Improved Detection of Five Major Gastrointestinal Pathogens by Use of a Molecular Screening Approach

Richard F. de Boer,* 1 Alewijn Ott,2 Barbara Keszyts,2 and Anna M. D. Kooistra-Smid1,2

Department of Research & Development1 and Department of Medical Microbiology,2 Laboratory for Infectious Diseases, Groningen, Netherlands

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The detection of bacterial and parasitic gastrointestinal pathogens through culture and microscopy is laborious and time-consuming. We evaluated a molecular screening approach (MSA) for the detection of five major enteric pathogens: Salmonella enterica, Campylobacter jejuni, Giardia lamblia, Shiga toxin-producing Escherichia coli (STEC), and Shigella spp./enteroinvasive E. coli (EIEC), for use in the daily practice of a clinical microbiology laboratory. The MSA consists of prescreening of stool specimens with two real-time multiplex PCR (mPCR) assays, which give results within a single working day, followed by guided culture/microscopy of the positive or mPCR-inhibited samples. In the present 2-year overview, 28,185 stool specimens were included. The MSA was applied to 13,974 stool samples (49.6%), whereas 14,211 samples were tested by conventional methods only (50.4%). The MSA significantly increased the total detection rate compared to that of conventional methods (19.2% versus 6.4%). The detection of all included pathogens, with the exception of S. enterica, significantly improved. MSA detection frequencies were as follows: C. jejuni, 8.1%; G. lamblia, 4.7%; S. enterica, 3.0%; STEC, 1.9%; and Shigella spp./EIEC, 1.4%. The guided culture/microscopy was positive in 76.8%, 58.1%, 88.9%, 16.8%, and 18.1% of mPCR-positive specimens, respectively. Of all mPCRs, only 1.8% was inhibited. Other findings were that detection of mixed infections was increased (0.9% versus 0.02%) and threshold cycle ($C_T$) values for MSA guided culture/microscopy-positive samples were significantly lower than those for guided culture/microscopy-negative samples. In conclusion, an MSA for detection of gastrointestinal pathogens resulted in markedly improved detection rates and a substantial decrease in time to reporting of (preliminary) results.

Infectious gastroenteritis (IG) is one of the most common diseases worldwide, killing millions of individuals each year (3, 16). In industrialized countries, IG remains a major public health burden, although mortality is low. In the Netherlands, with a population of 16.5 million, the yearly IG incidence is approximately 4.5 million (42). Although most episodes of IG are brief and do not require medical attention, the economic and social burdens of IG are significant (38).

The etiology of IG includes viral, parasitic, and bacterial pathogens. Most medical microbiology laboratories use conventional diagnostic procedures, such as culture and microscopy, for routine detection of enteric pathogens. These procedures include enrichment steps, use of selective culture media, biochemical identification, serotyping, and resistance profiling. Final results are obtained after 3 to 4 days, making these procedures laborious and time-consuming. Furthermore, the detection of pathogens in stool specimens by culture is complicated. For instance, bacteria belonging to the normal gastrointestinal flora can present with the same colony morphology as enteric pathogens (13, 30). The resultant misidentification increases hands-on time and delay in reporting of a definite negative result. Other problems are the viable but nonculturable state of Campylobacter jejuni (24, 29) and the limited viability of shigellae outside the human body (35). These may compromise the sensitivity of culture.

Conventional laboratory diagnosis of gastrointestinal parasites consists of microscopy and/or stool antigen tests. Microscopy in particular has disadvantages, as the detection and correct identification of parasites depend upon the experience and skills of the microscopist. Also, due to intermittent shedding of protozoa the sensitivity can be low, and therefore examination of multiple samples is required (8).

The workload involved in examining stool samples is high. Our laboratory receives around 15,000 stool specimens annually, with daily numbers varying greatly between seasons (42). Furthermore, the majority of stool specimens do not yield a positive result (42). Therefore, methods which quickly identify the negative specimens would facilitate routine screening. Antigen detection is a fast and effective alternative for several enteric pathogens (11, 15, 23, 27, 46). However, this method has a limited sensitivity, requires one test per pathogen, and is not available for all relevant pathogens.

Molecular methods provide a means for sensitive and rapid detection of enteric pathogens. However, broad application remains limited due to their assumed high costs, inhibition caused by fecal constituents (22), and the need for specialized laboratories. Due to the high throughput of stool screening and the number of possible enteric pathogens, implementation of a molecular approach which uses multiplexing of targets is mandatory (9, 10, 20, 25, 26, 36, 43, 44, 47). For the detection of enteric pathogens it has been proven feasible to use molec-
ular methods with improved performance and turnaround time (TAT) (7, 25, 32).

Since December 2006, we have implemented a molecular screening approach (MSA) for the simultaneous detection of five pathogens: *Salmonella enterica*, *Campylobacter jejuni*, *Giardia lamblia*, Shiga toxin-producing *Escherichia coli* (STEC), and *Shigella* spp./enteroinvasive *E. coli* (EIEC). This MSA, involving two internally controlled real-time multiplex PCRs (mPCRs), is now daily practice. The present report describes our experiences with the MSA over a 2-year period (2007 to 2008).

(Protocol for the results was presented at the 19th European Congress of Clinical Microbiology and Infectious Diseases, Helsinki, Finland, 18 May 2009.)

**MATERIALS AND METHODS**

mPCR technical validation. All primers and probes used for detection of the five enteric pathogens were previously described (4, 21, 34, 43, 45), and for some we previously performed extensive clinical validations (32, 33, 40). For validation of the mPCR, a total of 147 bacterial/fungal strains were used in selectivity testing. These included target organisms for inclusivity testing (33 *S. enterica*, 6 *C. jejuni*, 35 STEC [including all six variants], 24 *Shigella* spp., and 13 EIEC strains) and other nontarget gastrointestinal species (*n = 36*) for cross-reactivity testing. For determining the limit of detection (LOD), spiking experiments were performed in a background of two pooled fecal matrices (watery and unformed).

We made 10-fold dilution series simultaneous to performing enumeration of samples by viable count on the appropriate medium for each target organism (with the exception of *G. lamblia*).

The clinical performance of the two internally controlled real-time mPCRs was compared (partly retrospectively) with those of conventional culture and microscopy on a total of 826 clinical stool specimens received at our laboratory for detection of bacterial and/or parasitic enteric pathogens.

**Patient specimens.** Our laboratories, in 10 different locations, serve a population of about 1 million inhabitants, including both community and hospitalized patients. From January 2007 through December 2008 a total of 28,185 stool samples were received for detection of bacterial and/or parasitic enteric pathogens. The samples were from patients with IG included in their differential diagnosis. The mean age was 40 years (range, 0 to 104 years); 7,392 (37.4%) patients were males, and 12,368 (62.6%) were females.

Real-time mPCR was, like conventional microscopy, performed only at the central principal location of our laboratory, whereas routine bacterial culture was performed at all locations, following one standard operating procedure (see below).

Conventional culture and microscopy. Culturing of *S. enterica* and *Campylobacter* spp. was carried out as described previously (32). Briefly, culture of *S. enterica* consisted of selenite enrichment and selective culturing on salmonella-shigella (SS) medium and Hektoen enteric agar (HEA) medium at 35°C, biochemical identification, and group-specific *Salmonella* serological identification.

For *Campylobacter* species, routine culture consisted of selective culture on *Campylobacter* selective agar (48 h at 42°C) and charcoal cefoperazone desoxycholate agar (72 h at 35°C) under microaerophilic conditions. Identification was carried out by Gram staining, biochemical determination, determination of the absence of aerobic growth at 42°C, and resistance profiling. Culture of *Shigella* spp. was carried out as for *S. enterica*, with the exception of serological identification with species-specific *Shigella* (*S. sonnei*, *S. boydii*, *S. flexneri*, and *S. dysenteriae*) agglutination sera. Culture for *E. coli* O157 was carried out on specific request only, or in cases of a bloody sample or a history of bloody diarrhea, and consisted of selective culture on sorbitol MacConkey (SMAC) agar (48 h at 35°C). Identification of the non-sorbitol-fermenting colonies was carried out by performing an indole reaction and serological typing (with serogroup O157 antigens). All biochemically and serologically identified *Salmonella* and *Shigella* strains and *E. coli* O157 were confirmed using the Vitek 2 system (bioMérieux, Boxtel, Netherlands). All culture and identification media were from Mediaproducts BV, Groningen, Netherlands, whereas the *Salmonella* and *Shigella* agglutination sera were from Remel Europe Ltd., Dartford, United Kingdom. The *E. coli* O157 agglutination serum was from Oxoid, Basingstoke, Hampshire, England. Resistance profiling was performed with the Vitek 2 system.

Microscopy was performed on specific request for parasitological testing. Ova and cysts were detected using a Ridley concentrate (28) or the triple feces test (TFT) as described previously (41). Modified acid-fast staining for detection of cryptospordia on the formol-ether concentrate was performed on specific request.

**Molecular screening approach.** (i) **Specimen preparation.** Fecal suspensions were prepared according to the preenrichment protocol for stool samples, release 1.0 (bioMérieux), and stored at −20°C until DNA extraction on the next day. A selenite enrichment broth from the same stool specimen was inoculated and incubated for approximately 16 h at 35°C. The remaining part of the specimen was stored at 4°C until further culture, depending on the real-time mPCR result.

(ii) **DNA extraction.** DNA was extracted from the fecal suspension and selenite enrichment broth using the automated NucliSens easyMAG (bioMérieux) according to the manufacturer’s instructions. Briefly, 100 μl of fecal suspension and 50 μl of selenite enrichment broth were used as input. In addition, approximately 6,000 copies of the phocine herpesvirus 1 (PhHV), which served as an internal control (IC), were copurified. DNA was eluted in 110 μl of elution buffer. An aliquot (1 ml) from the selenite enrichment broth was stored at −20°C. The remaining selenite enrichment broth was stored at room temperature until further culture, depending on the real-time mPCR result. Every extraction run included a negative and a positive extraction control (NEC and PEC, respectively). The latter consisted of a pooled fecal suspension that was spiked with all target organisms that could be detected with the mPCR.

(iii) **Real-time mPCR.** Real-time amplification was carried out on an AB 7500 sequence detection system (Applied Biosystems, Nieuwerkerk a/d IJssel, Netherlands). The mPCR mixtures (25 μl) consisted of 1× TaqMan Universal PCR master mix (Applied Biosystems), 2.5 μl bovine serum albumin (Roche Diagnostics, Almere, Netherlands), 5 μl of template DNA, 2.5 μl of primer, 0.25 μl of probe, 200 nM of each dye, recorded an increase in fluorescent signal and hence a positive reaction. A real-time mPCR was considered inhibited when the Ct value of less than 40 cycles was recorded, and/or when the component tab, which displays the spectral contribution of each dye, recorded an increase in fluorescent signal and hence a positive reaction. A real-time mPCR was considered inhibited when the Ct value for the PhHV exceeded 36.07 cycles for mPCR-1 and 36.38 cycles for mPCR-2 (i.e., the mean Ct value for uninhibited specimens ± 2 standard deviations).

(v) **MSA guided culture/microscopy.** For all real-time mPCR-positive and -inhibited specimens, culture and microscopy were immediately started from the stored stool specimens by routine conventional procedures. Culture/microscopy was performed on mPCR-negative specimens. However, stool specimens specifically ordered for enteric parasites were screened using microscopy regardless of the outcome of the MSA.

All stool samples received on a Friday and during the weekend were cultured for *Campylobacter* spp. irrespective of mPCR outcome, as a delay of more than 1 day (caused by not performing the mPCR during the weekend) would result in a significantly lower potential to obtain a positive result for the MSA.

From October 2007 onward, an additional confirmation for STEC mPCR-positive samples was performed: from the cultured SMAC agar five *E. coli* colonies were subcultured and sent to the National Institute for Public Health and the Environment (RIVM, Bilthoven, Netherlands) for genotyping and serotyping of non-O157 *E. coli* strains.

**Statistical analysis.** We used chi-square to test whether the MSA detected more specific pathogens than conventional culture/microscopy and to compare the yield of MSA guided culture/microscopy with that of conventional culture/microscopy (NCSS 2007, Keysville, UT). Median values of subgroups were compared using the Wilcoxon rank-sum test (NCSS 2007). With both tests statistical significance was indicated by a two-tailed test (*P < 0.05*).

**RESULTS**

mPCR technical validation. The primer/probe sets for the detection of *S. enterica*, *C. jejuni*, *G. lamblia*, STEC, and *Shigella* spp./EIEC showed 100% inclusivity and exclusivity (data
TABLE 1. Results of the technical validation of the real-time mPCR on clinical stool specimens

<table>
<thead>
<tr>
<th>Pathogen</th>
<th>Target or result</th>
<th>Real-time mPCR result</th>
<th>Conventional diagnostic result</th>
<th>No. of specimens</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1st target</td>
<td>2nd target</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1st target</td>
<td>2nd target</td>
<td></td>
</tr>
<tr>
<td>Single infection</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S. enterica</td>
<td>Pos</td>
<td>Pos</td>
<td></td>
<td>79</td>
</tr>
<tr>
<td>C. jejuni</td>
<td>Pos</td>
<td>Neg</td>
<td></td>
<td>9b</td>
</tr>
<tr>
<td>G. lambia</td>
<td>Neg</td>
<td>Pos</td>
<td></td>
<td>4</td>
</tr>
<tr>
<td>STEC</td>
<td></td>
<td></td>
<td></td>
<td>139</td>
</tr>
<tr>
<td>Shigella spp./EIEC</td>
<td>Pos</td>
<td>Pos</td>
<td></td>
<td>19</td>
</tr>
<tr>
<td>Mixed infection</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S. enterica + STEC</td>
<td>Pos</td>
<td>Pos</td>
<td>Pos</td>
<td>1</td>
</tr>
<tr>
<td>S. enterica + Shigella</td>
<td>Pos</td>
<td>Pos</td>
<td>Pos</td>
<td>1</td>
</tr>
<tr>
<td>C. jejuni + G. lambia</td>
<td>Pos</td>
<td>Pos</td>
<td>Pos</td>
<td>1</td>
</tr>
<tr>
<td>C. jejuni + STEC</td>
<td>Pos</td>
<td>Pos</td>
<td>Pos</td>
<td>1</td>
</tr>
<tr>
<td>C. jejuni + Shigella spp./EIEC</td>
<td>Pos</td>
<td>Pos</td>
<td>Pos</td>
<td>1</td>
</tr>
<tr>
<td>STEC + G. lambia</td>
<td>Pos</td>
<td>Pos</td>
<td>Pos</td>
<td>1</td>
</tr>
<tr>
<td>Negative results</td>
<td>Neg</td>
<td>Neg</td>
<td>485</td>
<td></td>
</tr>
</tbody>
</table>

* Pos, positive; Neg, negative.

b Discrepancy analysis was performed on nine samples, using mPCR guided culture and confirmatory real-time PCR targeting the invA gene. Three samples were positive by guided culture and confirmatory PCR, and six samples were confirmatory PCR positive only.

c Discrepancy analysis was performed on 25 samples, using mPCR guided culture and confirmatory real-time PCRs targeting a Campylobacter spp.-specific region of the 16S rRNA gene and the glyA gene specific for C. coli. Of nine mPCR-positive samples the conventional culture had been positive for C. coli (seven times) and C. lari (two times). Reanalysis, using confirmatory PCRs, revealed four samples to contain C. jejuni and five samples to contain both C. jejuni and C. coli (mixed infection). Of 16 samples that were negative by conventional culture, 7 samples were positive by mPCR guided culture and Campylobacter spp. PCR, whereas 9 samples were Campylobacter spp. PCR positive only.

available upon request). Spiking experiments in a background of two pooled fecal matrices revealed that the analytical sensitivities of the assays were in the range of 10² to 10⁴ CFU/g of feces (data available upon request). Analysis of 828 clinical stool specimens revealed 338 samples positive by real-time mPCR, while only 278 samples were positive for the targeted organisms by conventional methods (Table 1). The overall sensitivity compared to that of the conventional methods was 98.2% (273 of 278), and the inhibition rate was approximately 5%. Based on these results, our laboratory implemented an MSA for these pathogens for use in daily practice. As the mPCR showed an almost 100% sensitivity in comparison to that of conventional methods, culture/microscopy for mPCR-negative specimens was not performed.

Performance of the MSA and conventional methods. The MSA was used on 13,974 samples (49.6%), whereas 14,211 samples were screened using conventional methods (50.4%). The MSA screened samples on all five pathogens, whereas conventional methods targeted either all pathogens or parasitic or bacterial pathogens only, depending upon the physician’s request.

The results for detection by the MSA and conventional methods are shown in Table 2.

Overall, 3,602 fecal samples (12.8%) were positive. Of these, 2,689 were positive by the MSA (19.2% of MSA samples), whereas 913 were positive using conventional methods (6.4%), resulting in a significant increase in pathogen detection frequency using the MSA (chi-square test, P < 0.001). PCR inhibition was observed in 1.8% of the stool specimens for both mPCR-1 (n = 246) and mPCR-2 (n = 255).

Detection of the individual pathogens by the MSA. Using the MSA, the detection frequencies of the five pathogens screened for were 8.1%, 4.7%, 3.0%, 1.9%, and 1.4% for C. jejuni, G. lamblia, S. enterica, STEC, and Shigella spp./EIEC, respectively (Table 2). The 1,136 C. jejuni-positive samples included 5 mPCR-inhibited samples that were positive in the MSA guided culture for C. jejuni. Also included was one C. jejuni guided culture-positive sample received on a Friday, which remained negative by mPCR. This sample was also mPCR positive for G. lamblia (concomitant infection). The 662 G. lamblia-positive samples included one mPCR-inhibited sample that was positive for G. lamblia in the MSA guided microscopy. The 424 S. enterica-positive samples included one mPCR-inhibited sample that was positive for S. enterica in the MSA guided culture.

Detection of pathogens by conventional methods. Using conventional methods, the detection frequencies were 5.3%, 3.3%, 3.1%, 0.2%, and 0.2% for C. jejuni, G. lamblia, S. enterica,

TABLE 2. Results of the molecular screening approach (MSA) versus conventional methods

<table>
<thead>
<tr>
<th>Pathogen</th>
<th>Total</th>
<th>No. (%) positive</th>
<th>No. of specimens with indicated MSA result</th>
<th>No. of specimens with indicated result by conventional methods</th>
<th>No. (%) positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. jejuni</td>
<td>13,956</td>
<td>1,136 (8.1)</td>
<td>869</td>
<td>246 (1.8)</td>
<td>7,004</td>
</tr>
<tr>
<td>G. lamblia</td>
<td>13,952</td>
<td>662 (4.7)</td>
<td>294</td>
<td>246 (1.8)</td>
<td>9,054</td>
</tr>
<tr>
<td>S. enterica</td>
<td>13,974</td>
<td>420 (3.0)</td>
<td>376</td>
<td>246 (1.8)</td>
<td>7,061</td>
</tr>
<tr>
<td>STEC</td>
<td>13,928</td>
<td>268 (1.9)</td>
<td>45</td>
<td>255 (1.8)</td>
<td>2,157</td>
</tr>
<tr>
<td>Shigella spp./EIEC</td>
<td>13,929</td>
<td>199 (1.4)</td>
<td>36</td>
<td>255 (1.8)</td>
<td>6,983</td>
</tr>
</tbody>
</table>

* Included were 6,981 stool specimens that were received at our laboratory with a specific request for the detection of parasitic pathogens.

b The total number of samples positive for MSA includes the inhibited samples that were guided culture/microscopy positive.

c Conventional methods consist of routine culture or microscopy (direct or TFT).
Shigella O157, and Shigella spp., respectively (Table 2). The 15 Shigella-positive samples included S. flexneri (n = 9) and S. sonnei (n = 6).

All individual pathogens were significantly more often detected with the MSA than by using conventional methods (chi-square test, P < 0.001), with the exception of S. enterica (P = 0.66).

**MSA guided culture and/or microscopy.** The MSA guided culture/microscopy yielded a positive result in 76.8% (869 of 1,132), 58.1% (294 of 506), 88.9% (376 of 423), 16.8% (45 of 268), and 18.1% (36 of 199) of mPCR-positive specimens for C. jejuni, G. lamblia, S. enterica, STEC, and Shigella/EIEC, respectively (Table 2). It appeared that the MSA guided culture improved recovery rates for Campylobacter jejuni in comparison to conventional culture; there was a significant increase in the number of C. jejuni isolates when using the MSA guided culture approach (chi-square test: P < 0.002). The chi-square test was not performed for G. lamblia, as the total number of positive samples screened with both mPCR and guided microscopy (n = 506) was substantially lower than the total number of mPCR-positive samples (n = 662). This discrepancy was caused due to differences in test request policies of physicians; for the 156 additional mPCR-positive samples, microscopy was not specifically requested. For all other screened pathogens no significant differences in isolate yield were found between the MSA guided culture and conventional culture.

**E. coli** colonies of 33 mPCR STEC-positive samples proved to be positive for non-O157 STECs. Thus, the MSA resulted in a significant increase in the number of guided culture-positive samples in comparison to culture for STEC O157 (n = 12) exclusively (Fisher’s exact test, P < 0.0001). Among the 33 successfully isolated STEC non-O157 strains, 19 different O serogroups were observed, in addition to 4 O-nontypeable STEC isolates. Most common were O63 (n = 4), O113 (n = 4), O103 (n = 3), O26 (n = 2), O51 (n = 2), O92 (n = 2), and O145 (n = 2).

The 36 Shigella isolates that were isolated with guided culture from mPCR-positive samples included S. sonnei (n = 20), S. flexneri (n = 12), S. dysenteriae (n = 1), and Shigella spp. (n = 3).

**Detection of multiple enteric pathogens in individual stool samples.** Multiple enteric pathogens in individual samples were detected significantly more often with the MSA than using conventional methods, with regard to the five screened pathogens (chi-square test, P < 0.0001). Conventional culture/microscopy detected only 3 individual samples with multiple enteric pathogens (0.02%), whereas the MSA detected 130 samples (0.9%) (Table 3). Mixed infections with C. jejuni were detected most frequently (n = 77), while co-infections with Shigella spp./EIEC were detected most infrequently (n = 41).

Of all C. jejuni-positive samples detected with the MSA, a total of 6.8% were co-infections. This fraction was higher for G. lamblia (7.6%) and S. enterica (10.6%). Interestingly, for Shigella spp./EIEC and STEC this fraction was much higher (20.6% and 19.4%, respectively).

Of the 130 co-infections detected with the MSA, a total of 22 (16.9%) could be confirmed with the subsequently performed MSA guided culture/microscopy (Table 3).

<table>
<thead>
<tr>
<th>Targets</th>
<th>No. of pathogens detected by:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MSA</td>
</tr>
<tr>
<td>Two pathogens</td>
<td></td>
</tr>
<tr>
<td>C. jejuni + STEC</td>
<td>20</td>
</tr>
<tr>
<td>C. jejuni + G. lamblia</td>
<td>19</td>
</tr>
<tr>
<td>C. jejuni + Shigella spp./EIEC</td>
<td>17</td>
</tr>
<tr>
<td>C. jejuni + S. enterica</td>
<td>16</td>
</tr>
<tr>
<td>S. enterica + STEC</td>
<td>13</td>
</tr>
<tr>
<td>G. lamblia + STEC</td>
<td>11</td>
</tr>
<tr>
<td>G. lamblia + Shigella spp./EIEC</td>
<td>10</td>
</tr>
<tr>
<td>S. enterica + G. lamblia</td>
<td>8</td>
</tr>
<tr>
<td>S. enterica + Shigella spp./EIEC</td>
<td>6</td>
</tr>
<tr>
<td>Shigella spp./EIEC + STEC</td>
<td>5</td>
</tr>
<tr>
<td>Total</td>
<td>125</td>
</tr>
</tbody>
</table>

a Included was one C. jejuni guided culture-positive sample received on Friday that remained negative by mPCR.

b Total number of samples with multiple enteric pathogens detected.

**Distribution of C_T values per screened enteric pathogen.** The distribution of C_T values of mPCR-positive specimens on which the MSA guided culture/microscopy was performed is shown per pathogen in Fig. 1. For all pathogens, C_T values of samples in which the MSA guided culture/microscopy result remained negative were significantly (all P < 0.0001) higher than in samples with positive guided culture/microscopy.

**Other microbiological findings.** A total of 1,083 (3.8%) additional pathogenic microorganisms were identified in the 28,185 stool specimens examined by MSA and routine diagnostic procedures. The following microorganisms were identified: non-C. jejuni Campylobacter spp. (n = 32), Clostridium difficile toxin (n = 244), Plesiomonas shigelloides (n = 1), adenovirus (n = 27), rotavirus (n = 47), norovirus (n = 108), Dientamoeba fragilis (n = 605), Cyclospora cayetanensis (n = 5), Entamoeba histolytica (n = 1), Enterobius vermicularis (n = 4), Hymenolepis nana (n = 4), a Taenia sp. (n = 3), Ascaris lumbricoides (n = 1), and Trichuris trichiura (n = 1).

**DISCUSSION**

We here present the first study comparing the conventional culture and microscopy with a molecular screening approach (MSA) for detection of S. enterica, C. jejuni, G. lamblia, STEC, and Shigella/EIEC in stool specimens.

The mPCR proved to have a superior performance in comparison to conventional methods in the technical validation phase. The technical validation that we performed was evaluated against the largest number of stool specimens to date in comparison to previous studies (10, 25). Also, this screening
panel is the most comprehensive one, covering five major enteric pathogens. Hence, our laboratory implemented the MSA in daily practice and discontinued routine culture/microscopy for all mPCR-negative samples.

Implementation of the MSA increased the detection frequency of GI pathogens 3-fold (19.2% compared to 6.4% with conventional methods). However, part of the increase may be due to the fact that the MSA screened for five pathogens in all samples, whereas physicians did not request screening on all pathogens for all samples that were examined with conventional methods. Undetected *G. lamblia* infections in stool specimens that were screened only for bacterial targets have previously been reported (37). As *G. lamblia* is highly prevalent, it seems appropriate to screen all stool specimens routinely for this pathogen.

We observed a significant increase by the MSA in the detection of all targeted pathogens with the exception of *S. enterica*. Previous studies also reported improvements in the detection rate of enteric pathogens by molecular assays (1, 7, 18, 25, 36, 37, 40). The reason why *S. enterica* was the only pathogen not detected more frequently by MSA than by culture (3.0%) may be the use of a sensitive selective enrichment broth. Therefore, even low loads of *S. enterica* can be detected by culture. A previous comparative evaluation study regarding the detection of *S. enterica* with real-time PCR, directly on stool suspensions, had sensitivities ranging...
from 85% to 91% in comparison to culture, including selective enrichment (31).

The performance of the MSA guided culture/microscopy varied substantially between pathogens. Guided culture confirmed 89% of S. enterica and 77% of C. jejuni mPCR-positive samples. The MSA guided culture of C. jejuni increased the yield of isolates compared to that of conventional culture. Schuurman et al. also reported this phenomenon, which is most likely due to higher awareness of the lab technician of the presence of Campylobacter colonies in mPCR-positive samples (32).

Of STEC and Shigella/EIEC mPCR-positive samples, only 17 to 18% could be confirmed. Other studies have reported higher success rates for STEC isolation by culture if serotypes other than O157 are also searched for (14, 15, 40). But in the routine laboratory, STECs other than O157 cannot be detected by means other than PCR aimed at the stx1 and/or stx2 genes. Furthermore, the mPCR has an analytical sensitivity that is at least 1 to 2 log10 greater than that of culture on SMAC medium (34). Although the yield of STEC was low when routine culture of the O157 subtype was used, picking E. coli colonies for further testing may significantly increase STEC isolation, as non-O157 isolates clearly predominate over the O157 serogroup. Others found non-O157 STEC proportions of 64%, 78%, and 80% (see references 2, 5, and 40, respectively).

For Shigella spp., the sensitivity and specificity of the enteric media used for culture (SS and HEA) might also be suboptimal, as described previously (6). Another factor explaining the low isolation rate of mPCR-positive stool specimens is the fact that mPCR targets the ipaH gene, which is plasmid bound and is carried by all Shigella spp. as well as by EIEC. As there are no routine methods for culturing EIEC, part of the mPCR-positive stool specimens could contain EIEC. Other molecular assays may differentiate Shigella spp. from EIEC (17, 19).

The performance of the MSA guided microscopy for G. lamblia was similar to that in earlier reports, with 53%, 63%, and 61% microscopy-confirmed samples (see references 1, 7, and 37, respectively).

This study revealed a significant increase in the detection of multiple enteric pathogens in stool samples by mPCR. This was also observed in another study (1). Interestingly, coinfections with C. jejuni were relatively infrequent, whereas Shigella/EIEC and STEC were more often found as a second pathogen. Possibly, the latter organisms may also be part of the normal intestinal flora or colonize the gut longer after infection than C. jejuni does. Amar et al. observed that Salmonella spp., non-jejuni/non-coli Campylobacter spp., enteraggregative E. coli, and rotavirus A were the agents most often associated with at least one other pathogen. Cryptosporidium spp., G. lamblia, and C. jejuni/E. coli were detected less frequently as coinfectants (1). Median C_T values of guided culture/microscopy-confirmed samples were significantly lower than for nonconfirmed specimens. However, except for S. enterica there was a large overlap of C_T values between the two groups of samples. A considerable proportion of samples had high C_T values. As we previously showed that our PCRs were free of contamination (32, 33, 34), we believe these high C_T values truly reflect the presence of these pathogens. Small amounts of pathogen DNA in a patient’s stool might correlate with (previous) disease or asymptomatic infection/colonization, as described previously for G. lamblia (12). Further studies are needed to elucidate the clinical relevance of the various positive real-time mPCR findings.

Although the hands-on time and time to generate final results were not recorded during this study, MSA is undoubtedly faster than conventional methods in generating (preliminary) results. Because MSA guided culture is started only after positive mPCR testing, subtyping of the pathogen and susceptibility testing take longer (32).

Another benefit of MSA is that this procedure could be implemented cost efficiently in our laboratory compared to conventional methods. The MSA procedure also permits the possibility of expanding the screening panel in order to detect additional enteric pathogens, with only little additional hands-on time and costs. An expansion including other highly prevalent pathogenic parasitological targets might replace routine microscopy, creating a single “stool” workflow. A disadvantage of replacing routine microscopy by MSA is the inability to detect nonanticipated cysts and ova. However, these are rare in the Netherlands (only 0.06% in the present study) and microscopy can still be added on suspicion of a parasitic infection.

In conclusion, this study demonstrated that MSA for gastrointestinal pathogens in a routine clinical microbiology laboratory improved the performance of testing (speed and sensitivity). This study illustrates the common presence of enteric pathogens in stool specimens, but the significance of some of these findings remains to be established.

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