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Oligonucleotide Probe for Detection and Identification of *Campylobacter pylori*

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We have developed a novel and practical DNA-RNA hybridization assay for the detection and identification of *Campylobacter pylori* in the gastric mucosa. This technique utilizes a [^{32}P]ddATP-labeled synthetic oligonucleotide probe complementary to a nucleotide sequence present in *C. pylori* 16S rRNA. This probe is very sensitive and reacted with all 23 strains of *C. pylori* tested. It is also highly specific, since there was no cross-reactivity with the heterologous organisms *Campylobacter coli*, *C. fetus* subsp. *fetus*, *C. jejuni*, and *C. laridis* or with *Escherichia coli*. Hybridization of the oligonucleotide probe with *C. pylori* RNA was completely inhibited by treatment of the membrane filters with RNase but not DNase. Although a gastric mucosa tissue homogenate slightly inhibited the hybridization, as few as 10^4 *C. pylori* cells could be detected even in the presence of 5 mg of gastric mucosa. Gastric biopsy specimens obtained from patients referred for upper gastrointestinal tract endoscopy were tested for *C. pylori* infection by direct oligonucleotide hybridization, and the results were compared with those of bacteriological cultures, the urease test, and histological observations. A comparison of the urease test and the oligonucleotide hybridization results showed an excellent correlation between the two methods. The clinical usefulness of this oligonucleotide-RNA hybridization method is discussed.

Recent studies have revealed that the gastric mucosae of patients undergoing gastroduodenoscopy for upper abdominal complaints are often colonized by *Campylobacter pylori*. The presence of *C. pylori* was closely associated with histologically proven gastritis and peptic ulcers (for a review, see reference 1). Since eradication of this organism from the gastric mucosa alleviates symptoms, early detection and treatment is important (6). The urease test is the conventional method for detecting *C. pylori* in biopsy specimens, but false-positive and false-negative results have been reported (2, 3, 5, 8). In this paper, we describe an improved method of detection and identification of *C. pylori* using a novel and specific oligonucleotide-RNA hybridization technique.

MATERIALS AND METHODS

Bacterial strains. All strains with ATCC numbers were purchased from the American Type Culture Collection (ATCC; Rockville, Md.). The sources of the other strains are described in footnotes *b* through *h* of Table 1. Brain heart infusion agar supplemented with 5% sheep blood (GIBCO Diagnostics, Madison, Wis.) was used for the growth of all of the *Campylobacter* strains. The plates were incubated at 37°C in microaerophilic conditions provided by a Campy Pak envelope (BBL Microbiology Systems, Cockeysville, Md.) in a GasPak jar (BBL) without catalyst.

Synthetic oligonucleotide probe. The oligonucleotide probe sequence 5'-d(GGACATAGGCTGATCTCTTAGC) is complementary to the 16S rRNA sequences of *C. pylori* reported by Romaniuk et al. (10). This oligonucleotide was synthesized on a DNA synthesizer (Applied Biosystems 380 A) and purified by polyacrylamide gel electropholesis. The 3' end of the oligomer was labeled with [α-^{32}P]ddATP (~3,000 Ci/mmol; Amersham Corp., Arlington Heights, Ill.) and terminal deoxynucleotidyl transferase (3'-end labeling kit; Amersham) under the conditions suggested by the supplier. The specific activity of the labeled oligonucleotide probe was 8.8×10^6 to 1.8×10^8 cpm/μg.

Preparation of dot blot. Fresh bacterial cells were suspended in 0.9% NaCl at an optical density at 660 nm of 1 and then dissolved and diluted with 5 M guanidine thiocyanate (Fulka Chemical Corp., Ronkonkoma, N.Y.)-0.1 M EDTA (pH 7.0)-10 mM dithiothreitol solution (GED solution). One hundred microliters of bacterial cell lysate, unless otherwise noted, was filtered onto a nylon membrane filter (Gene Screen Plus; E. I. du Pont de Nemours & Co., Inc., Wilmington, Del.) by vacuum aspiration with a microsample filtration manifold (Minifold I; Schleicher & Schuell, Dassel, Federal Republic of Germany), and then the filters were air dried. In the experiments shown in Fig. 2, filters were treated with DNase (DNase type 1 [Boehringer Mannheim Biochemicals, Indianapolis, Ind.] [46 U/ml] in 50 mM Tris hydrochloride, 10 mM $MgSO_4$, 0.1 mM dithiothreitol, and 50 μg of bovine serum albumin fraction v per ml) or RNase (RNase A [Boehringer Mannheim] at 20 μg/ml of 2× SSC [1× SSC is 0.15 M NaCl plus 15 mM sodium citrate]) at 37°C for 30 min. The filters were then washed three times with 2× SSC at room temperature for 5 min and allowed to air dry before hybridization.

To determine a possible inhibitory effect of gastric mucosa on the assay (see Fig. 3), cultured cells of *C. pylori* ATCC 43579 were mixed with different amounts of rat gastric tissue and homogenized in 5 M GED solution with a polytron homogenizer, filtered onto a membrane filter, and then

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TABLE 1. Specific hybridization of synthetic oligonucleotide probe and chromosomal DNA probes with strains of *C. pylori*

Test strain	Result[a] by		
	Synthetic oligonucleotide probe	Chromosomal DNA probe of *C. pylori*: ATCC 43504	Chromosomal DNA probe of *C. pylori*: ATCC 43526
C. pylori			
ATCC 43504	+	+	+
ATCC 43526	+	+	+
ATCC 43579	+	+	+
3158[b]	+	+	+
3162[b]	+	+	+
3164[c]	+	+	+
3265[b]	+	+	+
3267[b]	+	+	+
3268[b]	+	+	+
3270[b]	+	+	+
3271[b]	+	+	+
3274[b]	+	+	+
3275[b]	+	+	+
17[d]	+	+	+
19[d]	+	+	+
20[d]	+	+	+
26[d]	+	+	+
35[d]	+	+	+
37[d]	+	+	+
TX30A[e]	+	+	+
60190[f]	+	+	+
133C[g]	+	+	+
NCTC 11638[h]	+	+	+
C. coli ATCC 33559	−	VW	ND[i]
C. fetus subsp. *fetus* ATCC 27374	−	−	ND
C. jejuni ATCC 33560	−	VW	ND
C. laridis ATCC 35221	−	VW	ND
E. coli HB101	−	−	ND

[a] The autoradiograph of the dot blot was scored as follows: +, strong, darkened dot (positive reaction); −, no visible reaction (negative reaction); and VW, very weak reaction (cross-reaction). For details, see Materials and Methods.
[b] Strains isolated in Peru and obtained from D. L. Shungu and C. Gill.
[c] Strains isolated in Australia and obtained from Shungu and Gill.
[d] Strains isolated in Japan and obtained from T. Kim.
[e] Isolated in the United States and obtained from D. R. Morgan.
[f] Isolated in England and obtained from Morgan.
[g] Isolated in Peru and obtained from Morgan.
[h] Isolated in Australia and obtained from Morgan.
[i] ND, Not done.

hybridized with the probe. The amount of rat gastric tissue used ranged from 0 to 5 mg, since the average clinical biopsy sample is usually smaller than 5 mg.

Biopsy specimens of gastric mucosa were homogenized in 0.5 ml of 5 M GED solution and filtered onto a membrane filter as described above.

Hybridization method. The hybridization and subsequent washing were performed by the method of Farr et al. (4). The optimum times and temperatures for the incubations were determined in preliminary experiments. Briefly, the prehybridization solution (0.08 ml/cm^2 of filter) contained 5 M tetramethylammonium chloride, 50 mM Tris hydrochloride (pH 7.5), 2 mM EDTA, 0.3% sodium dodecyl sulfate, 100 μg of denatured salmon sperm DNA per ml, and 5× Denhardt solution (1× Denhardt solution is 0.02% bovine serum albumin, 0.02% polyvinylpyrrolidone, and 0.02% Ficoll). This solution was transferred to a plastic bag containing the filter, and the plastic bag was sealed and incubated with constant agitation for 30 to 60 min at 50°C. Radioactive probes were then added to the bags at a concentration of 10 ng/ml. The bags were incubated at 50°C for 2 h. After hybridization, the filters were washed twice in 2× SSPE (1× SSPE is 150 mM NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA [pH 7.0])–0.1% sodium dodecyl sulfate at room temperature for 10 min and then twice in the hybridization solution without salmon sperm DNA and Denhardt solution at 55°C for 15 min. Hybridization with the probe was detected by autoradiography with X-Omat AR films (Eastman Kodak Co., Rochester, N.Y.) exposed for 1 to 2 days at −70°C with intensifying screens. For liquid scintillation counting, the filters were cut into squares, placed into scintillation vials, and counted in Hydrofluor scintillant (National Diagnostics, Manville, N.J.).

Chromosomal DNA probe and whole-cell dot blot hybridization. The preparation of bacterial chromosomal DNA probes and whole-cell dot blot hybridization procedures were performed as previously described (9). Briefly, chromosomal DNA was labeled with [α-^{32}P]dCTP by using the nick translation procedure. The labeled chromosomal DNA probes were denatured at 100°C in 10 mM Tris hydrochloride (pH 8.0)–1 mM EDTA for 5 min immediately before use. Bacterial cell suspension (5, 10, and 50 μl; optical density at 660 nm = 0.1) was filtered onto a membrane filter by using a microsample filtration manifold. Filters were treated twice with 0.5 N NaOH for 2 min to release and fix the DNA to the filters and then twice with 1 M Tris hydrochloride (pH 7.5) for 2 min. The filters were allowed to air dry. The prehybridization solution (0.08 ml/cm^2 of filter) contained 50% formamide, 1% sodium dodecyl sulfate, 1 M NaCl, and 10% dextran sulfate. The solution was transferred to a sealable plastic bag containing the filter and incubated with constant agitation for at least 15 min at 42°C. Denatured salmon sperm DNA and denatured radioactive probe were added to the bags before hybridization at concentrations of 100 μg and 10 ng/ml, respectively. The bags were incubated overnight at 42°C. After hybridization, the filters were washed twice in 2× SSC (pH 7.0) at room temperature for 5 min, twice in 2× SSC and 1% sodium dodecyl sulfate at 65°C for 30 min, and then twice in 0.1× SSC at room temperature for 30 min.

Bacteriological culture and urease test. Biopsy specimens from endoscopies were inoculated onto 5% sheep blood brain heart infusion agar and incubated as described above. Biochemical identification of *C. pylori* was performed according to the *Manual of Clinical Microbiology* (7). For the urease test, specimens were inoculated in a urease test tube (BBL) and incubated at room temperature for 4 h. Positive samples turned medium red.

RESULTS

We synthesized three different oligonucleotide sequence probes and tested their hybridization specificities for three strains of *C. pylori*, four strains of other *Campylobacter* species, and one strain of *Escherichia coli*. One of these oligonucleotides, 5′-d(GGACATAGGCTGATCTCT TAGC), hybridized specifically with *C. pylori* under the hybridization and washing conditions described in Materials and Methods (Fig. 1). No cross-reactivity was found. Treatment of the filters with RNase but not DNase completely inhibited the hybridization, indicating that this oligonucleotide hybridized with bacterial RNA (Fig. 2). The specificity of the oligonucleotide probe was tested and compared with that of chromosomal DNA probes by using 20 clinical

FIG. 1. Species-specific and dose-response hybridization studies with the oligonucleotide probe. Bacterial cells were suspended in saline, adjusted to an optical density at 660 nm of 1, and then diluted with 5 M GED solution. A 100-μl sample of each dilution was filtered onto a membrane filter and hybridized with the ^{32}P-labeled oligonucleotide probe. For details, see Materials and Methods.

isolates of *C. pylori* which were isolated in Peru, Australia, the United States, England, and Japan and a panel of unrelated bacteria. These data are summarized in Table 1. The oligonucleotide probe reacted with all *C. pylori* strains, with no cross-reactions to other bacterial strains, whereas the whole-chromosomal DNA probe showed weak cross-reactions with some strains of other *Campylobacter* species. Next, the sensitivity of this oligonucleotide probe was tested in the presence of rat gastric tissue. Cultured *C. pylori* cells were added to a sample of rat gastric tissue and homogenized in 5 M GED solution by using a polytron homogenizer. The homogenates were then filtered onto a membrane filter and hybridized with the probe. Although the tissue homogenates slightly inhibited the hybridization, as few as 5×10^3 to 1×10^4 *C. pylori* cells per assay were detected by this method (Fig. 3).

FIG. 2. Hybridization of the oligonucleotide probe with bacterial RNA. A cell suspension of *C. pylori* ATCC 43579 (optical density at 660 nm = 0.1) was mixed with 4 volumes of 5 M GED solution, filtered onto a membrane filter, treated with RNase A or DNase, and then hybridized with the oligonucleotide probe. Hybridization counts were measured by liquid scintillation counting. For details, see Materials and Methods.

FIG. 3. Detection of *C. pylori* by the oligonucleotide probe in the presence of rat gastric tissue. Cultured cells of *C. pylori* ATCC 43579 were mixed with different amounts of rat gastric tissue homogenate, filtered onto a membrane filter, and then hybridized with oligonucleotide probe. The amounts of bacterial cells and rat gastric tissue on each dot blot were 0 to 10^6 cells and 0, 1, and 5 mgeq of tissue homogenates, respectively. For details, see Materials and Methods.

Biopsy specimens obtained from 14 patients referred for upper gastrointestinal tract endoscopy were tested for *C. pylori* infection by oligonucleotide hybridization, and the results were compared with those of the urease test, direct stain, and bacteriological culture (Table 2). There was high correlation between the results obtained by oligonucleotide hybridization and the urease test: seven of eight urease-positive specimens showed positive hybridization reactions, and seven of eight hybridization-positive specimens were urease positive.

DISCUSSION

In a recent report (9), we demonstrated that DNA-DNA hybridization with a dot blot format and ^{32}P-labeled probes is useful for rapid and accurate identification of intestinal *Bacteroides* species. Although in these studies the whole-chromosomal DNA probes were specific enough to distinguish one bacterial species from the others, more highly specific non-cross-reacting probes are needed for the direct detection of specific organisms in crude clinical specimens (12).

Since oligonucleotide probes are highly specific and easy to synthesize in large quantities, they are well suited for use

TABLE 2. Comparison of various assays for detecting *C. pylori* in clinical specimens

Patient no.	Clinical diagnosis by endoscopy	Result by: Oligonucleotide probe	Urease test	Warthin-Starry stain	Bacteriological culture
1	Normal	−	−	−	−
2	Gastritis	−	−	−	−
3	Gastritis	−	−	−	−
4	Gastritis	−	−	−	−
5	Gastritis	+	+	+	+
6	Gastritis	+	+	+	+
7	Gastritis	−	+	+	NT[a]
8	Gastritis	+	+	NT	+
9	Gastritis	−	−	−	−
10	Gastritis	+	−	−	−
11	Gastritis	+	+	NT	+
12	Duodenal ulcer	+	+	+	+
13	Duodenal ulcer	+	+	+	+
14	Duodenal ulcer	+	+	NT	−

[a] NT, Not tested.

in clinical laboratories. For example, Sommerfelt et al. (11) reported the clinical application of a highly specific synthetic oligonucleotide probe for the identification of enterotoxigenic *E. coli*. Although the design of oligonucleotide probes for *Campylobacter* spp. has been reported (A. Rashtchian, M. Abbott, D. H. Lovern, G. A. Mock, and M. M. Shaffer, Abstr. Annu. Meet. Am. Soc. Microbiol. 1986, C-90, p. 343), their clinical application has yet to be reported. Rashtchian et al. reported two synthetic oligonucleotide probes based on *C. jejuni* 16S rRNA sequences. One of these probes was unable to distinguish between *C. jejuni*, *C. coli*, and *C. laridis*, whereas the second probe hybridized to all *Campylobacter* species tested. Our results are the first, to our knowledge, to describe the construction of a species-specific 16S rRNA-targeted oligonucleotide probe for *C. pylori*.

A possible disadvantage of short oligonucleotide probes is that their sensitivity may be affected by minor nucleotide changes in the target sequences. However, our oligonucleotide probe hybridized to all of the 20 *C. pylori* strains which had been isolated in five different countries, with no detectable cross-reactions to other bacterial strains (Fig. 1; Table 1). A whole-bacterium-chromosomal DNA probe, however, showed weak homology with the DNAs of *C. pylori*, *C. coli*, *C. jejuni*, and *C. laridis* (Table 1), as previously reported by Romaniuk et al. (10).

A comparison of the urease test and the oligonucleotide hybridization results showed an excellent correlation between the two methods. Although the short half-life and the requirement for ^{32}P labeling restrict the hybridization reactions to laboratories equipped to use radioactive compounds, by adapting nonradioactive-labeling methods it should become feasible to use the oligonucleotide hybridization technique in clinical studies.

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