ORIGINAL ARTICLE INFECTIOUS DISEASES

Case–control comparison of bacterial and protozoan microorganisms associated with gastroenteritis: application of molecular detection

L. E. S. Bruijnesteijn van Coppenraet[1], M. Dullaert-de Boer[2], G. J. H. M. Ruijs[1], W. A. van der Reijden[3], A. G. M. van der Zanden[2], J. F. L. Weel[4] and T. A. Schuurs[4]

1) *Laboratory for Medical Microbiology and Infectious Diseases, Isala, Zwolle, The Netherlands,* 2) *Laboratory for Medical Microbiology and Public Health, Hengelo, The Netherlands,* 3) *Regional Laboratory for Medical Microbiology and Public Health, Haarlem, The Netherlands and* 4) *Centre for Infectious Diseases Friesland, Izore, Leeuwarden, The Netherlands*

Abstract

The introduction of molecular detection of infectious organisms has led to increased numbers of positive findings, as observed for pathogens causing gastroenteritis (GE). However, because little is known about the prevalence of these pathogens in the healthy asymptomatic population, the clinical value of these additional findings is unclear. A case–control study was carried out in a population of patients served by general practitioners in the Netherlands. A total of 2710 fecal samples from case and matched control subjects were subjected to multiplex real-time PCR for the 11 most common bacterial and four protozoal causes of GE. Of 1515 case samples, 818 (54%) were positive for one or more target organisms. A total of 49% of the controls were positive. Higher positivity rates in cases compared to controls were observed for *Campylobacter* spp., *Salmonella* spp., *Clostridium difficile,* enteroinvasive *Escherichia coli/Shigella* spp., enterotoxigenic *E. coli,* enteroaggregative *E. coli,* atypical enteropathogenic *E. coli* (EPEC), *Cryptosporidium parvum/hominis,* and *Giardia lamblia.* However, *Dientamoeba fragilis* and Shiga-like toxigenic *E. coli* were detected significantly less frequent in cases than in controls, while no difference in prevalence was found for typical EPEC and enterohemorrhagic *E. coli.* The association between the presence of microorganisms and GE was the weakest in children aged 0 to 5 years. Higher relative loads in cases further support causality. This was seen for *Campylobacter* spp., *Salmonella* spp., enterotoxigenic *E. coli,* and *C. parvum/hominis,* and for certain age categories of those infected with *C. difficile,* enteroaggregative *E. coli,* and atypical EPEC. For *D. fragilis* and Shiga-like toxigenic *E. coli*/enterohemorrhagic *E. coli,* pathogen loads were lower in cases. Application of molecular diagnostics in GE is rapid, sensitive and specific, but results should be interpreted with care, using clinical and additional background information.
Clinical Microbiology and Infection © 2015 The Authors. Published by Elsevier Ltd on behalf of European Society of Clinical Microbiology and Infectious Diseases.

Keywords: Case–control, diarrhea, gastroenteritis, multiplexed real-time PCR, pathogen load
Original Submission: 17 December 2014; **Revised Submission:** 3 February 2015; **Accepted:** 9 February 2015
D. Raoult
Article published online: XXX

Corresponding author: L. E. S. Bruijnesteijn van Coppenraet, Isala, Dr van Heesweg 2, 8025AB Zwolle, The Netherlands
E-mail: e.s.bruijnesteijn@isala.nl

Introduction

Infectious gastroenteritis (GE) is a common illness with an incidence varying around 280 per 1000 person-years in the Netherlands and 190 per 1000 person-years in England, depending on the exact definition of GE and on seasonal peaks [1]. The burden for general practitioners (GP) is substantial; in the Netherlands, eight of every 1000 persons will visit the GP for gastrointestinal (GI) complaints, accounting for a total of 128 000 visits each year [2].

According to national guidelines, GPs may decide to send in samples for microbiologic examination. In the past, these samples were analysed mainly by antigen detection and/or culture for bacterial causes of GE, and by microscopy detection for parasitic causes. Nowadays, the detection of infectious agents by molecular methods has become the routine

Clinical Microbiology and Infection © 2015 The Authors. Published by Elsevier Ltd on behalf of European Society of Clinical Microbiology and Infectious Diseases
This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/)
http://dx.doi.org/10.1016/j.cmi.2015.02.007

ARTICLE IN PRESS

diagnostic method in many medical microbiologic laboratories in the Netherlands. It has replaced standard stool culture, antigen detection and microscopy. In general, molecular detection is rapid, sensitive and specific, and it enables universal application for viruses, parasites and bacteria using only one sample.

Using real-time PCR, a significant increase of *Campylobacter jejuni* infections was found [3]. For *Salmonella* spp. and *Shigella* spp./enteroinvasive *Escherichia coli* (EIEC), improved sensitivities are also obtained [4]. For *Yersinia enterocolytica,* it is now feasible to discern the pathogenic strains, whereas routine culture cannot discriminate between pathogenic and nonpathogenic types [5]. This also holds true for *E. coli* pathotypes. For *Clostridium difficile,* the detection of the toxin-coding genes enable swift and more sensitive diagnosis compared to the cumbersome cytotoxin neutralization test or the enzyme immunoassay method [6].

Protozoa are more often diagnosed after implementation of molecular detection [7]. Conventional diagnostics for protozoa consists of microscopy, often with poor sensitivity. The quality of detection relies greatly on the personal expertise and the training of laboratory technicians. Furthermore, each of the protozoa have specific difficulties in microscopic detection. For instance, *Cryptosporidium* requires specific staining methods to be visualized. As is true for bacteria, the sensitive molecular technique enables direct detection of pathogenic types: no longer is *Entamoeba dispar* found; only *Entamoeba histolytica* is detected. Also, intermittent shedding, as seen in giardiasis, is no longer relevant in such a sensitive assay. Finally, fixation of feces is no longer necessary for the detection of *Dientamoeba fragilis* [7].

In addition to these practical advantages, application of molecular detection has led to discussions about the interpretation and relevance of positive results. What is the value of detecting a small bacterial load, the detection of "possibly pathogenic" protozoa, or the detection of a virulence- or toxin-coding gene instead of the toxin itself? Case–control studies can further elucidate these issues. However, case–control studies, in which a general population in a developed country is investigated for a panel of GE agents using molecular methods, are lacking.

In this study, stool samples from subjects with and without GI complaints were investigated using internally controlled multiplexed real-time PCR. The positivity rates and the relative detectable loads were analysed for the most common bacterial and protozoan GI agents associated with GE.

Methods

Study population

The study population consisted of patients who visited the GP for GI complaints and for whom microbiologic examination was requested (cases), and a matched group of persons without GI complaints (controls). Matching criteria were age group (<5, 5–20, 21–50 and >50 years of age), month of sample collection, sex and region. Case and control subjects were requested to participate in the study by filling out a questionnaire and providing a fresh stool sample. GI complaints were defined as diarrhoea and/or other abdominal discomfort for which an infectious cause is likely, as assessed by the GP. Written approval was obtained by the medical ethics review board, and data for all samples were encoded to ensure anonymity according to the board's requirements. Control subjects were either recruited by the GP (54%; consisting of patients visiting their GP for a variety of non-GI medical problems, all fitting criteria for an immunocompetent patient) or were recruited by the laboratory and included healthy volunteers (46%). Control subjects were excluded if they had experienced GI complaints within 4 weeks before sample collection. In total, 2802 stool samples of case and control subjects were collected from August 2010 through December 2012.

Processing of stool samples

The stool samples from case and control subjects were processed by the four participating laboratories, each from a different representative region in the Netherlands, and were all gathered from the regions in which the collaborating laboratories were located. Routine diagnostic analysis performed prospectively for case samples was executed using local protocols. At each laboratory, handling and storage at −80°C of aliquoted stool samples was performed identically. A centralized and independent analysis of all the case and control samples was executed in a blinded fashion by one of the laboratories. The results of that analysis are presented here.

One aliquot of 100 μg frozen stool was used for nucleic acid extraction. Briefly, feces was suspended in 400 μL STAR buffer (Roche), vigorously shaken on a Magnalyser (1 minute; Roche) and pelleted (3 minutes, 13 000 rpm). A total of 100 μL of supernatant was extracted on the MagnaPure96 (MP96; Roche) using the DNA and Viral NA small volume kit, and total nucleic acids were eluted in 100 μL.

Real-time PCR

Internally controlled multiplexed real-time PCR was performed for the following microorganisms: *Campylobacter* spp., *Salmonella* spp., pathogenic *Yersinia enterocolitica,* toxigenic *Clostridium difficile, Shigella*/EIEC, enterohemorrhagic *E. coli* (EHEC), Shiga-like toxigenic *E. coli* (STEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), atypical and typical enteropathogenic *E. coli, Entamoeba histolytica, Giardia lamblia, Cryptosporidium parvum/hominis* and *D. fragilis.* PCR reactions were performed in multiplex format with the internal control

Clinical Microbiology and Infection © 2015 The Authors. Published by Elsevier Ltd on behalf of European Society of Clinical Microbiology and Infectious Diseases, *CMI*, **xxx**, 1.e1–1.e11
This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/)

Please cite this article in press as: Bruijnesteijn van Coppenraet LES, et al., Case–control comparison of bacterial and protozoan microorganisms associated with gastroenteritis: application of molecular detection, Clinical Microbiology and Infection (2015), http://dx.doi.org/10.1016/j.cmi.2015.02.007

detection included in each reaction mix. Bovine serum albumin (20 mg/mL; Invitrogen, The Netherlands) was added to 15 μL 2 × TaqMan Fast Advance Master Mix (Life Technologies, USA). Oligos diluted in Gibco molecular-grade water (Life Technologies) and 10 μL of DNA extract were added to the master mix to form a total reaction volume of 30 μL. Detection was executed with the ABI7500 real-time thermocycler (Life Technologies) with the following program: 45 cycles of 95°C for 15 seconds and 60°C for 60 seconds. Primers and probes are listed in Table 1.

The performance of the multiplex assays regarding sensitivity, specificity, reproducibility and stability was extensively tested and confirmed using analytical panels and clinical materials as well as international proficiency panels, if available.

The samples were checked by comparing the (prospective) diagnostic results of the separate laboratories with the results found by the centralized analysis described here. In case of discrepant results, both the local prospective method as performed by the participating laboratories and the retrospective method described here were repeated using a new frozen aliquot. In case of unresolved discrepancies, the sample was excluded.

Statistical analysis

Statistical analysis was performed by SPSS software v18 (IBM, USA). Dichotomous variables were tested by Fisher's exact test. Categorical variables with more than 2 categories were tested by the chi-square test (SPSS) or Fisher's exact test in case of small groups (SISA). Continuous variables were tested by the Mann-Whitney *U* test (exact in case of small groups). An alpha value of 0.05 (two-tailed) was used as the significance level.

Results

Study population

A total of 2802 samples were collected. Ninety-two samples were excluded because of missing information, inconclusive results or an insufficient amount of material (Fig. 1). After repeating the test for 14 samples, no samples were excluded as a result of inhibition of the PCRs. A total of 1515 case and 1195 control subjects were included for analysis.

Matching criteria were all met, except for the month of collection. Often a 1- to 2-week delay was observed between collection of the case and of the control samples. The distribution of the collected samples over the year is depicted in Fig. 2, and age distribution is provided in Fig. 3.

Of 1221 case and 713 control subjects, a completely filled-out questionnaire was present, and for an additional 293 case

TABLE 1. Study primers and probes

Target organism	Toxin/coding	Gene	Amount per reaction (pmol)	Name of oligo	Sequence	Reference	Adapted from reference
ETEC	Heat-stable toxin I	*estA*	10	Fst1	5′-AGT GGT CCT GAA AGC ATG AAT RGT AG-3′	This study	8
			10	Rst1	5′-CCC GGT ACA AGC AGG ATT ACA-3′	This study	8
			4.5	Pst1-MGB-FAM	FAM-5′-TTA CTG CTG TGA ATT G-3′-MGB	This study	
ETEC	Heat-stable toxin II	*estB*	10	Fst2	5′-GCA AAA TCC GTT TAA CTA ATC TCA AA-3′	8	
			10	Rst2	5′-TTG CCA ACA TTA GCT TTT TCA TG-3′	This study	8
			4.5	Pst2-TQ-FAM	FAM-5′-TCC GTG AAA CAA CAT GAC GGG AGG-3′-BHQ	This study	
Escherichia coli LT gen.	Heat-stable toxin	*eltB*	10	Flt	5′-GGC AGG CAA AAG AGA AAT GG-3′	8	
			10	Rlt	5′-TCC TTC ATC CTT TCA ATG GCT T-3′	This study	8
			1.8	Plt-TQ-NED	NED-5′-TCA GGT CGA AGT CCC GGG CAG-3′-BHQ	This study	
Shigella spp./EIEC	Invasive plasmid	*ipaH*	10	Fipah	5′-CCT TTT CCG CGT TCC TTG-3′	This study	9
			10	Ripah	5′-CGG AAT CCG GAG GTA TTG C-3′	9	
			4.5	Pipah-MGB-NED	NED-5′-CCT TTC CGA TAC CGT C-3′-MGB	This study	9
EPEC	Intimin	*eaeA*	10	Feaea	5′-GGC GAT TAC GCG AAA GAT ACC-3′	10	
			10	Reaea	5′-CCA GTG AAC TAC CGT CAA AGT TAT TAC C-3′	10	
			4.5	Peaea-MGB-VIC	VIC-5′-CAG GCT TCG TCA CAG TTG CAG GC-3′-MGB	10	
EHEC (/*Shigella*)*	Shiga-like toxin I	*stx1*	10	Fstx1	5′-TGG CAT TAA TAC TGA ATT GTC ATC ATC-3′	11	
			10	Rstx1	5′-GCG TAA TCC CAC GSA CTC TT-3′	11	
			4.5	Pstx1-MGB-FAM	FAM-5′-TTC CTT CTA TGT GTC CGG CAG-3′-MGB	11	
EHEC (/*Shigella*)*	Shiga-like toxin II	*stx2*	10	Fstx2	5′-CCG GAA TGC AAA TCA GTC G-3′	This study	11
			10	Rstx2	5′-ACC ACT RAA CTC CAT TAA CGC C-3′	11	
			4.5	Pstx2-MGB-VIC	VIC-5′-ACT CAC TGG TTT CAT CAT A-3′-MGB	11	
Salmonella spp.	Tetrathionate reductase	*ttr*	10	ttr-6	5′-CTC ACC AGG AGA TTA CAA CAT GG-3′	12	
			10	ttr-4	5′-AGC TCA GAC CAA AAG TGA CCA TC-3′	12	
			4.5	Psalm-TQ-VIC	VIC-5′-CAC CGA CGG CGA GAC CGA CT-3′-BHQ1	This study	12

Continued

Clinical Microbiology and Infection © 2015 The Authors. Published by Elsevier Ltd on behalf of European Society of Clinical Microbiology and Infectious Diseases, *CMI*, **xxx**, 1.e1–1.e11
This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/)

Please cite this article in press as: Bruijnesteijn van Coppenraet LES, et al., Case–control comparison of bacterial and protozoan microorganisms associated with gastroenteritis: application of molecular detection, Clinical Microbiology and Infection (2015), http://dx.doi.org/10.1016/j.cmi.2015.02.007

TABLE 1. Continued

Target organism	Toxin/coding	Gene	Amount per reaction (pmol)	Name of oligo	Sequence	Reference	Adapted from reference
Campylobacter spp. (16S)	ssu-rRNA	*16S*	10	Fcamp2	5′-CAC GTG CTA CAA TGG CAT ATA CAA T-3′	This study	13
			10	CampR2	5′-GG CTT CAT GCT CTC GAG TT-3′	13	
			4.5	Pcamp3-MGB-FAM	FAM-5′-TAT GTC CCA GTT CGG ATT G-3′-MGB	This study	13
Yersinia enterocolytica	Heat-stable toxin	*yst*	10	Pr2a	5′-AAT GCT GTC TTC ATT TGG AGC-3′	14	
			10	Pr2c	5′-ATC CCA ATC ACT ACT GAC TTC-3′	14	
			4.5	Pyent2-TQ-NED	NED-5′-CAA GCA AGC TTG TGA TCC TCC G-3′- BHQ1	14	
Typical EPEC	Bundle-forming pilin	*bfpA*	10	bfpA_F1	5′-ATC ACA CCT GCG GTA ACG G-3′	15	
			10	bfpA_F2	5′-TCA CAC CGG CGG TAA CG-3′	15	
			10	bfpA_R	5′-CGA RAA AGG TCT GTC TTT GAT TGA-3′	15	
			4.5	bfpA_P	NED-5′-CAG CAA GCG CAA GCA CCA TTG C-3′-BHQ1	15	
EAEC	Transcriptional regulator	*aggR*	10	aggR_F	5′-CAA TAA GGA AAA GRC TTG AGT CAG A-3′	15	
			10	aggR_R1	5′-TCA AGC AAC AGC AAT GCT GC-3′	15	
			10	aggR_R2	5′-TTA TCA AGC AAT AGC AAT GCT GCT-3′	15	
			6.0	aggR_P	VIC-5′-CCT TAT GCA ATC AAG AAT-3′-BHQ1	15	
EAEC	Dispersin translocator	*aat*	10	pCVD432_F1	5′-GGG CAG TAT ATA AAC AAC AAT CAA TGG-3′	15	
			10	pCVD432_F2	5′-GGG CAG TAT ATA AAC AAC AAC CAG TG-3′	15	
			10	pCVD432_R	5′-GCT TCA TAA GCC GAT AGA AGA TTA TAG G-3′	15	
			1.5	pCVD432_P1	FAM-5′-TCT CAT CTA TTA CAG ACA GCC-3′-MGB	15	
			1.5	pCVD432_P2	FAM-5′-CTC ATC TAT TAC AGA CAG CAA T-3′-MGB	15	
Clostridium difficile	Toxin A	*tcdA*	10	FtcdA2	5′-TTG TAT GGA TAG GTG GAG AAG TCA G-3′	This study	16
			10	CD-tcdA-R	5′-AAT ATT ATA TTC TGC ATT AAT ATC AGC CCA T-3′	16	
			3.0	MGB1	FAM-5′-ATA TTG CTC TTG AAT ACA TAA A-3′-MGB	16	
			3.0	MGB2	FAM-5′-TAT TGT TCT TGA ATA CAT AAA AC-3′-MGB	16	
Entamoeba histolytica	ssu-rRNA	*18S*	10	Ehd-239F	5′-ATT GTC GTG GCA TCC TAA CTC A-3′	17	
			10	Ehd-88R	5′-GCG GAC GGC TCA TTA TAA CA-3′	17	
			3.0	Histolytica-96T	VIC-5′-TCA TTG AAT GAA TTG GCC ATT T-3′-MGB	17	
Giardia lamblia	ssu-rRNA	*18S*	3.7	Giardia-80F	5′-GAC GGC TCA GGA CAA CGG TT-3′	17	
			3.7	Giardia-127R	5′-TTG CCA GCG GTG TCC G-3′	17	
			3.0	Giardia-105T	FAM-5′-CCC GCG GCG GTC CCT GCT AG-3′-BHQ	17	
Cryptosporidium parvum/hominis	DNAJ-ike protein		15	Fcpar	5′-CTT TTT ACC AAT CAC AGA ATC ATC AGA-3′	17	
			15	Rcpar	5′-TGT GTT TGC CAA TGC ATA TGA A-3′	17	
			3.0	Pcpar-MGB-Ned	NED-5′-TCG ACT GGT ATC CCT ATA A-3′-MGB	17	
Dientamoeba fragilis	5.8S-rRNA	*5.8S*	4.5	Df124F	5′-CAA CGG ATG TCT TGG CTC TTT A-3′	18	
			4.5	Df221R	5′-TGC ATT CAA AGA TCG AAC TTA TCA C-3′	18	
			3.0	Df172Trev MGB	FAM-5′-CAA TTC TAG CCG CTT AT-3′-MGB	18	
Internal control phocine herpesvirus	Glycoprotein B	*gB*	4.5/10	PhHV-267s	5′-GGG CGA ATC ACA GAT TGA ATC-3′	19	
			4.5/10	PhHV-337as	5′-GCG GTT CCA AAC GTA CCA A-3′	19	
			0.3/4.5	PhHV-305tq	CY5–5′-TTT TTA TGT GTC CGC CAC CAT CTG GAT C-3′-BHQ2	19	

EAEC, enteroaggregative *E. coli;* EHEC, enterohemorrhagic *E. coli;* EIEC, enteroinvasive *E. coli;* EPEC, enteropathogenic *E. coli;* ETEC, enterotoxigenic *E. coli;* LT, heat-labile enterotoxin.
*(/*Shigella*): target gene is also present in several *Shigella* strains, but not encountered in the *Shigella* strains included in the specificity panel.

Clinical Microbiology and Infection © 2015 The Authors. Published by Elsevier Ltd on behalf of European Society of Clinical Microbiology and Infectious Diseases, *CMI*, **xxx**, 1.e1–1.e11
This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/)

Please cite this article in press as: Bruijnesteijn van Coppenraet LES, et al., Case–control comparison of bacterial and protozoan microorganisms associated with gastroenteritis: application of molecular detection, Clinical Microbiology and Infection (2015), http://dx.doi.org/10.1016/j.cmi.2015.02.007

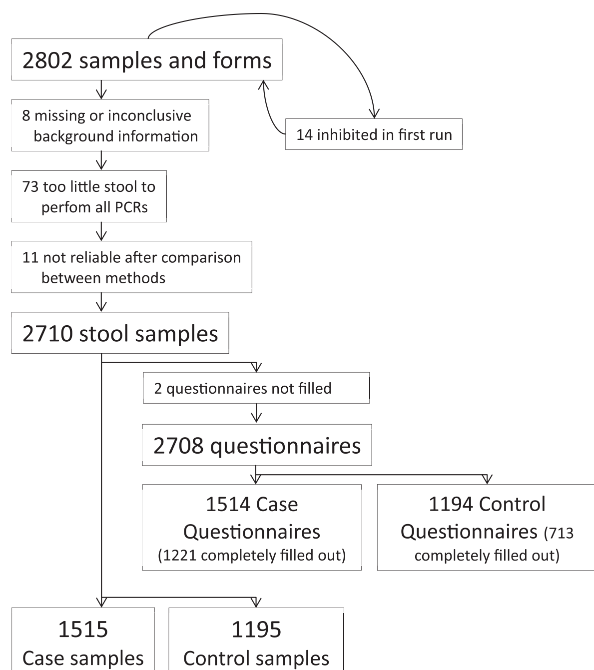

FIG. 1. Inclusion of samples and available background information.

and 481 control subjects, a partially filled-out questionnaire was available (Table 2). A total of 1137 case subjects (93.1%) reported having diarrhoea. Abdominal discomfort was the second most common symptom (69.7%). Other complaints—including the presence of mucus or blood in feces, vomiting and fever—were reported less frequently. Significantly more cases than controls reported recent travelling, antacid use and antibiotic use. Also, more household members with GE complaints were reported by the case subjects than by control subjects (Table 2).

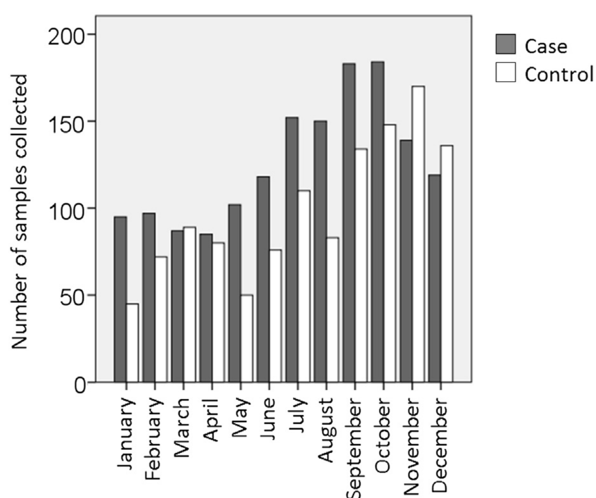

FIG. 2. Monthly distribution of collected stool samples from case and matched control subjects.

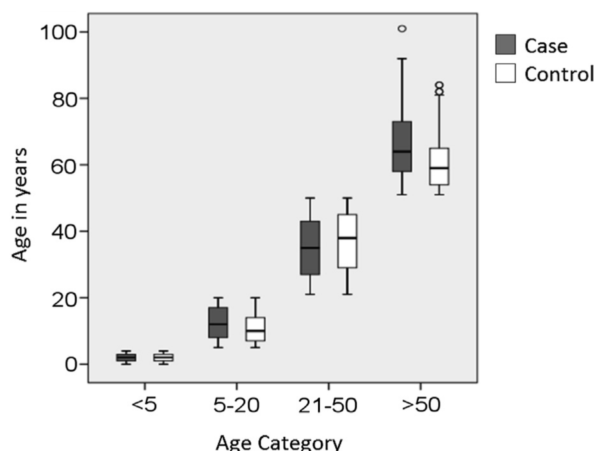

FIG. 3. Age distribution of collected stool samples by age category.

Positivity rates of GE-associated microorganisms

Of 1515 case samples, 818 (54%) were positive for one or more target organisms. Of 1195 control samples, 584 (48.9%) were positive (Table 3). A significantly greater positivity rate in case subjects compared to control subjects was observed for *Campylobacter* spp., *Salmonella* spp., *C. difficile*, *Shigella*/EIEC spp., ETEC, EAEC, atypical EPEC, *C. parvum/hominis* and *G. lamblia*. *D. fragilis,* however, was detected significantly less frequent in case subjects than in control subjects (25.7% and 37.3%, respectively; $p < 0.0001$). STEC was detected less often in case samples (borderline significance; p 0.067), whereas no significant difference in prevalence between case and control subjects was observed for typical EPEC and EHEC (Table 3).

For most targets, the positivity rate in case versus control subjects varied among age categories (Table 4). Frequently no differences between case and control subjects were seen for the youngest age categories (<5 and 5–20 years of age). For example, control subjects' carrying *C. difficile* was particularly high for children aged less than 5 years: 14.4% compared, to 10.5% of case subjects. In the older age categories, however, asymptomatic carriership decreased. A similar phenomenon was observed for *G. lamblia,* for which the age group 5 to 20 years had almost an identical portion of case subjects (7.7%) as control subjects (7.2%) who were found to be positive, whereas for the older age groups, more case subjects than control subjects were found to be positive.

Y. enterocolytica was only detected twice, both in case subjects. *E. histolytica* was not detected at all in this study.

In 216 case subjects (14.3%) and 107 control subjects (9.0%), more than one target organism was detected. Because *D. fragilis* was highly prevalent in this study, calculations were performed a second time with *D. fragilis* eliminated from the equation. This resulted in a total amount of 541 (35.7%) positive case samples, of which 124 (8.2%) were positive for more than one organism.

This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/)

Please cite this article in press as: Bruijnesteijn van Coppenraet LES, et al., Case–control comparison of bacterial and protozoan microorganisms associated with gastroenteritis: application of molecular detection, Clinical Microbiology and Infection (2015), http://dx.doi.org/10.1016/j.cmi.2015.02.007

ARTICLE IN PRESS

TABLE 2. Characteristics of study population and data retrieved from questionnaires

	Data retrieved from number of questionnaires		Case (*n* = 1515)		Control (*n* = 1195)			
Characteristic	Case	Control	No. positive	%	No. positive	%	p	Cases matched (%)
Age	1515	1185						
<5 years			152	10.4	104	8.8	NA	68.4
5–20 years			313	20.6	208	17.4	NA	66.5
21–50 years			557	36.8	445	37.6	NA	79.9
>50 years			493	32.2	428	36.1	NA	86.8
Sex	1515	1188						
Male			651	43.0	518	43.3	NA	79.6
Female			864	57.0	678	56.7	NA	78.5
Recent travel abroad	1514	1194	226	14.9	72	6.0	0.000	NA
Household members with gastroenteritis complaints	1282	713	221	14.6	43	3.6	0.000	NA
Antacid use	1501	1194	221	14.6	100	8.4	0.000	NA
Antibiotic use	1498	1192	99	6.5	29	2.4	0.000	NA
Diarrhoea	1221	NA	1137	93.1	NA	NA	NA	NA
No diarrhoea			84	6.9	NA	NA	NA	NA
<1 week diarrhoea			188	15.4	NA	NA	NA	NA
1–2 weeks' diarrhoea			313	25.6	NA	NA	NA	NA
>2 weeks' diarrhoea			636	52.1	NA	NA	NA	NA
Abdominal pain/cramps	1355	NA	944	69.7	NA	NA	NA	NA
Fever	1348	NA	185	13.7	NA	NA	NA	NA
Vomiting	1346	NA	175	13.0	NA	NA	NA	NA
Blood in stool	1345	NA	115	8.6	NA	NA	NA	NA
Mucus in stool	1343	NA	302	22.5	NA	NA	NA	NA

Partially filled out questionnaires are responsible for different counts of answers per question. Data regarding age and sex were retrieved from sample identifiers.
NA, not applicable.

TABLE 3. Overall positivity for the different target organisms

	Case (*n* = 1515)		Control (*n* = 1195)		
Organism	No. positive	%	No. positive	%	p (case vs. control)
Campylobacter spp.	154	10.2	33	2.8	0.000
Salmonella spp.	28	1.8	4	0.3	0.000
Pathogenic *Yersinia enterocolytica*	2	0.1	0	—	0.507
Clostridium difficile	64	4.2	21	1.8	0.000
Shigella/EIEC	14	0.9	0	—	0.000
EHEC	2	0.1	2	0.2	1.000
STEC	15	1.0	22	1.8	0.067
ETEC	48	3.2	8	0.7	0.000
EAEC	94	6.2	34	2.8	0.000
Atypical EPEC	144	9.5	84	7.0	0.022
Typical EPEC	10	0.7	10	0.8	0.655
Entamoeba histolytica	0	—	0	—	—
Giardia lamblia	85	5.6	33	2.8	0.000
Cryptosporidium parvum/hominis	46	3.0	10	0.8	0.000
Dientamoeba fragilis	390	25.7	446	37.3	0.000
One or more detections	818	54.0	584	48.9	0.008
Negative	697	46.0	611	51.1	
One or more detections excluding *D. fragilis*	541	35.7	230	19.2	0.000
Negative when excluding *D. fragilis*	974	64.3	965	80.8	
1 target organism excluding *D. fragilis*	417	27.5	204	17.1	0.000
2 target organisms excluding *D. fragilis*	90	5.9	21	1.8	
3 target organisms excluding *D. fragilis*	27	1.8	5	0.4	
4 target organisms excluding *D. fragilis*	7	0.5	0	—	

EAEC, enteroaggregative *Escherichia coli* (with *aggR* and/or *aat*); EHEC, enterohemorrhagic *E. coli* (with *eaeA* and *stx1* and/or *stx2*); EIEC, enteroinvasive *E. coli* (with *ipaH*); EPEC, enteropathogenic *E. coli* (typical with *eaeA* and *bfpA*, and atypical with only *eaeA*); ETEC, enterotoxigenic *E. coli* (with *lt* and/or *st*); STEC, Shiga-like toxigenic *E. coli* type (with *stx1* and/or *stx2*).

For the control samples, the total amount of positive findings was now 230 (19.2%); 26 samples (2.2%) showed multiple target organisms (Tables 3 and 4).

Ct values

Translating the Ct-value ranges for the different target organisms to relative loads, the organisms that showed significantly higher relative loads in cases than in controls were *Campylobacter* spp., *Salmonella* spp., ETEC, typical EPEC, *C. parvum/hominis* and *G. lamblia* (although statistical significance was not reached; p 0.084) (Fig. 4). For *Salmonella* spp., Ct values of controls were never below 33. For other target organisms, higher loads in case subjects were found only in specific age categories: *C. difficile* (age group 21–50), EAEC (age group 21–50) and atypical EPEC (age group <5). Frequently, similar trends were seen but did not reach significance or could not be

Clinical Microbiology and Infection © 2015 The Authors. Published by Elsevier Ltd on behalf of European Society of Clinical Microbiology and Infectious Diseases, *CMI*, **xxx**, 1.e1–1.e11
This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/)

Please cite this article in press as: Bruijnesteijn van Coppenraet LES, et al., Case–control comparison of bacterial and protozoan microorganisms associated with gastroenteritis: application of molecular detection, Clinical Microbiology and Infection (2015), http://dx.doi.org/10.1016/j.cmi.2015.02.007

TABLE 4. Positivity for all target organisms by age category

Organism	Case								Control								p (case vs. control)*			
	<5 (*n* = 152)		5–20 (*n* = 313)		21–50 (*n* = 557)		>50 (*n* = 493)		<5 (*n* = 104)		5–20 (*n* = 208)		21–50 (*n* = 445)		>50 (*n* = 428)					
	No. positive	%	No. positive	%	No. positive	%	No. positive	%	No. positive	%	No. positive	%	No. positive	%	No. positive	%	<5	5–20	21–50	>50
Campylobacter spp.	7	4.6	28	8.9	60	10.8	59	12.0	0	—	6	2.9	14	3.1	13	3.0	0.044	0.006	0.000	0.000
Salmonella spp.	1	0.7	7	2.2	10	1.8	10	2.0	1	1.0	2	1.0	1	0.2	0	—	1.000	0.328	0.028	0.002
Pathogenic *Yersinia enterocolytica*	0	—	1	0.3	1	0.2	0	—	0	—	0	—	0	—	0	—	—	1.000	1.000	—
Clostridium difficile	16	10.5	10	3.2	15	2.7	23	4.7	15	14.4	2	1.0	4	0.9	0	—	0.436	0.136	0.059	0.000
Shigella/EIEC	1	0.7	2	0.6	7	1.3	4	0.8	0	—	0	—	0	—	0	—	1.000	0.519	0.019	0.128
EHEC	0	—	1	0.3	0	—	1	0.2	0	—	0	—	2	0.4	0	—	—	1.000	0.197	1.000
STEC 1 and 2	0	—	2	0.6	8	1.4	5	1.0	1	1.0	2	1.0	11	2.5	8	1.9	0.406	0.653	0.252	0.402
ETEC	3	2.0	2	0.6	29	5.2	14	2.8	1	1.0	1	0.5	4	0.9	2	0.5	0.648	1.000	0.000	0.009
EAEC	11	7.2	12	3.8	39	7.0	32	6.5	5	4.8	4	1.9	17	3.8	7	1.6	0.601	0.301	0.037	0.000
Atypical EPEC	33	21.7	23	7.3	50	9.0	38	7.7	12	11.5	16	7.7	27	6.1	28	6.5	0.044	0.867	0.095	0.524
Typical EPEC	6	3.9	0	—	1	0.2	3	0.6	4	3.8	1	0.5	0	—	5	1.2	1.000	0.399	1.000	0.483
Entamoeba histolytica	0	—	0	—	0	—	0	—	0	—	0	—	0	—	0	—	—	—	—	—
Giardia lamblia	14	9.2	24	7.7	24	4.3	23	4.7	4	3.8	15	7.2	10	2.2	4	0.9	0.135	1.000	0.081	0.001
Cryptosporidium parvum/hominis	12	7.9	9	2.9	21	3.8	4	0.8	5	4.8	2	1.0	2	0.4	1	0.2	0.446	0.213	0.000	0.380
Dientamoeba fragilis	49	32.2	145	46.3	113	20.3	83	16.8	36	34.6	122	58.7	160	36.0	124	29.0	0.787	0.007	0.000	0.000
One or more detections	101	66.4	206	65.8	283	50.8	228	46.2	66	63.5	140	67.3	207	46.5	166	38.8	0.689	0.776	0.182	0.023
Negative	51	33.6	107	34.2	274	49.2	265	53.8	38	36.5	68	32.7	238	53.5	262	61.2				
One or more detections excluding *D. fragilis*	79	52.0	99	31.6	198	35.5	165	33.5	44	42.3	44	21.2	77	17.3	63	14.7	0.161	0.009	0.000	0.000
Negative when excluding *D. fragilis*	73	48.0	214	68.4	359	64.5	328	66.5	60	57.7	164	78.8	368	82.7	365	85.3				
1 target organism excluding *D. fragilis*	59	38.8	82	26.2	149	26.8	127	25.8	40	38.5	38	18.8	65	14.6	59	13.8				
2 target organisms excluding *D. fragilis*	15	9.9	13	4.2	33	5.9	29	5.9	4	3.8	5	2.4	9	2.0	3	0.7	0.056	0.088	0.000	0.000
3 target organisms excluding *D. fragilis*	5	3.3	3	1.0	14	2.5	5	1.0	0	—	1	0.5	3	0.7	1	0.2				
4 target organisms excluding *D. fragilis*	0	—	1	0.3	2	0.4	4	0.8	0	—	0	—	0	—	0	—				

EAEC, enteroaggregative *Escherichia coli* (with *agg*R and/or *aat*); EHEC, enteroheamorrhagic *E. coli* (with *eae*A and *stx*1 and/or *stx*2); EIEC, enteroinvasive *E. coli* (with *ipa*H); EPEC, enteropathogenic *E. coli* (typical with *eae*A and *bfpA*, and atypical with only *eae*A); ETEC, enterotoxigenic *E. coli* (with *lt* and/or *st*); STEC, Shiga-like toxigenic *E. coli* type (with *stx*1 and/or *stx*2).
*Significance (Fisher's exact test) included for cases vs. controls per age category.

This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/)

Please cite this article in press as: Bruijnesteijn van Coppenraet LES, et al., Case–control comparison of bacterial and protozoan microorganisms associated with gastroenteritis: application of molecular detection, Clinical Microbiology and Infection (2015), http://dx.doi.org/10.1016/j.cmi.2015.02.007

ARTICLE IN PRESS

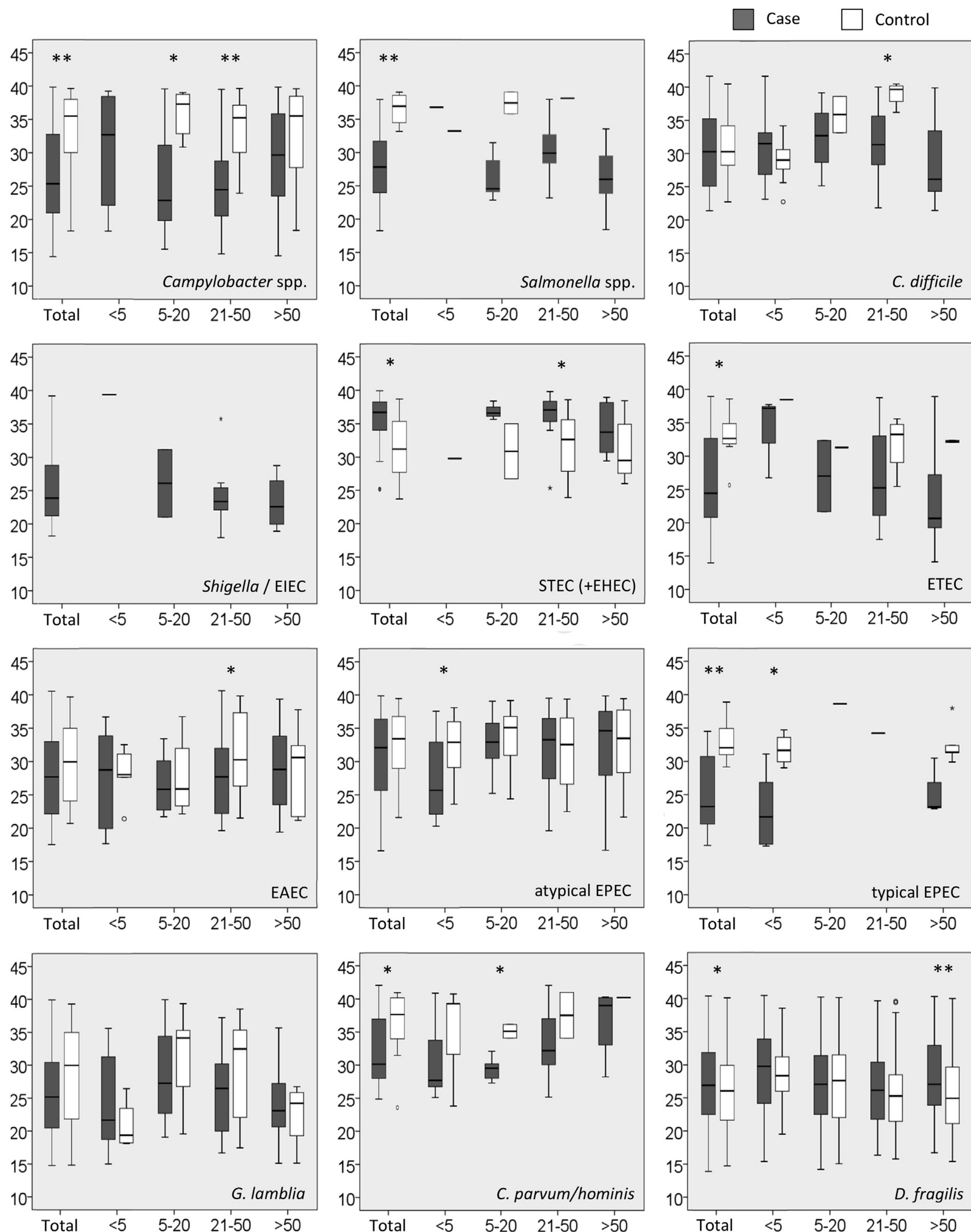

FIG. 4. Distribution of Ct values in case versus control subjects per age category. Difference between case and control subjects is significant at *p < 0.05 and **p < 0.005.

Clinical Microbiology and Infection © 2015 The Authors. Published by Elsevier Ltd on behalf of European Society of Clinical Microbiology and Infectious Diseases, CMI, xxx, 1.e1–1.e11
This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/)

Please cite this article in press as: Bruijnesteijn van Coppenraet LES, et al., Case–control comparison of bacterial and protozoan microorganisms associated with gastroenteritis: application of molecular detection, Clinical Microbiology and Infection (2015), http://dx.doi.org/10.1016/j.cmi.2015.02.007

ARTICLE IN PRESS

calculated at all as a result of the small number of positive findings (Fig. 4). Interestingly, the lower prevalences of STEC and *D. fragilis* (Table 3) were accompanied by significantly lower relative loads in case subjects compared to control subjects (Fig. 4).

Discussion

Comparison of positivity rates of the normal patient population, routinely tested in our laboratories, with those of the study population revealed similar results, thus ruling out selection bias (results not shown). Also, the occurrence of diarrhoea was only 93.1% in the case population. The criteria for GE were assessed by the GPs and therefore was in complete concordance with the variability of stool samples that are routinely processed in the participating laboratories.

In total, 54.0% of case subjects and 48.9% of control subjects were positive for one or more target organisms. Although all target organisms were expected to be more prevalent in case subjects than in control subjects, no difference in occurrence was observed for typical EPEC and EHEC. Remarkably, *D. fragilis* ($p < 0.0001$) and STEC (p 0.067) were detected even less frequently in case subjects compared to control subjects.

As a result of the high prevalence of *D. fragilis* in both case (25.7%) and control (37.3%) subjects, as well as its questionable pathogenic status, the total positivity rates were recalculated without *Dientamoeba:* 35.7% total positivity was found for case and 19.2% for control subjects. The reduction in positivity was observed among all age categories. However, in the <5-year category, the total asymptomatic positivity excluding *D. fragilis* was still 42.3% (Table 4). The total detection yield excluding *D. fragilis* for case subjects was also highest in the <5 age category, at 52%. Therefore, the statistical significant association of a positive detection with clinical illness is smallest in young children. The differences in detection rates between case and control subjects are more evident in the two older age categories, and therefore, causal relations between pathogen and complaints are stronger in the age categories 21–50 years and >50 years.

Few case–control studies have been published with comparable patient populations that investigated the occurrence of a panel of GI-associated organisms. One study in Great Britain used molecular detection methods on stored samples (1993–1996) from a population that had been previously tested using conventional methods [20]. The most important finding of Amar and colleagues [20] was the increased amount of positive detections both in case and control subjects for all target organisms that were included in the molecular reinvestigation. The second comparable study (1996–1999) was from the Netherlands and primarily used conventional methods [21]. Compared to control subjects, higher occurrences were found in case subjects for *Salmonella* spp., *Campylobacter* spp., *Cryptosporidium* and the viruses tested. Interestingly, the positivity rates for most pathogens tested (excluding *Shigella*/EIEC, *C. parvum/hominis* and *D. fragilis*) were in the same range as in our study. One would expect to find higher occurrences using molecular techniques, as has been shown in other studies [4,5]. A possible explanation is that since the de Wit study [21] was performed, the incidence of intestinal infections has decreased as a result of increased hygienic measures and awareness in the food industry and in the general population.

Furthermore, both Amar et al. [20] and de Wit et al. [21] reported a high prevalence of target organisms in control subjects, which seems to be in concordance with the results presented here. In the study of de Wit et al., Dientamoeba was observed at a nonsignificantly higher frequency in controls—a finding that was clearly significant in our study using molecular techniques.

In addition to prevalence, our case–control study also provides data on relative pathogen loads. Pathogen load is proposed to be a second informative factor to determine causality [22]. If a higher prevalence and a higher relative load (lower Ct values) in cases of GI complaints is expected to prove causality, then this is true for *Campylobacter* spp., *Salmonella* spp., ETEC and *C. parvum/hominis.* For *C. difficile,* EAEC and atypical EPEC, this was seen only in specific age categories. The opposite is true for *D. fragilis* and STEC, in which both load and prevalence were lower in case subjects (prevalence STEC was not significant).

C. difficile exemplifies an intestinal pathogen with a higher relative load in case subjects for those age categories in which higher prevalences were found. In the youngest age categories, the occurrence in control subjects was comparable to that in case subjects, as were the differences in pathogen load. High numbers of *C. difficile*–positive detection in children without GI complaints are known [23], while asymptomatic carriership among the elderly seems much less frequent [24]. This correlates with our findings, which found decreasing relative load with age in control subjects together with increasing differences in pathogen load between case and control subjects with age (Fig. 4).

Neither increased prevalence nor increased pathogen load in case subjects was seen for STEC. Nevertheless, this *E. coli* pathotype may cause severe clinical disease and has been identified in many GI (pseudo)outbreaks, most often related to food [25]. However, even in highly pathogenic STEC lineages, asymptomatic infection has been previously recognized [26]. Also, colonization of *E. coli* pathotypes in the large intestine

This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/)

Please cite this article in press as: Bruijnesteijn van Coppenraet LES, et al., Case–control comparison of bacterial and protozoan microorganisms associated with gastroenteritis: application of molecular detection, Clinical Microbiology and Infection (2015), http://dx.doi.org/10.1016/j.cmi.2015.02.007

ARTICLE IN PRESS

does not necessarily activate pathogenic properties, while infestation of the small intestine does [22]. Hence, substantial occurrences of *E. coli* pathotypes may have been expected in control subjects. However, in our study population, we found STEC (including EHEC) to be more prevalent in the control group than in the case group (borderline significance, p 0.067). Also, the Ct value range in control samples was significantly lower compared to cases (p 0.003; when including EHEC, p 0.008). This was true for both *stx*1-and *stx*2-positive strains. When these results are investigated in more detail and subtyping is performed, we hope to provide an explanation for the lack of disease association that we observed. These findings indicate that the molecular detection of *stx*1 and *stx*2 genes of *E. coli* pathotypes does not directly point to disease causality.

Another striking detection rate was observed for *D. fragilis*. Lower prevalences were found in case subjects. This was observed in all age groups, although a statistical difference was lacking in the youngest age category. Pathogen load was similar for all age categories except for the group of subjects aged >50 years, where relative loads were lower in cases. Doubts still exist about the pathogenic significance of *D. fragilis* [27]. Many publications report that *D. fragilis* is present in subjects with GI complaints, but little is known about the true prevalence in the healthy population [28]. Because cases arise from the healthy population and are thus superimposed on the prevalence in the healthy population, the prevalence of a pathogen will always be higher in cases than in the healthy population. In our study, both the lower prevalence in cases and the lack of differentiation in median Ct values between case and control subjects do not support pathogenicity. Moreover, Röser and colleagues [29] described a randomized double-blind, placebo-controlled treatment trial. Metronidazole significantly reduced the positivity in *D. fragilis*–infected children, whereas clinical complaints were unchanged and remained indistinguishable to the placebo-treated group. However, clinical relevance of intestinal *D. fragilis* infection cannot be ruled out completely and may still exist for individual cases.

In conclusion, for *Campylobacter* spp., *Salmonella* spp., ETEC and *C. parvum/hominis,* and for certain age categories of *C. difficile,* EAEC and atypical EPEC, increased prevalence and pathogen load in cases clearly suggest causality. However, because a large overlap in Ct value ranges exists for all target organisms except for *Salmonella* spp., pathogen load is unreliable to determine disease causality in practical use. Positive molecular detection results of STEC/EHEC or *D. fragilis* do not directly point to causality. The results of this study emphasize that detection of a GI pathogen must be accompanied by clinical data, and preferably other background and diagnostic data to have sufficient clinical meaning. The advantages of molecular detection in terms of enhanced sensitivity and speed is helpful in diagnosing microbiologic causes of GE, but positive findings must be interpreted with care.

Transparency declaration

The authors declare that they have no conflicts of interest.

Acknowledgements

We thank A. Ott for statistical expertise, D. de Jong for data analysis, Roche Diagnostics for financial support of nucleic acid extraction and Life Technologies for financial support of the PCR reactions.

References

[1] de Wit MA, Koopmans MP, Kortbeek LM, Wannet WJ, Vinjé J, van Leusden F, et al. Sensor, a population-based cohort study on gastroenteritis in the Netherlands: incidence and etiology. Am J Epidemiol 2001;154:666–74.

[2] de Wit MA, Koopmans MP, Kortbeek LM, van Leeuwen NJ, Bartelds AI, van Duynhoven YT. Gastroenteritis in sentinel general practices, The Netherlands. Emerg Infect Dis 2001;7:82–91.

[3] de Boer RF, Ott A, Güren P, van Zanten E, van Belkum A, Kooistra-Smid AM. Detection of *Campylobacter* species and *Arcobacter butzleri* in stool samples by use of real-time multiplex PCR. J Clin Microbiol 2013;51:253–9.

[4] de Boer RF, Ott A, Kesztyüs B, Kooistra-Smid AM. Improved detection of five major gastrointestinal pathogens by use of a molecular screening approach. J Clin Microbiol 2010;48:4140–6.

[5] Fredriksson-Ahomaa M, Korkeala H. Low occurrence of pathogenic *Yersinia enterocolitica* in clinical, food, and environmental samples: a methodological problem. Clin Microbiol Rev 2003;16:220–9.

[6] Pancholi P, Kelly C, Raczkowski M, Balada-Llasat JM. Detection of toxigenic *Clostridium difficile*: comparison of the cell culture neutralization, Xpert *C. difficile*, Xpert *C. difficile*/Epi, and Illumigene *C. difficile* assays. J Clin Microbiol 2012;50:1331–5.

[7] Bruijnesteijn van Coppenraet LE, Wallinga JA, Ruijs GJ, Bruins MJ, Verweij JJ. Parasitological diagnosis combining an internally controlled real-time PCR assay for the detection of four protozoa in stool samples with a testing algorithm for microscopy. Clin Microbiol Infect 2009;15:869–74.

[8] Lothigius A, Janzon A, Begum Y, Sjöling A, Qadri F, Svennerholm AM, et al. Enterotoxigenic *Escherichia coli* is detectable in water samples from an endemic area by real-time PCR. J Appl Microbiol 2008;104:1128–36.

[9] Vu DT, Sethabutr O, Von Seidlein L, Tran VT, Do GC, Bui TC, et al. Detection of *Shigella* by a PCR assay targeting the *ipa*H gene suggests increased prevalence of shigellosis in Nha Trang, Vietnam. J Clin Microbiol 2004;42:2031–5.

[10] Karns JS, Van Kessel JS, McClusky BJ, Perdue ML. Incidence of *Escherichia coli* O157: H7 and *E. coli* virulence factors in US bulk tank milk as determined by polymerase chain reaction. J Dairy Sci 2007;90:3212–9.

[11] Schuurman T, Roovers A, van der Zwaluw WK, van Zwet AA, Sabbe LJ, Kooistra-Smid AM, et al. Evaluation of 5′-nuclease and

Clinical Microbiology and Infection © 2015 The Authors. Published by Elsevier Ltd on behalf of European Society of Clinical Microbiology and Infectious Diseases, *CMI*, **xxx**, 1.e1–1.e11
This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/)

Please cite this article in press as: Bruijnesteijn van Coppenraet LES, et al., Case–control comparison of bacterial and protozoan microorganisms associated with gastroenteritis: application of molecular detection, Clinical Microbiology and Infection (2015), http://dx.doi.org/10.1016/j.cmi.2015.02.007

ARTICLE IN PRESS

hybridization probe assays for the detection of shiga toxin-producing *Escherichia coli* in human stools. J Microbiol Methods 2007;70:406–15.

[12] Malorny B, Paccassoni E, Fach P, Bunge C, Martin A, Helmuth R. Diagnostic real-time PCR for detection of *Salmonella* in food. Appl Environ Microbiol 2004;70:7046–52.

[13] Lund M, Nordentoft S, Pedersen K, Madsen M. Detection of *Campylobacter* spp. in chicken fecal samples by real-time PCR. J Clin Microbiol 2004;42:5125–32.

[14] Zheng H, Wang J, Sun Y, Jiang B. Clinical isolation and characterization of *Yersinia enterocolitica* in China using real-time PCR and culture method. Digestion 2007;75:199–204.

[15] Friesema IH, de Boer RF, Duizer E, Kortbeek LM, Notermans DW, Norbruis OF, et al. Etiology of acute gastroenteritis in children requiring hospitalization in the Netherlands. Eur J Clin Microbiol Infect Dis 2012;31:405–15.

[16] Knetsch CW, Bakker D, de Boer RF, Sanders I, Hofs S, Kooistra-Smid AM, et al. Comparison of real-time PCR techniques to cytotoxigenic culture methods for diagnosing *Clostridium difficile* infection. J Clin Microbiol 2011;49:227–31.

[17] Verweij JJ, Blangé RA, Templeton K, Schinkel J, Brienen EA, van Rooyen MA, et al. Simultaneous detection of *Entamoeba histolytica, Giardia lamblia*, and *Cryptosporidium parvum* in fecal samples by using multiplex real-time PCR. J Clin Microbiol 2004;42:1220–3.

[18] Verweij JJ, Mulder B, Poell B, van Middelkoop D, Brienen EA, van Lieshout L. Real-time PCR for the detection of *Dientamoeba fragilis* in fecal samples. Mol Cell Probes 2007;21:400–4.

[19] Niesters HG. Clinical virology in real time. J Clin Virol 2002;25(Suppl. 3):S3–12.

[20] Amar CF, East CL, Gray J, Iturriza-Gomara M, Maclure EA, McLauchlin J. Detection by PCR of eight groups of enteric pathogens in 4,627 faecal samples: re-examination of the English case–control Infectious Intestinal Disease Study (1993–1996). Eur J Clin Microbiol Infect Dis 2007;26:311–23.

[21] de Wit MA, Koopmans MP, Kortbeek LM, van Leeuwen NJ, Vinjé J, van Duynhoven YT. Etiology of gastroenteritis in sentinel general practices in the Netherlands. Clin Infect Dis 2001;33:280–8.

[22] Robins-Browne RM, Levine MM. Laboratory diagnostic challenges in case/control studies of diarrhea in developing countries. Clin Infect Dis 2012;55(Suppl. 4):S312–6.

[23] Morris O, Tebruegge M, Pallett A, Green SM, Pearson AD, Tuck A, et al. *Clostridium difficile* in children: a review of existing and recently uncovered evidence. Adv Exp Med Biol 2013;764:57–72.

[24] Arvand M, Moser V, Schwehn C, Bettge-Weller G, Hensgens MP, Kuijper EJ. High prevalence of *Clostridium difficile* colonization among nursing home residents in Hesse, Germany. PLoS One 2012;7:e30183.

[25] Kaper JB, Nataro JP, Mobley HL. Pathogenic *Escherichia coli*. Nat Rev Microbiol 2004;2:123–40.

[26] Balabanova Y, Klar S, Deleré Y, Wilking H, Faber MS, Lassen SG, et al. Serological evidence of asymptomatic infections during *Escherichia coli* O104: H4 outbreak in Germany in 2011. PLoS One 2013;8:e73052.

[27] Johnson EH, Windsor JJ, Clark CG. Emerging from obscurity: biological, clinical, and diagnostic aspects of *Dientamoeba fragilis*. Clin Microbiol Rev 2004;17:553–70.

[28] Barratt JL, Harkness J, Marriott D, Ellis JT, Stark D. A review of *Dientamoeba fragilis* carriage in humans: several reasons why this organism should be considered in the diagnosis of gastrointestinal illness. Gut Microbes 2011;2:3–12.

[29] Röser D, Simonsen J, Stensvold CR, Olsen KE, Bytzer P, Nielsen HV, et al. Metronidazole therapy for treating dientamoebiasis in children is not associated with better clinical outcomes: a randomized, double-blinded and placebo-controlled clinical trial. Clin Infect Dis 2014;58:1692–9.

This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/)

Please cite this article in press as: Bruijnesteijn van Coppenraet LES, et al., Case–control comparison of bacterial and protozoan microorganisms associated with gastroenteritis: application of molecular detection, Clinical Microbiology and Infection (2015), http://dx.doi.org/10.1016/j.cmi.2015.02.007