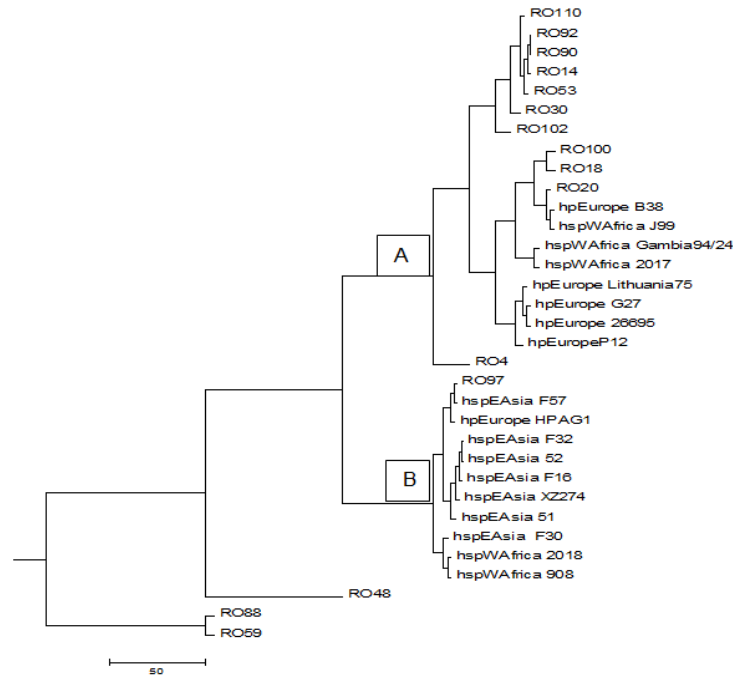


Fig. 3. Dendrogram of genetic relationships of 33 strains of *H. pylori* at the loci of HpA, HpD and HpE
 RO – Regional strains

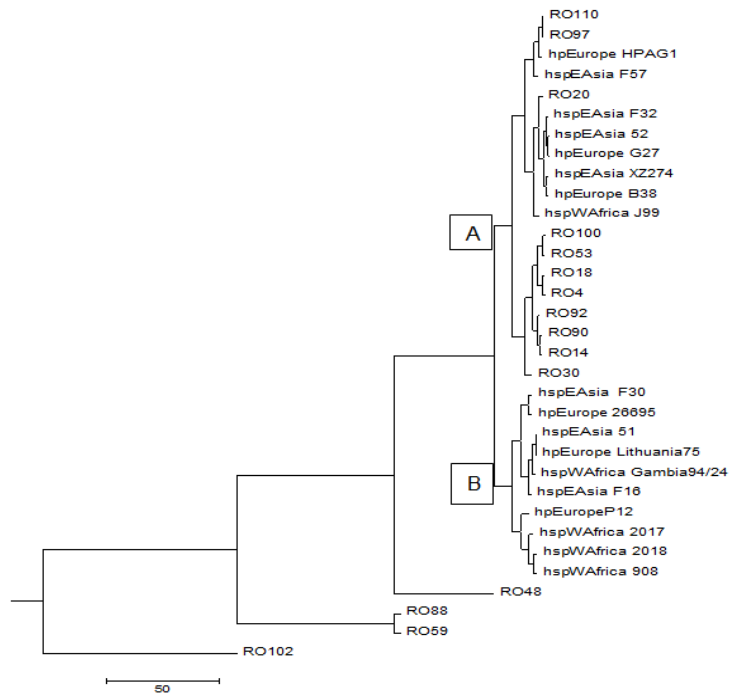


Fig. 4. Dendrogram of genetic relationships of 33 strains of *H. pylori* at the loci of HpA, HpD and HpG
 RO – Regional strains

4. CONCLUSION

We propose the simple, low cost method for differentiation of *H. pylori* isolates and further comparing with the NCBI database. MLVA typing is a faster and more standardized method for studying the genetic relatedness and is available for every standard PCR laboratory. At preliminary stage it is sufficient to use only 3 VNTR loci for the differentiation of *H. pylori* strains.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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