Multiplex-Touchdown PCR Assay for the Detection and Genotyping of Helicobacter pylori from Artificially Contaminated Sheep Milk

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ABSTRACT

Helicobacter pylori (Hp) is an organism commonly present worldwide in the human population, sometimes causing serious illnesses such as duodenal and gastric ulcers, adenocarcinoma of the stomach, and low-grade B-cell mucosa-associated lymphoid tissue lymphoma of the stomach. This article describes a multiplex-touchdown PCR method for the identification and genotyping (vacA—s1/m1, s1/m2, and s2/m2—and cagA genes) of Hp directly from sheep milk artificially contaminated with Hp strains from human gastric biopsies and with Hp ATCC 43504. The strains from humans carried s1/m2 cagA+ and s2/m2 cagA− allelic combinations, while the ATCC strains carried an s1/m1 cagA+ allelic combination. The technique showed a sensitivity of 15 CFU/ml for species identification and of 1,500 CFU/ml for the detection of genes encoding for VacA and CagA. It has proven to be specific and rapid, and the authors suggest that it be used as a rapid screening method to ensure that sheep milk is uncontaminated with this organism.

Helicobacter pylori (Hp) is one of the most common causes of chronic bacterial infections in humans (28). Recent evidence has suggested that it is involved in the occurrence and development of gastric adenocarcinoma (7), lymphoproliferative disorders, and gastric mucosa-associated lymphoid tissue lymphoma (17). The number of asymptomatic Hp carriers is quite high and varies significantly according to the geographic location considered (9).

The routes of infection have not yet been firmly established, and different modes of transmission have been suggested (13), although the most commonly accepted hypothesis is that infection takes place through fecal-oral transmission (27). Subsequent to fecal contamination, Hp may occur in food and water supplies (18). Several investigators have considered Hp a foodborne pathogen on the basis of some of its microbiological and epidemiologic characteristics. A high prevalence of infection has been reported to exist within closed family groups (21) and among individuals living in institutions (13), suggesting that transmission of the microorganism occurs indirectly by eating “the same food at the same table” (18). Hp has also been isolated in drinking and irrigation water (11, 15), and it is believed that vegetables and foods of animal origin, such as sheep milk (4) and cow milk (10), act as vehicles of transmission. Another factor to be taken into account is the ability of Hp to survive in complex foodstuffs such as milk (20) and in ready-to-eat foods such as lettuce, tofu, and chicken (18).

Finally, given its taxonomic proximity to Campylobacter jejuni, a typical emerging foodborne pathogen, it is hypothesized that both Hp and C. jejuni have a similar transmission pattern and are thus foodborne pathogens (28).

The isolation of Hp from foods, particularly when they present high loads of accompanying microflora, is exacting and time-consuming, since it requires selective media containing several antibiotics, microaerophilic conditions, and long incubation periods (7 days) (23). Under adverse environmental conditions such as desiccation, lack of protection against oxygen, and exposure to antimicrobial agents, Hp may produce viable nonculturable forms (5). The isolation of Hp from foods by means of conventional microbiological techniques is not able to detect these viable nonculturable forms yielding false-negative results for Hp while the viable nonculturable forms remain infectious (13). Hence, several investigators have proposed molecular assays for the detection of Hp in water and different food items (26).

It is well known that Hp genotypes that have specific virulence markers are particularly pathogenic for humans. One such marker is the vacA gene that encodes for the vacuolating cytotoxin VacA and may present three allelic variants: s1/m1, s1/m2, and s2/m2 (3). Another virulence marker is the cagA (cytotoxin-associated gene A), which encodes for the CagA protein (2, 24). The presence of cagA is associated with duodenal ulceration, gastric mucosal atrophy, and gastric cancer (3). For public health purposes, evaluation of a food containing Hp will thus have to include genotyping of isolates. A rapid and sensitive PCR assay for the identification and genotyping of Hp has recently been
TABLE 1. MT-PCR primers

<table>
<thead>
<tr>
<th>Primer</th>
<th>DNA region(s) amplified</th>
<th>Primer sequence</th>
<th>Amplicon size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NHP-F</td>
<td>16S rRNA</td>
<td>5′-GCAATCAGCCGTAGTAATGTC-3′</td>
<td>521</td>
</tr>
<tr>
<td>NHP-R</td>
<td></td>
<td>5′-GCTAAGAGATACGCTACTTCC-3′</td>
<td></td>
</tr>
<tr>
<td>VacAS-F</td>
<td>vacA</td>
<td>5′-ATGGAAATAACAAACCACAC-3′</td>
<td>259 (type s1)</td>
</tr>
<tr>
<td>VacAS-R</td>
<td>Region s</td>
<td>5′-CTGCTTAGATGCGCAGGAC-3′</td>
<td>286 (type s2)</td>
</tr>
<tr>
<td>VacAM-F</td>
<td>vacA</td>
<td>5′-CAATCTGTGTTAACTAGCGCAGG-3′</td>
<td>567 (type m1)</td>
</tr>
<tr>
<td>VacAM-R</td>
<td>Region m</td>
<td>5′-GCGTCAATATATTTCCAAGG-3′</td>
<td>642 (type m2)</td>
</tr>
<tr>
<td>CagA-F</td>
<td>cagA</td>
<td>5′-GATAACAGCAGCGCTTTGGAGAGGA-3′</td>
<td>393</td>
</tr>
<tr>
<td>CagA-R</td>
<td></td>
<td>5′-CCATGAATTTTGTATCCGTTGG-3′</td>
<td></td>
</tr>
</tbody>
</table>

*From Faundez et al. (8) and Lu et al. (15).*

A total of 1,000 ml of tanked raw sheep milk was used for the tests. The milk was put into sterile refrigerated containers (about 4°C) and immediately transferred to our laboratory, where it was used for the tests. A total aerobic mesophilic count in plate count agar (Oxoid) was performed on 25 ml of the sample incubated at 32°C for 48 h. The milk was used for the experimental contaminations and to prepare the negative controls.

**MATERIALS AND METHODS**

**Bacterial strains and sample preparation.** Nine human *Hp* strains (Hp-nat18 and Hp-spad1 through Hp-spad8) from nine gastric biopsy samples and *H. pylori* ATCC 43504 (Promochem LGC, UK), a human strain, were used to artificially contaminate samples of tanked sheep milk from a local farm. Isolation of the microorganism was performed according to the procedures described by Elizalde et al. (6) with a small modification. Nine antral biopsies from nine patients were put into sterile saline solution, homogenized, streaked onto freshly prepared brain heart infusion agar (Oxoid Basingstoke, Hampshire, UK) plates supplemented with 7% horse blood (Liofilchem, Teramo, Italy) and 10 mg/liter of vancomycin, 30 mg/liter of trimethoprim, 30 mg/liter of cefalosporin, 30 mg/liter of amphotericin B (Sigma-Aldrich, Milano, Italy), and incubated for 3 to 10 days at 37°C under microaerophilic conditions (Anaerocult C mini, Merck, Darmstadt, Germany). Growing bacteria were identified as *Hp* on the basis of the morphology of the colonies, Gram staining, and oxidase, catalase, and urease production (5).

The isolated strains (*Hp*-nat18 and *Hp*-spad1 through 8) were cultured on Wilkins-Chalgren anaerobe agar (Oxoid) containing 5% defibrinated horse blood (Oxoid) and 30 mg/liter of colistin methanesulfonate, 100 mg/liter of cycloheximide, 30 mg/liter of nalidixic acid, 30 mg/liter of trimethoprim, and 10 mg/liter of vancomycin (all from Sigma).

Lyophilized *Hp* ATCC 43504 was reconstituted according to the supplier’s instructions.

After a 7-day incubation at 37°C under microaerophilic conditions (Anaerocult C mini, Merck), the bacterial cells (strains *Hp*-nat18, *Hp*-spad1 through 8, and ATCC 43504) were harvested from the plates and suspended in 3 ml of sterile saline solution (0.85% NaCl). One milliliter was used for cell counting, 1 ml was used for the identification and genotyping of the strains by MT-PCR, and 1 ml was used for the contamination of 10 groups (each per strain) of 9 ml of tanked raw sheep milk samples, obtained as described below.

**Sheep milk sample.** A total of 1,000 ml of tanked raw sheep milk from a local farm was used for the tests. The milk was put into sterile refrigerated containers (about 4°C) and promptly transferred to our laboratory, where it was used for the tests. A total aerobic mesophilic count in plate count agar (Oxoid) was performed on 25 ml of the sample incubated at 32°C for 48 h. The milk was used for the experimental contaminations and to prepare the negative controls.

**Negative controls.** Several negative controls were prepared. One consisted of uncontaminated milk (10 ml) that tested negative for *Hp* using the protocol described by Dore et al. (4). The other negative controls were prepared with sheep milk samples that had separately been artificially contaminated, following the procedure described above, with *Escherichia coli* (ATCC 25922), *Listeria monocytogenes* (ATCC 15313), and *Staphylococcus aureus* (ATCC 13565) cultured in tryptone soya agar (Oxoid) for 24 h at 37°C under aerobic conditions and *Campylobacter jejuni* subsp. *jejuni* (ATCC 29428), cultured in Columbia sheep blood agar (bioMérieux, Rome, Italy) for 48 h at 42°C under microaerophilic conditions (CampyGen, Oxoid).

The bacterial cells were harvested from each plate and suspended in 2 ml of sterile saline solution, separately. One milliliter of each suspension was used for the bacterial count, as detailed elsewhere (14), and 1 ml was used for the contamination of 10 sheep milk samples.

**DNA extraction.** A GenomicPrep Cells and Tissue DNA Isolation Kit (Amersham Biosciences, GE Healthcare, Milan, Italy) was used to extract bacterial DNA from 1 ml of each *Hp* strain for the 10 suspensions used to experimentally contaminate the milk.

Bacterial DNA from 1 ml of each contaminated milk sample was extracted with a DNeasy Tissue Kit (QIAGEN, Milano, Italy) by slightly modifying the supplier’s instructions. Briefly, after centrifugation at 15,400 × g for 10 min, 180 μl of alanine aminotransferase buffer and 20 μl of lysozyme (Sigma) were added to the pellet and incubated at 37°C for 20 min.

**MT-PCR.** The identities of *Hp* ATCC 43504 and of the strain species previously isolated and biochemically identified as *Hp* were confirmed using a primer pair encoding for 16S rRNA that amplified a product of 521 bp (15) (Table 1).

The strains were genotyped using two sets of primers for the detection of the vacA gene (s1/m1, s1/m2, and s2/m2) and one set of primers for the detection of the cagA gene, as reported in Table 1 (8).

Samples (2 μl) of each extract were amplified in 50 μl of reaction mixture containing 10× HotMaster *Taq* buffer (10 mM Tris-HCl, pH 8.5, 50 mM KCl, 25 mM MgCl₂), 200 μM each of the dNTPs, 0.5 μM of each primer, and 1.25 U of HotMaster *Taq* DNA polymerase (Eppendorf AG, Hamburg, Germany). After preliminary incubation at 94°C for 2 min, a 35-cycle amplification protocol was implemented as follows: the first 10 cycles consisted
of denaturation at 94°C for 1 min, followed by annealing at 55°C for 2 min and elongation at 72°C for 1.5 min. Beginning with cycle 10, a touchdown PCR protocol was begun by reducing the annealing temperature by 1°C for each cycle. When the target temperature of 50°C was reached at cycle 15, the PCR was continued with these cycling parameters until cycle 35 was reached.

The MT-PCR products were visualized under UV transillumination following electrophoresis on 1.5% agarose gel stained with ethidium bromide and using the Gene Ruler 50-bp DNA Ladder (MBI Fermentas, Milano, Italy) as a reference standard. Each test was repeated three times.

Sensitivity of the MT-PCR. To evaluate the sensitivity of the technique, MT-PCR was performed on 10-fold dilutions (up to \(10^{-10}\)) of the raw sheep milk that had been artificially contaminated with the \(H.\) pylori strain \(nat18\). The dilutions were performed in raw sheep milk, and bacterial DNA was extracted from each dilution.

Specificity of the MT-PCR. To evaluate the specificity of the technique, MT-PCR was performed on the uncontaminated milk sample and on each milk sample contaminated with the bacterial strains that were used to prepare the negative controls.

RESULTS

The total aerobic mesophilic count of the sheep milk was \(1.6 \times 10^7\) CFU/ml. The mean value of bacterial counts for the \(H.\) pylori suspension was determined to be \(1.5 \times 10^6\) CFU/ml. The bacterial counts of the suspensions used as negative controls were \(1.7 \times 10^7\) CFU/ml for \(S.\) aureus, \(5.8 \times 10^7\) for \(L.\) monocytyogenes, \(5.1 \times 10^7\) for \(E.\) coli, and \(1.6 \times 10^6\) for \(C.\) jejuni. The uncontaminated milk was used as a negative control tested negative for \(H.\) pylori (Fig. 1). All of the MT-PCR assays that were performed on the \(H.\) pylori strains and on the artificially contaminated milk identified the various species amplified a product of 521 bp (Fig. 1). The nat18 \(H.\) pylori strain, along with the spad 2, 5, and 8 \(H.\) pylori strains, carried an s1/m2 and a cagA\(^+\) allelic combination. The spad1, 3, 4, 6, and 7 \(H.\) pylori strains carried an s2/m2 and a cagA\(^-\) allelic combination. Finally, the \(H.\) pylori ATCC 43504 strain carried an s1/m1 and a cagA\(^+\) allelic combination (Fig. 1). These genotypes were confirmed in each case by PCR using single primer pairs (Table 1). The MT-PCR assays performed on the negative controls were consistently negative (Fig. 1). The MT-PCR showed a sensitivity of \(10^{-3}\) CFU for each PCR test performed when using the species identification protocol with the primer pair encoding for 16S rRNA (15 CFU/ml) and of \(10^{-2}\) CFU for each PCR test performed (1,500 CFU/ml) when using the gene detection protocol encoding for VacA and CagA (Fig. 2).

FIGURE 1. Results of PCR assays for detection and genotyping of three representative strains of \(H.\) pylori from raw sheep milk samples and results of specificity evaluation. M, DNA ladder (50 bp); 1, strain \(H.\) pylori nat18; 2, \(H.\) pylori ATCC 43504; 3, strain \(H.\) pylori spad1; 4, Escherichia coli ATCC 25922; 5, \(L.\) monocytyogenes ATCC 15313; 6, \(S.\) aureus ATCC 13565; 7, Campylobacter jejuni subspecies jejuni ATCC 29428; 8, strain \(H.\) pylori nat18 from contaminated sheep milk; 9, strain \(H.\) pylori ATCC 43504 from contaminated sheep milk; 10, strain \(H.\) pylori spad1 from contaminated sheep milk; 11, uncontaminated sheep milk; 12, distilled water.

FIGURE 2. Results of sensitivity of the MT-PCR assay for the detection of \(H.\) pylori from milk samples. M, DNA ladder (50 bp); C, control: strain \(H.\) pylori nat18; S, \(H.\) pylori nat18 strain suspension (1.5 \(\times\) \(10^5\) CFU/ml) in raw sheep milk artificially contaminated; 1 through 11, 10-fold dilutions (until \(10^{-10}\)) of raw sheep milk artificially contaminated with \(H.\) pylori nat18 strain; 13, distilled water.

DISCUSSION

\(H.\) pylori is a significant human pathogen that has been reported to contaminate food such as milk (4, 10) and vegetables or water (12). The sheep milk produced in Italy represents a substantial part of the total global milk production, and it is used, often raw, to make high-quality cheeses, some of which have been officially acknowledged with European Commission labels.

This article describes an MT-PCR assay that can be used for the detection and characterization of \(H.\) pylori in artificially contaminated raw sheep milk. The technique has proven to be rapid (about 24 h) and specific. The specificity of the method was assessed on samples of milk artificially contaminated with foodborne pathogens generally found in sheep milk (1, 16, 25). Samples were contaminated with \(C.\) jejuni to compare the specificity of the primers used on phylogenetically similar organisms (28). We have shown that the method has good specificity, since the primers did not amplify other genetic material in the uncontaminated milk or in the samples artificially contaminated with several bacteria (Fig. 1). We have also shown that the technique is highly sensitive (Fig. 2).

Compared to other methods used for \(H.\) pylori detection in environmental and food samples as described elsewhere (19), the MT-PCR described in this article presents the advantage of detecting and characterizing \(H.\) pylori from microbiologically and rheologically complex foodstuffs in a single step. This molecular approach for the detection of \(H.\) pylori in
foods may substantially curb the material costs of control procedures by ensuring high sensitivity and quick results. The characterization of the genes encoding for the virulence factors (Fig. 1) provided important information with respect to the sanitary assessment of food items because of the greater pathogenicity of certain Hp genotypes such as vacA + s1/m1 cagA + and vacA + s1/m2 cagA +. As is well known, the main limitation of PCR assays is their inability to distinguish live organisms from dead organisms. PCR techniques can, however, be used to screen foodstuffs, thus making it necessary to use conventional isolation methods only on those samples that test positive by MT-PCR.

Future investigations on milk samples from sheep, goats, and cows will be performed to determine whether Hp is present and to obtain some information on the epidemiology of Hp infection in humans.

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