JOURNAL OF CLINICAL MICROBIOLOGY, Apr. 1997, p. 995–998 Vol. 35, No. 4
0095-1137/97/$04.00+0

Complex Polysaccharides as PCR Inhibitors in Feces: *Helicobacter pylori* Model

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Received 23 August 1996/Returned for modification 26 November 1996/Accepted 3 January 1997

A model was developed to study inhibitors present in feces which prevent the use of PCR for the detection of *Helicobacter pylori*. A DNA fragment amplified with the same primers as *H. pylori* was used to spike samples before extraction by a modified QIAamp tissue method. Inhibitors, separated on an Ultrogel AcA44 column, were characterized. Inhibitors in feces are complex polysaccharides possibly originating from vegetable material in the diet.

PCR is a technique for in vitro amplification of specific DNA segments. It is extremely sensitive, allowing the detection of minimal quantities of target DNA, and is therefore a valid tool for diagnosis. Unfortunately, there are a number of technical factors which can cause the amplification reaction to fail: e.g., batch-related variability of reagents (especially primers and *Taq* polymerase), mismanipulations during extraction of DNA from the samples, and malfunction of the thermal cycler. In addition, many of the reagents traditionally used for extraction of DNA from specimens, e.g., EDTA, detergents such as sodium dodecyl sulfate, and chaotropes such as guanidinium HCl, inhibit the enzymes used for amplification.

Sample preparation procedures can also contribute in diverse ways to the reliability of target detection within clinical samples. A variety of components which can be present in samples are known for their ability to inhibit PCR, for example, heme and its metabolic products (9, 18) as well as acidic polysaccharides (2, 3, 6).

Fecal samples remain the most difficult specimens for DNA extraction and amplification. Resolution of this problem would render PCR a valuable diagnostic tool because *Helicobacter pylori* cells present in stools may not be culturable. The present study attempted to characterize inhibitors of *H. pylori* DNA amplification and to develop a simple, fast, and low-cost technique to eliminate them from fecal samples in order to make possible the detection by PCR of *H. pylori* in stool samples.

This study was based on the amplification of a particular DNA fragment constructed for use as an internal control in the detection of *H. pylori* by PCR. The use of this standardized target provided a valuable model to study the PCR inhibitors present in feces.

Clinical samples. Fecal samples were obtained from healthy subjects (four adults and eight children) known to be *H. pylori* negative as determined by serology and/or urea breath tests. A total of 16 fecal samples were studied: three samples obtained on different days from two adults and one sample from each of the others. Consent was obtained from participants in this study.

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Construction of a target DNA. The target DNA was obtained with a PCR MIMIC construction kit (Clontech). The technique is based on a nonhomologous internal control (21, 22). PCR MIMIC is a cDNA fragment used as an internal standard in PCR amplification during which one set of specific primers is used to amplify both the target cDNA and the PCR MIMIC DNA fragment. Instead of amplifying a fragment of 298 bp delimited by the sequence complementary to the primers as described by Hammar et al. (8), a fragment of 602 bp with the same sequence at each of its extremities was constructed.

The final purified PCR MIMIC was diluted to 100 amol/μl in a solution of 50 μg of ultrapure glycogen/ml with the following conversion factor: 1 ng of a 500-bp DNA fragment/μl is equal to 3×10^3 amol/μl. A stock solution at a concentration of 100 amol/μl was kept at −20°C before use to guarantee its stability.

This target DNA, which routinely served as the internal control in *H. pylori* detection by PCR, was used to study the presence of PCR inhibitors in feces. For each assay the same amount of target DNA was used (5 μl of a 1/100 dilution of the stock solution, which corresponds to 1.4 ng of *H. pylori* DNA).

DNA extraction and purification from feces. For DNA extraction, the QIAamp tissue method (Qiagen, Hilden, Germany) was used with minor modifications. This method is based on selective binding of DNA to a silica gel membrane, allowing the adsorption of nucleic acids to silica gel surfaces in the presence of high concentrations of chaotropic salts. Carbohydrates and proteins are not adsorbed and are removed. Nucleic acids are eluted under low-salt conditions.

This method involves two phases: sample lysis in a buffer provided by the manufacturer and designed to enhance selective nucleic acid adsorption to the silica gel-based QIAamp membrane and DNA purification with a QIAamp spin column.

Following the manufacturer's instructions, 25 mg of fecal sample was added to a lysis buffer and homogenized by vortexing. Twenty microliters of a proteinase K solution (19.23 mg/ml) was added, followed by incubation at 55°C for 1.5 h. A second buffer provided in the kit was added, and the sample was incubated at 70°C for 10 min. Next, 210 μl of ethanol was added, followed by centrifugation at 14,000 × *g* for 10 s. The supernatant was then applied to the QIAamp spin column and centrifuged at 6,000 × *g* for 1 min. The QIAamp spin column was placed in a 2-ml collection microtube, and the tube con-

taining the filtrate was discarded. The column material was washed five times (200 μl each) with the washing buffer provided in the kit. Finally, the DNA was eluted with 200 μl of distilled water preheated to 70°C.

PCR. PCR was performed as described previously (17). Briefly, reactions were performed in a volume of 50 μl with a 10× PCR buffer [670 mM Tris-HCl (pH 8.8), 160 mM $(NH_4)_2SO_4$, 0.1% Tween]; 1.5 mM $MgCl_2$; 200 μM (each) nucleotides A, T, G, and C; and 1 U of *Taq* polymerase (Eurobio, Les Ulis, France), with 5 μl of purified sample and with specific primers (8) at 1 μM each. PCR consisted of 39 cycles of 1 min at 94°C, 1 min at 50°C, and 1 min at 72°C with a first cycle of 5 min at 94°C, 1 min at 50°C, and 1 min at 72°C and a final cycle of 1 min at 94°C, 1 min at 50°C, and 5 min at 72°C. Bands were visualized on a 1.5% agarose gel stained with ethidium bromide.

Analysis of PCR inhibitors. PCR inhibitors present in several fecal specimens were analyzed. For this purpose, 2.4 ml of QIAamp eluate of 300 mg of feces was concentrated by using 0.1 volume of sodium acetate and 2.5 volumes of absolute ethanol. The mixture was placed at −80°C for 1 h and then centrifuged at 14,000 × *g* for 30 min. The pellet was suspended in 300 μl of Tris-borate-EDTA buffer. PCR was then performed to confirm that the inhibitors were still present.

The molecular weights (MW) of the inhibitors were determined with an Ultrogel AcA44 (IBF, Villeneuve la Garenne, France), which separates fractions with an MW between 10,000 and 130,000. The gel column was calibrated with a 200-μl mixture containing 50 μg of the monoclonal antibody MOF11 (MW, 150,000) (kindly provided by J.-H. Bézian, Université Bordeaux 2, Bordeaux, France), 25 μg of bovine serum albumin (MW, 67,000) (Sigma, S. Quentin Fallavier, France), and 25 μg of ovalbumin (MW, 45,000) (Pierce, Rockford, Ill.). The protein concentration in the 30 fractions collected for calibration was then determined by the method of Pierce.

The remaining 200 μl of concentrated inhibitors was then passed through the separation gel and 20 200-μl fractions were obtained. The target DNA was added to each fraction, and the fractions were then tested by PCR to determine which ones contained inhibitors.

Characterization of inhibitors. Inhibitors were characterized by a process of elimination. The various treatments were carried out on the same sample in parallel with an appropriate control. After each experiment, a constant amount of target DNA was added to all samples before amplification.

The first treatment consisted of heating the eluate to eliminate heat-labile proteins (100°C, 30 min). Then the eluate was extracted with chloroform to eliminate glycolipids. A third treatment consisted of treating the eluate with DNase I to hydrolyze any DNA present in the feces which could inhibit the *Taq* polymerase.

Another possible class of inhibitors is complex polysaccharides. To detect their presence, two methods, enzymatic hydrolysis and a dot assay, were used as follows. First, the enzymatic hydrolysis was performed with α-glucosidase and β-glucosidase (Boehringer Mannheim, Meylan, France) according to the manufacturer's instructions. Briefly, 10 μl of 3 M sodium acetate was added to 200 μl of eluate to obtain pH 5.0. Then, 50 μl of a 0.1-mg/ml solution of β-glucosidase (in 0.1 M acetate buffer, pH 5.0) was added. The mixture was incubated at 25°C for 1 h. After incubation, 10 μl of 1 M Tris (pH 7.0) was added. Then, 50 μl of a 0.1-mg/ml solution of α-glucosidase (in 0.1 M phosphate buffer, pH 7.0) was added, and the mixture was incubated at 25°C for 1 h. Finally, 10 μl of 3 M sodium acetate was added to stop the reaction. The pH was verified at each step of the reaction. The action of each enzyme was tested separately, and an assay was performed using bovine serum albumin instead of the enzyme as a control. After the enzymatic treatment, an amplification reaction of each sample was performed in the presence of a constant amount of target DNA. Second, in order to confirm the presence of carbohydrates, samples of each fraction were spotted on thin-layer chromatography silica gel 60 plates (Merck, Nogent-sur-Marne, France). A mixture of gangliosides was used as a positive control. A reagent made of H_2SO_4-orcinol was sprayed on the silica plates in order to visualize any complex polysaccharides present. Resorcinol-HCl for sialic acid detection and ninhydrin for protein differentiation were also used.

TABLE 1. Results of PCR obtained from fecal specimens after treatment with QIAamp tissue method

Sample[a]	PCR result for[c]:		
	Pure sample	1:2 dilution	1:10 dilution
Adults[b]			
1			
a	−	+	
b	−	+	
c	+		
2			
a	−	−	+
b	−	±	+
c	−	±	+
3	−	+	
4	−	+	
Children			
5	−	±	+
6	+		
7	−	±	+
8	+		
9	−	+	
10	+		
11	+		
12	+		

[a] Eluate spiked with a constant amount of target DNA.
[b] Eluate obtained on different days (a to c).
[c] −, negative; ±, weak; +, positive.

Preliminary experiments using stool samples spiked with *H. pylori* DNA indicated that the QIAamp tissue method was able to remove part, if not all, of the inhibitors in some samples. The same experiment using stool samples spiked with our target DNA instead of whole *H. pylori* DNA was performed in a more systematic way on 16 samples obtained from 12 subjects (four adults and eight children) (Table 1). For five of the eight children, the QIAamp tissue method proved to be sufficient to remove the PCR inhibitors from stool samples. For the others, as well as for the adult samples (except one), samples had to be diluted further in order to produce a clear positive signal. Results varied from sample to sample. For some samples, a simple 1:2 dilution was sufficient to eliminate the inhibitors, while for others, a 1:10 dilution was necessary. These inhibitors were therefore not present in all feces, nor was their concentration constant in each subject from day to day.

A series of tests was then started in order to determine the composition of the inhibitors. Heating of the preparation in order to destroy heat-labile proteins was unsuccessful in removing inhibitors. Chloroform extraction, which removes lip-

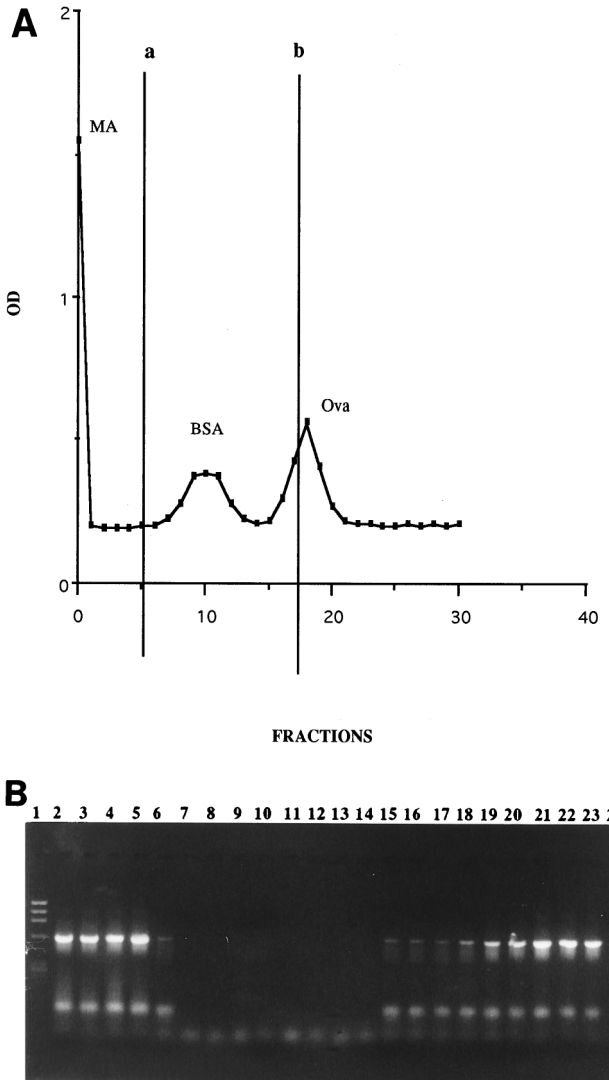

FIG. 1. (A) Optical density (OD) of fractions eluted from an Ultrogel AcA44 column showing peaks of protein standards and range of fractions containing inhibitors (a through b). MA, monoclonal antibody; BSA, bovine serum albumin; Ova, ovalbumin. (B) Results of the PCR on the eluate obtained from one fecal sample after passage through an Ultrogel column. Lanes: 1, marker ϕX174-RF DNA *Hae*III digest; 2 through 6 and 15 through 22, fractions which did not contain PCR inhibitors, indicated by the presence of a band at 602 bp corresponding to the MW of the target DNA; 7 through 14, fractions which contained PCR inhibitors, indicated by the absence of PCR products; 23, positive control (target DNA); 24, negative control.

ids, did not remove the inhibitors either. The possibility that DNA originating from other bacteria which inhibit the *Taq* polymerase could be present was also explored. However, treatment with DNase I did not block the inhibitors. The final possibility considered was that of complex polysaccharides, which behave similarly to DNA. To test this hypothesis, inhibitors were concentrated and purified. When passed through a gel column (Ultrogel) which separates fractions by MW, the fractions containing the inhibitors had an MW between 50,000 and 80,000. The results of gel column calibration and MW determination of the fractions containing the inhibitors are shown in Fig. 1. These fractions were subjected to enzymatic hydrolysis with α- and β-glucosidases. Two of five adult samples subjected to this treatment then became PCR positive.

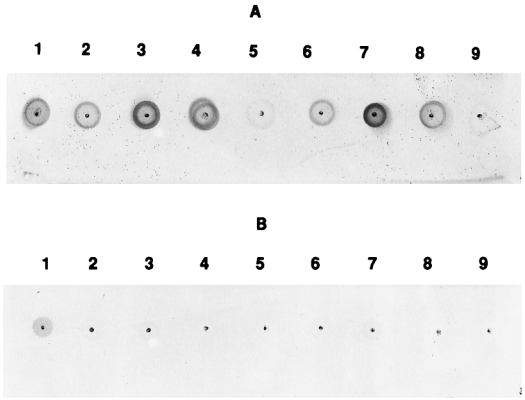

FIG. 2. Dot assay on thin-layer chromatography plates. (A) Positive H_2SO_4-orcinol reaction, indicating the presence of carbohydrates; (B) negative ninhydrin reaction, indicating the absence of amino acids. Dot 1, positive control (ganglioside mixture); dots 2 to 5, fecal sample X; dots 6 to 9, fecal sample Y. Dots 2 and 6, eluate obtained after DNA extraction by the QIAamp tissue method; dots 3 and 7, the same eluate after concentration; dots 4 and 8, fraction containing inhibitors (after Ultrogel separation); dots 5 and 9, fraction without inhibitors (after Ultrogel separation).

The thin-layer assay showed a positive reaction when visualized with H_2SO_4-orcinol, but not with HCl-resorcinol or ninhydrin (Fig. 2). This indicates that carbohydrates, and not amino acids, were present in the inhibitory fractions. Furthermore, the lack of reaction with HCl-resorcinol demonstrates that complex polysaccharides containing sialic acid, such as gangliosides, were not present.

Feces constitute complex biological samples which cause problems when PCR is used as a diagnostic method, not only because of the presence of numerous types of bacteria but also because of the different kinds of food degradation products present (7). Before the QIAamp tissue method was tested, DNA from adult fecal specimens was subjected to various extraction methods, but the results were always negative.

In this study, a simple and fast method to obtain DNA from fecal specimens in spite of the presence of inhibitors was developed. This method could prove useful for routine extraction of DNA from feces compared to other long, complex, and fastidious methods of DNA extraction proposed in the past, such as the following: phenol-chloroform extraction (1, 5), a complicated method involving extraction with 1,1,2-trichloro-1,2,2-trifluoroethane, precipitation with polyethylene glycol 6000, digestion with proteinase K in the presence of detergent, precipitation with cetyltrimethyl ammonium bromide, extraction with phenol-chloroform, and alcohol precipitation (12, 25); treatment with Chelex 100 followed by solvent extraction (11, 13, 24); immunomagnetic extraction followed by treatment with different kinds of buffers (11, 15, 20); treatment with hexadecyltrimethyl ammonium bromide, a detergent that has been reported to effectively remove PCR inhibitors from feces, followed by a phenol-chloroform extraction (10, 16); and purification of nucleic acids with silica particles in the presence of guanidium thiocyanate (23). The present method does not involve the use of toxic or hazardous reagents. Alcohol precipitation is not required, and purified DNA is ready for direct use after elution. Multiple samples can be processed easily in less than 3 h without any complicated manipulations. The selective binding characteristics of the QIAamp tissue method achieved only partial removal of PCR inhibitors. In samples where inhibitors persisted, diluting (1:2 or 1:10) was sufficient to remove their effects, rendering the PCR positive.

In samples still containing inhibitors after the QIAamp tissue method extraction, the inhibitors were not degraded by heat, removed by chloroform extraction, or hydrolyzed by the action of DNase. The lack of color change when H_2SO_4-orcinol reacted with specimen fractions obtained after concentration indicates that the inhibitors were carbohydrates. Since the MWs of the compounds were between 50,000 and 80,000, it can be concluded that they are complex polysaccharides which do not contain sialic acid, because they do not react with resorcinol-HCl. The fact that these compounds behaved similarly to DNA when extracted with the QIAamp tissue method indicates that they are negatively charged. Since these inhibitors are frequently absent from the feces of children, their concentrations in adult specimens vary, even for the same subject on different days, and because their MWs also vary, these complex polysaccharides could be of vegetable origin and linked to the diet of the subject. It has been previously demonstrated that acidic and neutral polysaccharides can contaminate plant genomic DNA preparations and inhibit both restriction endonuclease treatments (4, 22) and PCR (22). An alternative explanation, less plausible in this situation, could be the production of extracellular polysaccharides by bacterial species present in feces. It has been shown (14, 19) that a number of gram-negative bacteria are able to produce such compounds, which are extremely inhibitory to DNA restriction and DNA modifying enzymes (6).

Despite the very high concentration of enteropathogenic bacteria in stools, it has always been difficult to use PCR to detect these microorganisms because of inhibitors. Even after spiking feces with 8×10^7 *H. pylori* cells per g, we were not able to detect this bacterium with PCR, which demonstrates the magnitude of the problem.

In vivo, *H. pylori* should be present in feces in much lower numbers, since it probably cannot multiply in the intestinal environment. Additionally, degenerative forms with potentially altered DNA are likely to occur, rendering an extremely sensitive detection technique mandatory. The technique proposed here will undoubtedly contribute to achieving this goal, as preliminary attempts have shown. The complex polysaccharide inhibitors are presently being further characterized.

We thank Kathryn Mayo for revision of the English of this text.

L.M. was the recipient of a Junta Nacional de Investigação Científica e Técnológica fellowship from Portugal.

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