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Feasibility of a Molecular Screening Method for Detection of *Salmonella enterica* and *Campylobacter jejuni* in a Routine Community-Based Clinical Microbiology Laboratory▽

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Conventional diagnostic methods for the detection of *Salmonella enterica* and *Campylobacter jejuni* are laborious and time-consuming procedures, resulting in final results, for the majority of specimens, only after 3 to 4 days. Molecular detection can improve the time to reporting of the final results from several days to the next day. However, molecular assays for the detection of gastrointestinal pathogens directly from stool specimens have not made it into the routine clinical microbiology laboratory. In this study we have assessed the feasibility of a real-time PCR-based molecular screening method (MSM), aimed at *S. enterica* and *C. jejuni*, in the daily practice of a routine clinical microbiology laboratory. We have prospectively analyzed 2,067 stool specimens submitted for routine detection of gastrointestinal bacterial pathogens over a 7-month period. The MSM showed 98 to 100% sensitivity but routine culture showed only 77.8 to 86.8% sensitivity when an extended "gold standard" that included all culture-positive and all MSM-positive specimens, as confirmed by an independent secondary PCR of a different target gene, was used. An overall improvement in the rate of detection of both pathogens of 15 to 18% was observed. Both approaches performed nearly identically with regard to the specificity, positive predictive value, and negative predictive value, with the values for MSM being 99.7%, 93.1 to 96.6%, and 99.8 to 100%, respectively, and those for routine culture being 100%, 100%, and 97.6 to 99.5%, respectively. Finally, the final results were reported between 3 and 4 days earlier for negative specimens compared to the time of reporting of the results of routine culture. Positive specimens, on the other hand, required an additional 2 days to obtain a final result compared to the time required for routine culture, although preliminary MSM PCR-positive results were reported, on average, 2.9 to 3.8 days before the final routine culture results were reported. In conclusion, MSM can be incorporated into the daily practice of a routine clinical microbiology laboratory with ease. Furthermore, it provides an improvement in the screening for *S. enterica* and *C. jejuni* and substantially improves the time to the reporting of negative results.

Infectious gastroenteritis is still a major public health burden in developed countries, although the related mortality is low (14). In The Netherlands, the total number of gastroenteritis cases in the population was estimated to be 4.5 million in 1999 (36), with an estimated annual cost of €345 million to Dutch society (34). *Salmonella enterica* and *Campylobacter jejuni* are the most prevalent enteric bacterial pathogens responsible for infectious gastroenteritis in The Netherlands, with reported incidences of 24/100,000 population and 36 to 37.3/100,000 population for *S. enterica* and *C. jejuni*, respectively (9, 10, 35, 36). *C. jejuni* seems to be more prevalent in older children (ages 5 to 14 years) and young adults (ages 15 to 29 years), whereas *S. enterica* tends to infect young children more often (10). Most infections with *S. enterica* and *C. jejuni* usually result in mild, self-limiting gastrointestinal disease. However, both organisms can give rise to more severe disease forms and complications, like typhoid fever, which is caused by *S. enterica* serovars Typhi and Paratyphi, or Guillain-Barré syndrome, which is a neurodegenerative disorder triggered by molecular mimicry of *C. jejuni* lipo-oligosacchrides and neural gangliosides (33, 38). About 1 in 1,000 *C. jejuni* infections develops into Guillain-Barré syndrome (3).

Routine diagnostic procedures for the screening of fecal samples for *S. enterica* and *C. jejuni* consist of selective culture methods, preceded by enrichment for *S. enterica*, and followed by biochemical identification, partial serotyping (*S. enterica*), and resistance profiling. These procedures are laborious and time-consuming, and it can take up to 4 days or more to obtain a final result. Furthermore, several diagnostic problems are encountered when stool specimens are screened for these pathogens. For instance, *S. enterica* grows with a suspicious colony morphology on salmonella-shigella medium (SS) and Hektoen enteric agar (HEA) plates, but bacteria present in the normal human gastrointestinal flora also show the same colony type (11, 31). These false-positive findings result in increases in hands-on times and the delay of the reporting of the final result for these negative specimens. Another type of problem may be caused by the viable but nonculturable state of *C. jejuni* (29, 30). This may compromise the sensitivity of culture approaches. Furthermore, the majority of stool specimens received by the clinical laboratory for examination for bacterial

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gastrointestinal pathogens do not yield a positive result. For instance, in The Netherlands, the average percentage of positive findings for bacterial gastrointestinal pathogens (i.e., *Salmonella* spp., *Campylobacter* spp., and *Shigella* spp.) was only 6.2% (36). The total workload involved in stool screening is high, as, for instance, our laboratory receives approximately 10,000 stool specimens annually, resulting in an average of about 40 stool specimens per day. Furthermore, the throughput of these specimens shows seasonal variance, with peaks at the end of winter and during the summer and early autumn (36). All of this makes screening of stool specimens for enteric pathogens a laborious procedure, especially during the seasonal peaks. Therefore, culture-independent methods may facilitate routine screening, especially when the bulk of the specimens, which are negative, can be eliminated quickly.

Real-time PCR provides a means for the accurate and fast detection of pathogens; however, only a limited number of reports describe the detection of *S. enterica* and/or *C. jejuni* directly from human stool specimens (13, 17, 23, 25). Furthermore, all of these reports performed the analysis with a (relatively) small number of fecal specimens (i.e., 25 to 145 specimens).

Therefore, to date, the applicability of (real-time) PCR for the diagnosis of gastroenteritis has remained limited. The major issue here is the inhibition caused by fecal constituents (7, 28, 37). However, this can be overcome by the use of amplification facilitators, like bovine serum albumin (2, 21) or thermostable DNA polymerases that are more resistant to inhibition (1). Furthermore, with the advances in real-time PCR technology, PCR diagnostics are no longer restricted to highly specialized (research and academic) laboratories but are increasingly becoming available to community laboratories as well.

The aim of the present study was to assess the feasibility of a molecular screening method (MSM) for *S. enterica* and *C. jejuni* in the routine practice of a community-based clinical microbiology laboratory. MSM-positive specimens were subsequently cultured for antibiotic resistance profiling and epidemiology. We prospectively analyzed 2,067 stool specimens sent to the laboratory for the detection of *S. enterica* and/or *C. jejuni* by both the standard approach and MSM.

MATERIALS AND METHODS

Bacterial and fungal strains. A panel of 33 *S. enterica* strains, 6 *C. jejuni* strains, and a total of 36 bacterial and fungal negative control strains representing other gastrointestinal pathogens and normal gastrointestinal flora was used in this study. The 33 *S. enterica* strains included the following serotypes; Agona, Albany, Anatum, Berta, Blockley, Bochum, Bovis Morbificans, Corvallis, Derby, Dublin, Enterica, Enteritidis (ATCC 13076), Goldcoast, Hadar, Infantis, London, Montevideo, Munster, Panama, Paratyphi A (two strains), Paratyphi B, Paratyphi var. Java, Poona, Rubislaw, Salamea, Saint Paul, Senftenberg, Stanley, Takoradi, Typhi, Typhimurium (ATCC 14028), and Virchow. The six *C. jejuni* strains comprised the following: ATCC 29428, CCUG 10935 (Penner serotype 1), CCUG 10936 (Penner serotype 2), CCUG 10937 (Penner serotype 3), CCUG 10938 (Penner serotype 4), and NTCC 11168 (Penner serotype 2). The 36 bacterial and fungal strains other than *S. enterica* and *C. jejuni* have been described previously (32).

All strains were from the strain database of the Laboratory for Infectious Diseases (LvI), unless stated otherwise (ATCC), and were grown on the appropriate media and under appropriate conditions for 16 to 48 h. After harvesting of the strains (approximately 10^9 CFU) into 1 ml of physiological saline, chromosomal DNA was extracted from the strains by heat lysis for 10 min at 95°C.

Study design and fecal specimens. All stool specimens used in this study were sent to the Department of Bacteriology of LvI for routine examination of enteric pathogens during the period from 27 June 2005 to 25 January 2006. A total of 2,067 stools specimens with sufficient sample volume to ensure reliable results for both routine culture and MSM were enrolled in the study. Of these 2,067 specimens, 2,055 stool specimens were eligible for use in the evaluation of the procedures for *S. enterica* (no *S. enterica* culture was performed for the remaining 12 specimens) and 2,009 stool specimens were eligible for use in the evaluation of the procedures for *C. jejuni* (no *C. jejuni* culture was performed for the remaining 58 specimens) (Table 1). All experiments were performed fully blinded, and the results of both routine culture and MSM were linked only when both approaches were completed.

Bacterial culture for *S. enterica*. Routine culture for enteric pathogens was carried out by the Department of Bacteriology of LvI and for *S. enterica* consisted of selenite enrichment and selective culturing on SS medium and HEA at 35°C, biochemical identification (urea, lysine decarboxylase, tryptone water, triple sugar iron agar, and *o*-nitrophenyl-β-D-galactopyranoside), and group-specific *Salmonella* (long/short polyvalent, A, B, b, C_1, C_2, D, d, Vi, E, F, and G) serological identification. All culture and identification media were from Mediaproducts BV (Mediaproducts BV, Groningen, The Netherlands), whereas the *Salmonella* agglutination sera were from Remel (Remel Europe Ltd., Dartford, United Kingdom). Resistance profiling was performed with the VITEK 2 system (bioMérieux, Boxtel, The Netherlands). All strains isolated were stored at −75°C so that they were available for further testing, if necessary.

Bacterial culture for *Campylobacter* species. 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 (6.0% O_2, 7.1% CO_2, 3.6% H_2, and 83.3% N_2) (Anoxomat system; Mart Microbiology BV, Lichtenvoorde, The Netherlands). Identification was carried out by Gram staining, oxidase testing, determination of hippurate hydrolysis, determination of the absence of aerobic growth at 42°C, and resistance profiling (disk diffusion method with ciprofloxacin, erythromycin, nalidixic acid, norfloxacin, tetracycline, and trimethoprim [Neo-Sensitabs 73412, 74012, 75812, 76212, 78417, and 79012, respectively; Rosco, Taastrup, Denmark] and cephalothin [kf30; Oxoid Limited, Basingstoke, United Kingdom]). Culture and identification media were from Mediaproducts BV. All strains isolated were stored at −75°C so that they were available for further testing, if necessary.

Molecular screening method for *S. enterica* and *C. jejuni*. (i) Specimen collection. Stool specimens, stored at 2 to 8°C after inoculation of routine bacterial cultures for enteric pathogens, were collected at the end of the day and brought to the Research and Development Department. A fecal suspension (33 to 50% [wt/vol]) was prepared and stored at −20°C until DNA extraction on the next day. A selenite enrichment broth from the same stool specimen was also inoculated and incubated for 16 h at 35°C, whereas the remaining portion of the stool specimen was stored at 2 to 8°C until further culture, depending on the real-time PCR result.

(ii) DNA extraction. DNA was extracted from the fecal suspension and selenite enrichment broth as described previously (32) by using NucliSens magnetic extraction reagents (bioMérieux) and a NucliSens miniMAG instrument (bioMérieux), according to the manufacturer's instructions. Approximately 6,000 copies of phocine herpesvirus 1 (PhHV) were added to the extraction mixture to serve as an internal control to monitor DNA extraction and PCR inhibition. An aliquot (1 ml) from the selenite enrichment broth was stored at −20°C, in case of inhibition of the PCR, whereas the remaining selenite enrichment broth was stored at room temperature until further culture, depending on the real-time PCR result.

(iii) Real-time PCR. Real-time amplification was carried out on an ABI PRISM 7700 sequence detection system (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands) by using the TaqMan Universal PCR master mix (Applied Biosystems). The primers and probes have been described previously for *S. enterica* (16), *C. jejuni* (5), and PhHV (32) and were purchased from Applied Biosystems. Probes were labeled (at the 5′ end) with 6-carboxyfluorescein (*S. enterica*), VIC (*C. jejuni*), or 2′-chloro-5′-fluoro-7′,8′-fused phenyl-1,4-dichloro-6-carboxyfluorescein (NED) (PhHV). Quenching of the 3′ end was conducted with either 6-carboxytetramethylrhodamine or a nonfluorescent quencher combined with a minor groove binder group. The reactions were set up as single PCRs for each individual target, with the reaction mixtures (25 μl) consisting of 1× TaqMan Universal PCR master mix (Applied Biosystems), 2.5 μg bovine serum albumin (Roche Diagnostics Nederland BV, Almere, The Netherlands), 5 μl of template DNA, the primers at 300 nM, and the probe at 100 nM (except for *S. enterica*, for which the probe was used at 400 nM). The reactions were run in a single reaction plate under the following conditions: 50°C for 2 min and 95°C

TABLE 1. Overall MSM, MSM-guided culture, routine culture, confirmatory, and inhibition results for all 2,067 tested stool specimens[a]

Organism(s) tested for	No. of specimens	MSM result		MSM-guided culture result		Routine culture result		Confirmatory PCR result		PCR inhibition	
		S. enterica	*C. jejuni*	*S. enterica*	*C. jejuni*	*S. enterica*	*C. jejuni*	*S. enterica*	*C. jejuni*	Primary	Secondary
S. enterica and	48	Pos	Neg	Pos	ND	Pos	Neg	ND	ND	Non Inh.	NA
C. jejuni	1	Pos	Neg	Pos	ND	Pos	Neg	ND	ND	Inh.	Non Inh.
	2	Pos	Neg	Pos	ND	Pos	Neg	ND	ND	Inh.	Inh.
	2	Pos	Neg	Pos	ND	Neg	Neg	ND	ND	Non Inh.	NA
	6	Pos	Neg	Neg	ND	Neg	Neg	Pos	ND	Non Inh.	NA
	4	Pos	Neg	Neg	ND	Neg	Neg	Neg	ND	Non Inh.	NA
	119	Neg	Pos	ND	Pos	Neg	Pos	ND	ND	Non Inh.	Non Inh.
	1	Neg	Pos	ND	Pos	Neg	Pos	ND	ND	Inh.	Non Inh.
	2	Neg	Pos	ND	Pos	Neg	Pos	ND	ND	Inh.	Inh.
	16	Neg	Pos	ND	Pos	Neg	Neg	ND	ND	Non Inh.	Non Inh.
	20	Neg	Pos	ND	Neg	Neg	Pos	ND	ND	Non Inh.	Non Inh.
	2	Neg	Pos	ND	Neg	Neg	Pos	ND	ND	Inh.	Non Inh.
	26	Neg	Pos	ND	Neg	Neg	Neg	ND	Pos	Non Inh.	Non Inh.
	7	Neg	Pos	ND	Neg	Neg	Neg	ND	Neg	Non Inh.	Non Inh.
	1	Pos	Pos	Neg	Pos	Neg	Pos	Neg	ND	Non Inh.	Non Inh.
	1	Pos	Pos	Pos	Pos	Pos	Pos	ND	ND	Non Inh.	Non Inh.
Subtotal	258										
	1669	Neg	Neg	ND	ND	Neg	Neg	ND	ND	Non Inh.	Non Inh.
	34	Neg	Neg	ND	ND	Neg	Neg	ND	ND	Inh.	Non Inh.
	31	Neg	Neg	ND	ND	Neg	Neg	ND	ND	Inh.	Inh.
	1	Neg	Neg	ND	ND	Neg	Neg	ND	ND	Inh.	NP
	3	Neg	Neg	ND	ND	Neg	Pos	ND	ND	Non Inh.	Non Inh.
	1	Neg	Neg	ND	ND	Neg	Pos	ND	ND	Inh.	Inh.
Subtotal	1,739										
S. enterica only	5	Pos	NA	Pos	NA	Pos	ND	ND	NA	Non Inh.	Non Inh.
	1	Pos	NA	Pos	NA	Pos	ND	ND	NA	Inh.	Non Inh.
	1	Pos	NA	Pos	NA	Neg	ND	ND	NA	Non Inh.	Non Inh.
	1	Pos	NA	Neg	NA	Pos	ND	ND	NA	Non Inh.	Non Inh.
	50	Neg	NA	ND	NA	Neg	ND	ND	NA	Non Inh.	Non Inh.
Subtotal	58										
C. jejuni only	3	NA	Pos	NA	Pos	ND	Pos	NA	ND	Non Inh.	Non Inh.
	1	NA	Pos	NA	Neg	ND	Neg	NA	Pos	Non Inh.	Non Inh.
	7	NA	Neg	NA	ND	ND	Neg	NA	ND	Non Inh.	Non Inh.
	1	NA	Neg	NA	ND	ND	Pos	NA	ND	Non Inh.	Non Inh.
Subtotal	12										
Total	2,067										

[a] Pos, positive; Neg, negative; ND, not done; Non Inh., not inhibited; NA, not applicable; Inh., inhibited; NP, not possible due to insufficient specimen volume.

for 10 min, followed by 40 cycles of 95°C for 15 s, 50°C for 15 s, and 60°C for 1 min. Amplification data were acquired and analyzed with Sequence Detector software (version 1.9.1; Applied Biosystems).

(iv) Real-time PCR interpretation and MSM-guided culture for *S. enterica* and *C. jejuni*. A real-time PCR was considered positive when a threshold cycle (C_T) of less than 40 cycles for *S. enterica* and/or *C. jejuni* was recorded. A real-time PCR was considered inhibited when the C_T value for the PhHV real-time PCR exceeded 35.40 cycles (i.e., the mean C_T value for uninhibited specimens ± 2 standard deviations). When a real-time PCR was inhibited, the specimen was retested in the next run by using a new DNA extract from the stored fecal suspension and selenite enrichment broth. For all real-time PCR-positive and inhibited specimens, cultures for *S. enterica* and/or *C. jejuni* were immediately started from the stored (2 to 8°C) fecal specimens by the same procedures described above for the routine culture of enteric pathogens. When an inhibited specimen showed a valid negative result upon the second real-time PCR analysis, the culture for that specimen was discontinued. Culture was not performed for all other PCR-negative specimens. All isolated strains were stored at −75°C so that they were available for further testing, if necessary.

Confirmatory real-time PCR assays. For confirmation of the results for the real-time PCR-positive and culture-negative (both routine and real-time PCR-guided) specimens, DNA was extracted from the stored fecal suspension (−20°C) and selenite enrichment broth (−20°C) and subjected to a real-time PCR targeting a different region of the designated organism's genome.

For *S. enterica*, the real-time PCR primer/probe set targeting the *ttrBCA* region described by Malorny et al. (27) was used in the same reaction mixture set up as described above for the *invA*, *mapA*, and PhHV assays, with the primers (Applied Biosystems) at 300 nM and the probe (5′-6-carboxyfluorescein, 3′-eclipse dark quencher; Eurogentec Nederland BV, Maastricht, The Netherlands) at 100 nM.

For *C. jejuni* confirmation, a primer set targeting a *Campylobacter* sp.-specific region of the 16S rRNA gene was used. The reaction mixture consisted of 1× SYBR green PCR master mix (Applied Biosystems), primers 5′-AGC GCA ACC CAC GTA-3′ and C1228R (25) at 300 nM (kindly donated by H. Wilke, Laboratory for Medical Microbiology Twente Achterhoek, Enschede, The Netherlands), 2.5 μg bovine serum albumin (Roche Diagnostics Nederland BV), and 5 μl of template DNA.

The reactions for confirmation of the results for both *S. enterica* and *C. jejuni* were run on a ABI 7500 real-time PCR system (Applied Biosystems) by applying the following thermoprofile: 2 min at 50°C and 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. The specific amplification of *C. jejuni* DNA was confirmed by melting curve analysis in comparison with the results for a positive control specimen (*C. jejuni* ATCC 29428).

Both assays showed identical sensitivities compared to the results of the real-time PCR assays targeting *invA* and *mapA*, which were used as the screening assays (data not shown).

For confirmation of *C. coli* detection by routine culture, a *C. coli*-specific real-time PCR targeting the *glyA* gene described by Jensen et al. (18) was used,

with the reaction set up identically to the reaction setups described above for the real-time PCR assays targeting *invA*, *mapA*, and the *ttrBCA* region. The reactions were run on an ABI 7500 real-time PCR system with the same thermoprofile used for the *S. enterica* and *C. jejuni* confirmation assays.

RESULTS

Selectivities of primer/probe sets for *S. enterica* and *C. jejuni*. The primer/probe sets for the *S. enterica invA* and *C. jejuni mapA* genes were tested for their selectivity, consisting of inclusivity and exclusivity, with DNA preparations from pure bacterial strains. The *S. enterica invA*-specific primer/probe set detected all 33 *S. enterica* strains, and no cross-reactivity with any of the non-*S. enterica* strains was observed (data not shown).

The *C. jejuni mapA* primer/probe set correctly identified all available strains of *C. jejuni* and showed no cross-reactivity with any of the non-*C. jejuni* strains (data not shown).

Although only a limited number of *S. enterica* and *Campylobacter* spp. were tested, no further testing was attempted since both tests have already been tested with 210 *Salmonella* strains and over 6,000 clinical *Campylobacter* isolates (5, 16).

LODs. The analytical sensitivities of the *S. enterica* and *C. jejuni* real-time PCR assays were assessed with spiked fecal specimens. For *S. enterica*, two fecal specimens (negative for *S. enterica* by culture and real-time PCR) were inoculated into selenite enrichment broth and spiked with 10-fold serial dilutions of *S. enterica* subsp. *enterica* serovar Typhimurium strain ATCC 14028, resulting in a concentration range of 1.8×10^9 to 0 CFU/g of feces for each fecal specimen. An unspiked fecal suspension (33 to 50% [wt/vol]) was also made and was stored at −20°C. DNA was extracted from the fecal suspensions and the enrichment broths as described above for the clinical stool specimens, and real-time PCR was subsequently performed with the isolated nucleic acids. Culture for *S. enterica* was also inoculated from the selenite enrichment broths for the lower 5 dilutions of the concentration range. Both real-time PCR and culture showed identical lower limits of detection (LODs) of 18 CFU/g of feces, although for one of the two stool specimens culture did not detect *Salmonella* serovar Typhimurium from the SS medium plate inoculated with 1.8×10^1 CFU/g (data not shown).

For *C. jejuni*, two fecal suspensions (33 to 50% [wt/vol]), prepared from two individual specimens (negative for *C. jejuni* by culture and real-time PCR), were spiked with serial dilutions of *C. jejuni* strain ATCC 29428, resulting in a range of from 2.4×10^7 to 2.4×10^1 CFU/ml of suspension. Depending on the consistency of the suspended stool specimen, this corresponds to a range of 4.8×10^6 to 7.2×10^6 to 4.8 to 7.2 CFU/g of feces. After real-time PCR analysis, the LODs for the *C. jejuni* real-time PCR were shown to be 4.8×10^2 to 7.2×10^2 CFU/g of feces (data not shown).

Detection of *S. enterica* and *C. jejuni* by real-time PCR and routine culture. A total of 2,067 clinical stool specimens were screened for *S. enterica* and *C. jejuni* by MSM, and for 2,055 and 2,009 of these specimens, routine culture for *S. enterica* and *C. jejuni*, respectively, was performed in parallel. Both sample and specimen receipt dates were available for 1,349 specimens and resulted in a mean transportation time of 1.7 days (range, 0 to 18 days; median, 1 day). For *S. enterica*, the routine culture result was regarded as positive only if a strain of *Salmonella* was isolated and not if other enteropathogens such as *Shigella* species were recovered from the SS medium and HEA plates. Routine culture for *Campylobacter* species was regarded as positive only when the isolated strain was typed as *C. jejuni*. This was mandatory due to the fact that the MSM was aimed only at *C. jejuni* and not at the other thermophilic *Campylobacter* species. Table 1 shows all the individual results of MSM, subsequent and routine culture, confirmatory PCR testing, and PCR inhibition for the 2,067 specimens tested.

TABLE 2. Test performance of MSM and routine culture compared to that of the extended gold standard

Application and result	No. of specimens with the following extended gold standard result[a]:		
	Positive	Negative	Total
S. enterica, MSM			
Positive	68	5	73
Negative	0	1,947	1,947
Unresolved	0	35	35
Total	68	1,987	2,055
S. enterica, routine culture			
Positive	59	0	59
Negative	9	1,987	1,996
Total	68	1,987	2,055
C. jejuni, MSM			
Positive	193	6	199
Negative	4	1,771	1,775
Unresolved	1	34	35
Total	198	1,811	2,009
C. jejuni, routine culture			
Positive	154	0	154
Negative	44	1,811	1,855
Total	198	1,811	2,009

[a] The extended gold standard result was defined as positive when the target organism was isolated by either routine culture or MSM-guided culture upon an MSM-positive result or when an MSM-positive result was confirmed by the species-specific confirmation PCR.

Performance of MSM and routine culture compared to that of an extended "gold standard." To independently compare the performance of the MSM and routine culture, an extended gold standard was defined to establish the status of the clinical stool specimens. True-positive specimens were defined as all specimens with a positive culture result for the targeted organism by either routine culture or MSM-guided culture and all additional specimens positive by both MSM and confirmatory real-time PCRs. True-negative specimens were defined as all specimens not regarded as true positive. Table 2 shows the performance of the MSM and routine culture for both *S. enterica* and *C. jejuni* in regard to this extended gold standard.

For both *S. enterica* and *C. jejuni*, the MSM showed the highest sensitivity, with sensitivities of 100% and 98.0%, respectively, whereas routine culture showed sensitivities of only 86.8% and 77.8%, respectively. With regard to specificity, both the MSM and routine culture showed excellent performance,

TABLE 3. Effect of delayed inoculation of MSM-guided culture compared to that of routine culture

Target and delay in no. of days to result between routine and MSM-guided culture	No. of specimens with the following culture results for routine culture/MSM-guided culture results[a]:		*P* value[b] for pairwise comparisons between the following different delays (days)[b]:				
	Pos/pos	Pos/neg	2	3	4	≥2	≥3
S. enterica							
1	27	1	0.99	0.99	1.0	0.47	0.99
2	16	0	NA	1.0	1.0	NA	1.0
≤2	43	1	NA	1.0	1.0	NA	0.99
3	5	0	NA	NA	1.0	NA	NA
≤3	48	1	NA	NA	0.99	NA	NA
4	10	0	NA	NA	NA	NA	NA
≥2	31	0	NA	NA	NA	NA	NA
≥3	15	0	NA	NA	NA	NA	NA
C. jejuni							
1	64	4	0.037	0.0029	0.20	0.0047	0.0088
2	26	7	NA	0.36	0.74	NA	0.99
≤2	90	11	NA	0.015	0.48	NA	0.045
3	14	7	NA	NA	0.30	NA	NA
≤3	104	18	NA	NA	0.76	NA	NA
4	20	4	NA	NA	NA	NA	NA
≥2	60	18	NA	NA	NA	NA	NA
≥3	34	11	NA	NA	NA	NA	NA

[a] Pos, positive; neg, negative.
[b] *P* values were determined by the two-sided Fisher's exact test. NA, not applicable.

with 99.7% specificity for both bacteria with the MSM and 100% specificity for both bacteria by routine culture. The positive predictive value (PPV) of the MSM was slightly lower, with PPVs of 93.1% and 96.9% for *S. enterica* and *C. jejuni*, respectively, compared to the results of routine culture (PPVs of 100% for both bacteria). With regard to the negative predictive value (NPV), both the MSM and routine culture showed excellent performance, with NPVs for *S. enterica* and *C. jejuni* of 100% and 99.8%, respectively, by MSM, and 99.5% and 97.6%, respectively, by routine culture. Finally, the diagnostic accuracies for *S. enterica* and *C. jejuni* were established to be 99.8% and 99.5%, respectively, for MSM and 99.6% and 97.8%, respectively, for routine culture.

To assess if the observed difference in sensitivity between routine culture and both the MSMs reached statistical significance, the data from Table 1, excluding those for the inhibited and nonconfirmed PCR-positive specimens, were analyzed by McNemar's test. This revealed that both MSMs were significantly more sensitive than routine culture ($P < 0.005$).

TATs for final results with MSM and routine culture. To assess the influence of the MSM on the turnaround time (TAT), the time to the final result was recorded for the specimens positive by both routine culture and MSM-guided culture for *S. enterica* ($n = 58$) and *C. jejuni* ($n = 127$) (concordant positive specimens), as well as for the concordant negative specimens ($n = 1{,}792$). The TAT for positive specimens was, on average, 6.9 ± 2.0 days (mean ± standard deviation; median, 7 days) for *S. enterica* by the MSM, with preliminary MSM PCR-positive results reported after, on average, 1.4 ± 0.8 days (median, 1 day), whereas routine culture needed 5.2 ± 1.8 days (median, 5 days). For *C. jejuni* the TAT was, on average, 6.3 ± 1.6 days (median, 6 days) for the MSM, with MSM preliminary PCR-positive results reported after 1.5 ± 0.9 days (median, 1 day) and routine culture results reported after 4.4 ± 1.3 days (median, 4 days). The results for the concordant negative specimens were reported by MSM after 1.5 ± 1.1 days (median, 1 day), whereas routine culture needed 3.9 ± 1.3 days (median, 4 days).

Effect of delayed inoculation for MSM-guided culture. Delayed inoculation of culture may prove detrimental for obtaining positive culture results, especially for *Campylobacter* species (9). As the MSM introduced a delay of at least 1 day between the inoculation of the routine culture and MSM-guided culture, we assessed the effect of the delayed inoculation caused by the MSM on the outcomes for these cultures in comparison with the routine culture result. Table 3 shows the delay between the inoculation of the routine culture and the MSM-guided culture for those specimens positive by both routine culture and MSM. Delayed inoculation did not have any significant effect on the outcome of the MSM-guided culture for *S. enterica*. For *C. jejuni*, on the other hand, a delay of more than 1 day resulted in a significantly lower potential to obtain a positive culture result for the MSM-guided cultures.

Analysis of discrepant results between MSM and routine culture. For nine cases identified as *C. jejuni* by the MSM, including one of the two cases where the *C. jejuni* strain isolated from the MSM approach could by specified only by PCR analysis, *Campylobacter* species other than *C. jejuni* were isolated by routine culture. These included *C. coli* ($n = 7$), and *C. lari* ($n = 2$). To further elucidate the status of these specimens, the isolates stored at −75°C were recultured and tested by PCRs specific for *C. jejuni*, *C. coli*, and *Campylobacter* species. In addition, the fecal DNA extracts from these specimens were also subjected to the same panel of PCRs. The results are shown in Table 4. The results showed that for three of the seven cases in which routine culture initially identified the

TABLE 4. Additional analysis of discrepant *C. jejuni* MSM/*Campylobacter* species routine culture results[a]

Discrepancy type and study identifier	Initial result by:		Result of additional PCR testing performed with:			Final conclusion
	MSM	Routine culture	MSM isolate	Routine culture isolate	Stool DNA extract	
MSM *C. jejuni* positive/routine culture non-*C. jejuni Campylobacter* spp. positive						
74	*C. jejuni*	*C. coli*	*C. jejuni*	NT	*C. jejuni*	*C. jejuni*
89	*C. jejuni*	*C. coli*	*C. jejuni*	NT	*C. jejuni*	*C. jejuni*
103	*C. jejuni*	*C. coli*	NSI	*C. coli*	*C. jejuni*/*C. coli*	Mixed infection
109	*C. jejuni*	*C. coli*	*C. coli*	*C. coli*	*C. jejuni*/*C. coli*	Mixed infection
183	*C. jejuni*	*C. coli*	*C. coli*	*C. jejuni*/*C. coli*	*C. jejuni*/*C. coli*	Mixed infection
385	*C. jejuni*	*C. lari*	*C. coli*	*C. coli*	*C. jejuni*/*C. coli*	Mixed infection
417	*C. jejuni*	*C. coli*	*C. jejuni*	NT	*C. jejuni*	*C. jejuni*
527	*C. jejuni*	*C. lari*	*C. jejuni*	*C. jejuni*	*C. jejuni*	*C. jejuni*
590	*C. jejuni*	*C. coli*	*C. coli*	NT	*C. jejuni*/*C. coli*	Mixed infection
MSM negative/routine culture positive						
331	Negative	*C. jejuni*	NSI	*C. coli*	Negative	*C. coli*
1492	Negative	*C. jejuni*	NSI	NT	*C. coli*	*C. coli*
1598	Negative	*C. jejuni*	NSI	*C. species*	*C. coli*	*C. coli*, presumably mixed with another *Campylobacter* sp.
1910	Negative	*C. jejuni*	NSI	*C. jejuni*	Negative	*C. jejuni*
MSM negative/routine culture non-*C. jejuni Campylobacter* spp. positive						
1302	Negative	*C. coli*	NSI	*C. coli*	*C. coli*	*C. coli*
1403	Negative	*C. coli*	NSI	NT	*C. coli*	*C. coli*
1873	Negative	*C. coli*	NSI	*C. coli*	*C. coli*	*C. coli*

[a] NSI, no strain isolated; NT, not tested due to the absence of growth of the stored isolate.

organism as *C. coli*, the additional PCR results showed no evidence for the presence *C. coli* in these specimens. Although the three strains originally isolated by routine culture could not be recultured from their storage at −75°C, it seems likely that they were in fact hippurate-negative *C. jejuni* strains. For the four other specimens originally identified as *C. coli* by routine culture, additional testing confirmed the presence of *C. coli* in these specimens. However, both *C. jejuni* and *C. coli* were identified by the additional PCRs in all four specimens, as well as in one of the stored isolates which originated from routine culture. From this it may be concluded that these cases are in fact mixed infections rather than single infections. For the two specimens originally identified as *C. lari* by routine culture, additional testing showed that one was in fact a mixed *C. jejuni*-*C. coli* infection, whereas the other was a single *C. jejuni* infection in which the strain was misidentified as *C. lari* due to a combination of a (false-) negative hipurate hydrolysis and nalidixic acid resistance phenotype.

Campylobacter spp. were also isolated by routine culture from seven specimens with negative MSM results. Identification of the strains in these seven specimens yielded *C. jejuni* on four occasions and *C. coli* on the remaining three. The isolated strains and the original stool DNA extracts from these specimens were subjected to the same testing strategy mentioned above for the specimens that were MSM positive and routine culture positive for non-*C. jejuni Campylobacter* spp., and the results are also shown in Table 4. Of the four cases identified by routine culture to be *C. jejuni*, the results for only one case could be confirmed by the additional PCR testing. In all three other cases, the PCRs identified *C. coli* rather than *C. jejuni*, although in one case another *Campylobacter* sp. which could not be identified as *C. coli* or *C. jejuni* may have been present. The last three specimens identified to contain *C. coli* by routine culture were confirmed to be *C. coli* positive by the additional PCR testing.

Other microbiological findings. Additional microorganisms other than *S. enterica* and *Campylobacter* spp. were identified in 194 of the 2,067 stool specimens examined by routine diagnostic procedures. Among these 194 specimens, the following groups of microorganisms were identified as single infections: pathogenic bacteria ($n = 20$), viruses ($n = 3$), and intestinal parasites ($n = 140$).

The following mixed infections were identified at the indicated frequencies: double infections by intestinal parasites, $n = 21$; double infections by a bacterial species and an intestinal parasite, $n = 6$; bacterial species and two intestinal parasites, $n = 1$; three intestinal parasites, $n = 2$; and four intestinal parasites, $n = 1$.

In addition, two other mixed infections involving *Salmonella* spp. and/or *C. jejuni* were identified during this study and consisted of one case of mixed infection with *S. enterica* serovar Typhi and *C. jejuni* and one case of mixed infection with serovar Typhi and serovar Saint Paul. Both of these cases were identified by the MSM and routine culture approaches.

DISCUSSION

In the present study we have assessed the feasibility of an MSM for the detection of the two gastrointestinal bacterial pathogens *S. enterica* and *C. jejuni*. Stool specimens were first screened by real-time PCR, and subsequently, cultures were inoculated only after a positive or inhibited MSM result was obtained. The MSMs proved to be significantly more sensitive that the traditional culture approaches. The rates of detection of *S. enterica* and *C. jejuni* improved by 15% to 18%. Previous studies have also shown improvements in the rates of detection of gastrointestinal pathogens (7, 17, 22, 24, 26).

In our study, only 4 specimens of the 266 true-positive specimens were detected by routine culture and not by the MSM approach, and all these specimens contained *C. jejuni*, based upon their initial identification. However, subsequent retesting of the isolated strains and/or stool DNA extracts showed no evidence for *C. jejuni* in three of these four specimens. This makes the results of the initial hippurate hydrolysis testing at least questionable. It has been reported by others that false-positive hippurate hydrolysis results can be obtained, and it has been hypothesized that this may be the result of the transfer of amino acids or peptides from the culture media or their production during the incubation (8, 19). If this is the case, the MSM would have missed only a single *C. jejuni*-positive stool specimen among the 195 confirmed cases, increasing the sensitivity and the NPV of the *C. jejuni* MSM to 99.5% and 99.9%, respectively. However, we cannot rule out the possibility that the initial identification was correct and that in fact these three specimens were mixed *C. jejuni*-*C. coli* infections.

The main advantage of the MSM is the speed in which definitely negative samples can be identified at a confidence higher than that for routine culture. During our study, the MSM generated negative results within a mean 1.5 days after the specimen was received by the laboratory, which is nearly 2.5 days earlier than the time to negative results by routine culture. These data confirm the hypothesized speed of the MSM in routine practice. With regard to generating final positive results, the MSM was slightly slower than routine culture (6.9 to 6.3 days and 5.2 to 4.4 days, respectively), resulting in average delays of 1.7 and 2.0 days for *S. enterica* and *C. jejuni*, respectively. A delay of 1 day is expected for both MSMs, as the MSMs have to wait for the PCR results before cultures can be inoculated. The further delay was most likely caused by the fact that VITEK 2 system identification and resistance profiling for *S. enterica* strains positive by MSM was usually performed overnight, since the isolates were identified in the afternoon. This was mandatory due to the fact that the MSM approach was carried out by a single technician, and therefore, all culture handlings took place after the PCR was performed. In addition, MSM cultures were prone to increased hands-on time due to the fact that they were performed by technicians who normally do not perform routine cultures for *S. enterica* and *C. jejuni*. When these MSM cultures are performed by routine technicians, it is, however, expected that the total delay will be no more than the 1 day introduced by PCR. On the other hand, due to the high specificities and PPVs of the MSMs, preliminary positive results based on the real-time PCR results can be reported to the attending physician with a high degree of confidence. These preliminary positive results were reported, on average, 3.8 and 2.9 days earlier than the final routine culture results for *S. enterica* and *C. jejuni*, respectively.

Another advantage of using the MSM approach is that it increases the overall sensitivity. The subsequent culturing upon a positive MSM result yielded in total 19 additional culture-positive specimens not detected by routine culture. This may be explained by the fact that a technician might be tempted to look more intensively for a suspicious colony because the MSM already flagged a specimen as positive, whereas in routine culture, the time pressure introduced by the overall workload may prevent such a more thorough search.

An interesting observation made during this study was the identification of several mixed *C. jejuni*-*C. coli* infections. Although in both separate approaches these mixed infections would not have been noticed, the discrepancy analysis was able to reveal their existence. Others have also reported mixed *C. jejuni*-*C. coli* infections in human patients with a similar prevalence (19, 24), as well as in cultures originating from food production animals or poultry and raw meat from retail sales outlets (8, 12, 20). Our results confirm the findings described in those previous reports and indicate that mixed *Campylobacter* infections do occur in about 1 to 5% of the laboratory-confirmed cases of *Campylobacter*-associated gastroenteritis.

Although PCR inhibition can pose a serious problem for the molecular-based screening of stool specimens, the methods used in our study proved to be insensitive to PCR inhibition. This is important if the MSM approach is to be implemented in the routine laboratory, as inhibited specimens still need to be cultured, whereas one of the main goals of the MSM is to reduce the TAT and the workload involved with stool cultures. Furthermore, the inhibition rates reported in this work (3.7% after initial testing, 1.8% after retesting) are regarded to be excellent. Others have reported inhibitions rates of 8 to 15% at similar input levels (4, 6, 15) and 1 to 63% at lower input levels (15, 28).

On the basis of the data presented here, the introduction of an MSM for routine processing of stool specimens in the clinical microbiology laboratory is feasible. However, to accommodate the introduction of an MSM in the routine laboratory, several adaptations to the format presented here may be necessary. First of all, to obtain an improved success rate with the subsequent isolation of *C. jejuni* from MSM-positive specimens, the delay in the inoculation of the stool specimens must be limited at most to 1 day after the specimen is received by the laboratory. To accommodate this, specimens received on a Friday and during the weekend should be inoculated for *C. jejuni* culture as soon as possible. On the other hand, the MSM approach could also be performed during the weekends and not only on weekdays, as was the case in our study. However, this will need a change in laboratory management, as most microbiology laboratories offer only limited services during the weekend. Furthermore, it is advisable to incorporate tests for other gastrointestinal pathogens into the MSM in an attempt to further decrease the culture workload involved with the testing of stool specimens. Multiplexing of the different MSMs seems to be mandatory because with the increase in the number of screened targets, the throughput per real-time PCR run is diminished, which may prove to be limiting from the perspective of the high workload needed to process all stool spec-

imens sent to the laboratory, especially during the seasonal peaks. Automation of the DNA extraction process may also be considered to decrease the hands-on time required by the technicians performing the MSM.

In conclusion, our data indicate that an MSM approach for the screening of stool specimens for gastrointestinal pathogens in a routine clinical microbiology laboratory is feasible and that the introduction of MSM will result in faster reporting of the final results for negative specimens, as well as preliminary positive results, and with only minimal delay in the reporting of the final results for positive specimens. In addition, the MSM will significantly improve the performance of the screening of stool specimens with regard to sensitivity, whereas the specificity, PPV, and NPV will also be improved, although to a smaller extent. We are currently conducting further research on automated extraction and multiplex real-time PCR to accommodate the introduction of an MSM for stool specimens in our routine laboratory.

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