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A Quantitative Polymerase Chain Reaction Assay for Rapid Detection of 9 Pathogens Directly From Stools of Travelers With Diarrhea

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BACKGROUND & AIMS: **Every year, 80 million tourists traveling to tropical and subtropical areas contract traveler's diarrhea (TD). Forty percent to 80% of cases are caused by bacteria, yet clinical diagnostic tests are available to identify only a few of the strains that cause TD. We aimed to develop a quantitative polymerase chain reaction (qPCR) assay to identify all major pathogens in stool samples.**

METHODS: **We developed a low-cost, high-throughput, multiplex qPCR assay for simultaneous detection of 9 bacterial pathogens in stool samples: *Salmonella*, *Yersinia*, *Campylobacter*, and *Vibrio cholerae*, as well as *Shigella* or enteroinvasive *Escherichia coli*, enterohemorrhagic *E coli*, enterotoxigenic *E coli* (ETEC), enteroaggregative *E coli* (EAEC), and enteropathogenic *E coli* (EPEC). The assay was validated using positive (n = 245) and negative (n = 243) control strains, as well as preselected positive and negative stool samples. In addition, stool samples were collected from 96 returning travelers with TD. The findings were compared with those from routine diagnostic tests.**

RESULTS: **The assay detected the bacterial strains with 100% sensitivity and specificity, compared with results from the reference tests. Of all stool samples collected from travelers with TD, EPEC was found in 47%, EAEC in 46%, ETEC in 22%, enterohemorrhagic *E coli* in 7%, *Campylobacter* in 6%, *Shigella* or enteroinvasive *E coli* in 2%, and *Salmonella* in 2%. Multiple pathogens were found in 37% of all samples.**

CONCLUSIONS: **We developed a low-cost, high-throughput qPCR assay for use in routine diagnostic analysis and research. It detects the pathogenic bacteria most commonly associated with TD in stool samples with 100% sensitivity and specificity, compared with reference methods. The assay requires 4 hours, whereas current detection methods require 1 to 7 days. At least 1 TD pathogen was identified in stool samples from 76% of returning travelers, whereas conventional methods found a pathogen in only 17%. The most commonly detected bacteria were EPEC, EAEC, and ETEC.**

Keywords: Gastroenteritis; Tourist; Fecal Sample; PCR Assay.

Two hundred million tourists from industrialized countries travel to tropical and subtropical areas every year,[1] and approximately 80 million of them contract diarrhea (30%–70%, according to the Centers for Disease Control[2]). Despite the high rate of traveler's diarrhea (TD), in the majority of patients the causative agents remain unidentified because of poor diagnostics.

Numerous studies have focused on a more detailed characterization of the TD pathogens, failing, however, to identify them in up to 50% of all cases.[3–5] The proportion remains similar even in many recent studies,[6,7] except for a few in which the percentage of unexplained cases has decreased to 6% to 31%.[8–11] In all these investigations, TD mainly is considered to be of bacterial origin. Viruses or parasites have been identified in 5% to 25% of cases.[3,12] Most studies have reported enterotoxigenic *Escherichia coli* (ETEC) to be the most common pathogen, followed by *Campylobacter*, *Salmonella*, and *Shigella*.[3,4,10] Recently, methods based on molecular techniques have been used, and other bacteria, such as enteroaggregative *E coli* (EAEC), have proved substantially more common than anticipated previously.[8,13]

The methods available for routine clinical stool analyses mostly consist of traditional cultures and immunoassays that are both slow and laborious and can identify only a few of the

Abbreviations used in this paper: EAEC, enteroaggregative *Escherichia coli*; EHEC, enterohemorrhagic *Escherichia coli*; EPEC, enteropathogenic *Escherichia coli*; ETEC, enterotoxigenic *Escherichia coli*; PCR, polymerase chain reaction; qPCR, quantitative polymerase chain reaction; TD, traveler's diarrhea.

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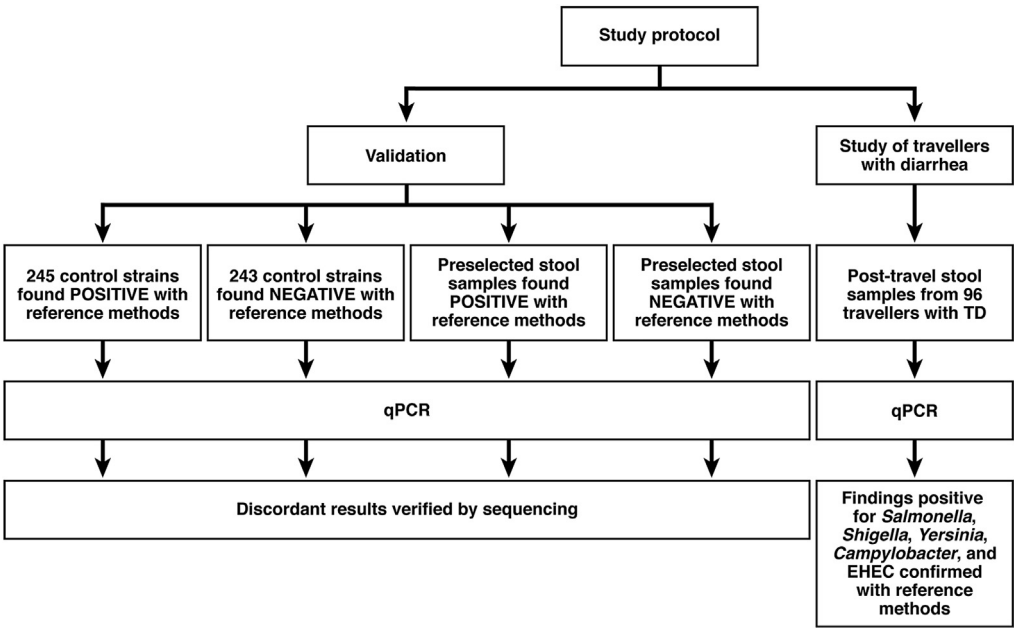

Figure 1. The study protocol including the 2 parts of this report: the validation process and study of patients with TD.

multitude of potential pathogens. At many laboratories this implies culturing *Salmonella*, *Campylobacter*, *Shigella*, and *Yersinia* and analyzing *Clostridium difficile* or enterohemorrhagic *E coli* (EHEC) on request. Approximately 90% of routine stool samples remain negative in traditional bacterial culture, which also makes the cost effectiveness of these analyses debatable. Newer studies using modern methodology based on polymerase chain reaction (PCR) are eagerly awaited because they may reveal a large number of causative agents, including the diarrheagenic *E coli* strains ETEC, EAEC, enteropathogenic *E coli* (EPEC), EHEC, and enteroinvasive *E coli*. However accurate the new PCR-based methods have proved, they have remained too laborious and time consuming to be adopted into common practice.[14]

We present a new multiplex quantitative PCR (qPCR) method that enables rapid detection of pathogens directly from stool samples, without cultures. Our assay uses a uniform assay design and covers 9 pathogens. The method is easy to perform, allows high-throughput analysis using a robotic platform, and provides results of a multitude of samples at low cost in a total of 4 hours. It can be applied to research as well as routine clinical work in developed countries. We report the results of validation experiments with preselected control samples and provide an account of how the method worked with immediate post-travel stool samples from travelers with diarrhea.

Materials and Methods

Ethics Statement

The study protocol was approved by the ethics committee of the Helsinki University Central Hospital. The study was conducted in accordance with the principles outlined in the Declaration of Helsinki. Written informed consent was obtained from all study subjects.

J.A. and J.K. have filed a patent application related to the new method.

Study Design

The study protocol is presented in Figure 1. The qPCR assay was first to be established with 1) preselected diarrheal and nondiarrheal bacterial control strains and with 2) preselected control stool samples analyzed in routine diagnostics. After this proof of concept, the assay was tested on 3) travelers with diarrhea providing stool samples immediately after their return home.

The Quantitative Polymerase Chain Reaction Assay

Design of the quantitative polymerase chain reaction assay. The PCR was designed to identify specific virulence genes, species-specific genes, or species-specific regions within established universal molecular clock genes (Table 1). The primers and probes were designed with Allele ID and Beacon Designer software (Palo Alto, CA) to recognize correct target genes and their global variants, including the Basic Local Alignment Search Tool search and secondary structure prediction using the National Center for Biotechnology Information (Bethesda, MD) database.

The qPCR was performed on the Mx3005P detection system (Stratagene, La Jolla, CA), and thermocycling was performed in the following conditions: initial denaturation and activation at 95°C for 15 minutes, 40 cycles of denaturation at 94°C for 1 minute, and annealing/extension at 60°C for 1 minute. Fluorescence was recorded at each annealing step. The 20-μL reaction contained 1× Multitect NoROX master mix (Qiagen, Venlo, the Netherlands), 1 μL of primer/probe mix (Table 1), and 0.5 μL template DNA.

For PCR analyses, bacterial cells were collected in 100 μL of water, boiled for 15 minutes, centrifuged at 13,000 rpm for 1 minute, and the supernatant (0.5 μL) was used in PCR reactions. Bacterial DNA was purified with the NucliSENS kit (Durham, NC) using the easyMAG automatic nucleic acid