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

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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.
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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

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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

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