Stability of randomly amplified polymorphic DNA fingerprinting in genotyping clinical isolates of Helicobacter pylori

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Abstract

AIM: H pylori genomes are highly diversified. This project was designed to genotype H pylori isolates by the polymerase chain reaction (PCR)-based randomly amplified polymorphic DNA (RAPD) fingerprinting technique and to verify its stability by Southern blotting and DNA sequencing.

METHODS: Clinical isolates of H pylori were cultured from gastric antra and cardia of 73 individuals, and genomic DNA was prepared for each isolate. RAPD was carried out under optimized conditions. 23S rDNA was regarded as an internal control, and a 361 bp rDNA fragment (RDF) was used as a probe to screen the RAPD products by Southern blotting. Ten RDFs from different clinical isolates and the flanking regions (both upstream and downstream) of four RDFs were amplified and sequenced.

RESULTS: H pylori isolates from different individuals had different RAPD profiles, but the profiles for isolates cultured from different gastric sites of a given individual were identical in all but one case. Isolates from 27 individuals were RDF positive by Southern blotting. Sequences of the RDFs and their flanking regions were almost the same between the RDF positive and negative isolates as determined by Southern blotting. There was no binding site for random PCR primer inside the sequences.

CONCLUSION: RAPD is very useful in genotyping H pylori grossly on a large scale. However, it seems unstable in amplification of low yield fragments, especially those that do not appear as visible bands on the agarose gel stained with EB, since the primer is partially matched to the template.

H pylori culture

Clinical isolates of H pylori cultured from gastric antra and cardia of 73 patients, 12 with gastric ulcer (GU), 18 with duodenum ulcer (DU), 8 with both GU and DU, and 25 with non-ulcer dyspepsia (NUD), were selected for this study. Each isolate preserved in the brain heart infusion broth (BHI, Gibco, Edinburgh, UK) supplemented with 100 mL/L horse serum and 200 mL/L glycerol was inoculated onto H pylori selective chocolate blood agar containing 40 g/L blood agar base No.2 (Oxoid, Basingstoke, UK) and 50 mL/L horse blood (Gibco, Edinburgh, UK). Antibiotics (Sigma, St. Louis, MO, USA) were used at the following concentrations: 3 mg/L vancomycin, 5 mg/L trimethoprim, 10 mg/L nalidixic acid and 2 mg/L amphotericin B. The plates were incubated in a microaerobic atmosphere (50 mL/L CO₂) in a CO₂ incubator (Forma Scientific, Marietta, OH, USA) at 37 °C for up to 5 days.

Extraction of genomic DNA

H pylori cells on the plates were harvested by using a sterile swab, and transferred into an Eppendorf tube containing 1.5 mL TE buffer (10 mmol/L Tris-HCl, pH 8.0; 1 mmol/L EDTA, pH 8.0). The suspension was centrifuged at 8 000 g for 10 min and washed once with TE buffer. The pellet was then suspended in 800 µL TE buffer was incubated with 10 µL of 100 g/L lysozyme (Sigma, St. Louis, MO, USA) at 37 °C for 30 min. The suspension was then lysed with 100 µL of 100 g/L sodium dodecyl sulfate (SDS) by incubating at 37 °C for another 30 min. Then 5 µL of proteinase K (10 g/L) (Boehringer, Mannheim, Germany) was added into the mixture and incubated at 56 °C for 1 h. Afterward, H pylori DNA was purified by extracting twice with equal volume of phenol and once with equal volume of chloroform, followed by centrifugation at 12 000 g for 10 min each time. The supernatant
Southern blot was carried out following the protocol provided in the Enhanced chemiluminescence (ECL) direct nucleic acid labeling and detection system (Amersham Pharmacia Biotech, Buckinghamshire, UK). In brief, after agarose gel electrophoresis of the RAPD products, the DNA was submitted to denaturation by immersing the gel (200 cm²) in 200 ml buffer containing 0.5 mol/L NaOH and 1.5 mol/L NaCl for 30 min. Then the gel was neutralized in 200 ml of 0.5 mol/L Tris-HCl and 1.5 mol/L NaCl (pH 7.5) for 30 min. Afterward, the DNA was transferred to the Hybond N⁺ membrane (Amersham Pharmacia Biotech, Buckinghamshire, UK) by using a capillary blotting apparatus. DNA fixation was carried out by exposing the membrane to an UV transilluminator (Vilber Lourmat, Marné la Vallee Cedex 1, France) for 5 min. RDF sequencing and digested by EcoRI from pT-Adv vector was used as a probe. The pre-hybridization and hybridization were performed in a buffer containing 0.5 mol/L NaCl and 50 g/L blocking agent in a hybridization oven at 42 °C for 1 h and 5 h respectively. After stringency washing (twice for 20 min in 6 mol/L urea, 4 g/L SDS and 0.1×standard saline citrate, SSC; twice for 5 min in 2×SSC), chemiluminescence signals were detected by exposing the hyperfilm to the membrane for 5 to 30 min in a dark room.

RESULTS

H pylori gene profiles identified by RAPD

For all H pylori isolates from the 73 individuals, bacterial growth, genomic DNA extraction and RAPD were carried out under the same conditions. RAPD products were analyzed by 10 g/L agarose gel electrophoresis (Figures 1 and 2), which showed that H pylori from gastric antra of different individuals had different RAPD profiles whereas the isolates from antrum and cardia of the same individual had almost identical RAPD profiles with only one exception (lanes 5C and 5A in Figure 1).

Figure 1 10 g/L agarose gel electrophoresis of RAPD products of H pylori isolates. M: DNA Hind III markers; 82, 86, 96, 28, 59, 48 and 60: Designations of H pylori isolates; A: Antrum; C: Cardia.

Probe preparation

The sequence of RDF from the reference strain NCTC11637 was 99 % identical to that reported in the GenBank. It was a 361 bp fragment of the 23S rDNA (from 2133 to 2493), which is just inside the loop (2134-2684) to form peptidyltransferase center in the ribosome. The fragment was then used as a probe in Southern blotting to investigate if the random PCR primer products of H pylori isolates from gastric antra of different individuals had different RAPD profiles whereas the isolates from antrum and cardia of the same individual had almost identical RAPD profiles with only one exception (lanes 5C and 5A in Figure 1).

Southern blotting

Firstly, the sensitivity of Southern blotting, in which the RDF was used as both target and probe, was determined by using the ECL system. The results showed that the sensitive limitation of this system was 4 pg. Then, the probe was used to screen the RAPD products of different H pylori isolates. Parts of the results are shown in Figure 2. H pylori isolates from 27 of the
73 individuals were RDF positive, and the darkness of the blots was quite different from one isolate to another. As the size of the RAPD fragment hybridized with the RDF was bigger (about 400 bp) than that of the RDF, at least one primer binding site would be outside the RDF region.

Figure 2 Results of agarose gel electrophoresis of RAPD products (A) and Southern blotting (B). A: 10 g/l agarose gel electrophoresis of RAPD products of H pylori isolates; M: 1 Kb DNA ladder; 73, 27, 98, 82, 88, 05, 90, 57, 23, 09, 15, 71, 40, 33 and 93: Designations of the H pylori isolates; N: H pylori reference strain NCTC11637; R: 1 ng RDF loaded in the well; O: Template blank control; B: Southern blotting results using the RDF as a probe, the dark blots on either side of Picture B corresponding to the 1 ng RDF in the agarose gel in Picture A.

Sequencing RDFs and their flanking regions
In order to know if there were sequence differences that might facilitate the binding of the random primer, the RDFs and their flanking regions from different H pylori isolates were sequenced. Firstly, the RDFs from 10 different H pylori isolates were cloned for sequencing. The isolates with designated numbers of 15, 22, 38, 58, 90 and 93 were clonal positive and the isolates 20, 41, 84 and 90 were RDF negative in Southern blotting. Blast analysis of the sequenced results exhibited that the RDFs from the 10 isolates were quite similar.

The random primers were first proposed for fingerprinting H pylori isolates by Akopyanz et al.[33]. They recommended the primer since it had a relative high CG content and could be used to distinguish different H pylori isolates. In fact, when one employs this primer, there will be numerous potentially amplifiable fragments and only a few of them are visible on agarose gels after EB staining. This depends on the matching extent of the primer to the genomic DNA. For the bright or visible bands, the primer may be completely or mostly matched, but for the light or invisible bands, the primer is probably partially matched.

In conventionally used PCR, the specificity depends mainly on the properties of the primers and the annealing temperature. However, in PCR-based RAPD, the random primer is only 10 mers and the annealing temperature is 36 °C. Therefore, specificity of amplification is relatively low. Since we could not find any primer binding site inside or outside the RDF region in this experiment, we propose that, for amplification of the RDF, the primer be partially matched to the genomic DNA, and thus, the profiles of RAPD and Southern blotting are diversified.

From our experience, RAPD depends highly on the quality and quantity of the template. If we make a comparison between two RAPD profiles of a given H pylori isolate, the concentration, purity and even the integrity of the DNA templates should be considered. The RAPD results will be quite different if the genomic DNA is prepared using two different methods. Sometimes different PCR machines might also give different results. So, when we employed this method, we had tried to treat every sample under the same conditions, and handled the DNA preparation carefully. Even so, RAPD seems to be unstable in amplification of low yield fragments, especially those that do not appear as visible binds in agarose gel electrophoresis.

RAPD has reasonable discriminatory power[39] and is effective for H pylori genotyping grossly on large scale. But stability of this method should be taken into consideration.

Although we found many strain specific RAPD profiles in this study, using the RAPD-based methods to screen H pylori strain specific genes are not recommended. To identify strain specific genes, the PCR sequencing techniques are more reliable and reproducible.

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