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Evaluation of the multiplex PCR Allplex-GI assay in the detection of bacterial pathogens in diarrheic stool samples

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ABSTRACT

Rapid and accurate detection of the pathogens that cause gastrointestinal infection is important for appropriate therapy and proper infection control. This study assesses the performance of a new molecular assay for simultaneous detection of 13 different gastrointestinal bacteria in stool specimens. Using the Allplex GI-Bacteria (AGI-BI/AGI-BII) assay, a total of 394 stool samples were tested and the results were compared with culturing on selective differential followed by identification by mass spectroscopy. Discordant results were analyzed by a different multiplex PCR method, the Fast-Track Diagnostics Bacterial gastroenteritis (FTD-BG). The routine method (RM) detected 109 (27.7%) positive samples and the Allplex-GI assay, 261 (66.2%). Analysis of discordant results revealed that the molecular assay detected 44 pathogens that were not detected by the RM, including 23 *Campylobacter* spp., 11 *Salmonella* spp, 3 *Y. enterocolitica*, 2 EIEC/*Shigella* spp, 2 *E. coli* 0157, 2 *C. difficile* and 1 *Aeromonas* spp. Five cases not detected by the molecular method were detected by the RM (3 *Aeromonas* spp, 1 *Salmonella* spp and 1 *Y. enterocolitica*). For all targets, the percentages of sensitivity and specificity were > 95%, except for *Aeromonas* spp., which were 81% and 99% respectively. This study suggests that Allplex-GI multiplex PCR is a sensitive and specific assay that enables a rapid and accurate diagnosis of bacterial gastrointestinal infections.

1. Introduction

Diarrhea is the second leading cause of morbidity worldwide after acute respiratory infection (Liu et al., 2011). Among infants, it is the leading cause of mortality in the world (Buss et al., 2015), the majority in children under 5 years old in developing countries (Walker et al., 2013). In 2009, the World Health Organization (WHO) estimated that 2 billion episodes of diarrhea caused 1.8 million deaths per year (Vocal et al., 2015). In developed countries, it represents a major economic burden because of the frequent need to hospitalize mainly critical patients, young children and the elderly (Mengelle et al., 2013). The rapid and accurate detection of the pathogens that cause gastrointestinal infection (GI) is therefore important for appropriate therapy and proper infection control to prevent the spread of disease (Binnicker, 2015).

GI can be due to bacteria, viruses or parasites and the clinical presentation is not usually indicative of a specific pathogen (Onori et al., 2014). Conventional diagnosis is culture-based in different atmospheres and at different temperatures for antigen and toxin detection or by single PCR (Dunbar, 2013), although these strategies have poor sensitivity, potentially long turnaround times and complicated laboratory workflows. This delay reduces the value of the etiologic diagnosis for patient management (Buss et al., 2015). Despite the array of techniques, the etiological agent is detected in only 60–80% of cases (Moreno and Vila, 2006).

Recently, several multiplex molecular assays have been developed for the detection of gastrointestinal pathogens directly from clinical stool samples. These tests make it possible to detect a large number of microorganisms in a short period of time and also have a fast turnaround time and high reproducibility combined with automatic platforms. The new multiplex real-time PCR Allplex™ GI-Bacteria Assay uses two panels (AGI-BI and AGI-BII) to simultaneously detect 13 gastrointestinal bacteria. The objective of this study was to assess the performance of these panels in the diagnosis of bacterial gastrointestinal infection. The results of this assay were then compared with the routine diagnostic methods used in our laboratory.

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2. Material and methods

2.1. Clinical specimens

The study was conducted at the University Hospital 12 de Octubre, a 1300 bed facility serving a population of 450,000 people in south Madrid (Spain). Between January and September 2016, a total of 394 fresh diarrheic stool samples that had been sent to our laboratory requesting bacterial culture were analyzed. All samples were analyzed by the Allplex™ GI-Bacteria (I) Assay (AGI-BI) and Allplex™ GI-Bacteria (II) Assay (AGI-BII) panels and compared with routine methods. Only 201 of 394 samples involved requests for *C. difficile* toxin detection. Ninety-four samples were characterized stool samples with known results and 300 were prospective stool samples. The characterized stool samples were positive by the routine method and refrigerated at 4 °C. Prospective samples were sequential raw stool samples received at ambient temperature, cultured and then stored at 4 °C. The multiplex PCR assay was performed < 4 days after refrigeration. When possible, an aliquot of each sample was stored at − 20 °C in order to analyze discordant results.

2.2. Routine diagnostic methods (RM)

Stool samples were plated on MacConkey agar, Salmonella-Shigella agar (Soria Melguizo, Spain), Sorbitol MacConkey agar, Campylobacter agar and Cefsulodin-Irgasan-Novobiocin™ agar (CIN) (in-house). Selenite broth (in-house) was also inoculated and plated on Salmonella-Shigella agar after overnight incubation at 35–37 °C. All media were incubated at 35–37 °C under aerobic conditions, except for Campylobacter agar, which was incubated at 42 °C under microaerophilic conditions (5–10% O_2). Suspicious colonies were identified by Matrix-Assisted Laser Desorption Ionization- Time of Flight (MALDI-TOF) (Bruker Daltonics, Bremen, Germany) and the MicroScan Walkaway System (Beckman Coulter Inc., California, USA). When *E. coli* O157 was suspected, an immunochromatographic assay RIDA® QUICK Verotoxin/O157 Combi (RBiopharm, Darmstadt, Germany) was performed and in-house PCR was used to confirm the presence of *stx1/stx2* gene toxins in positive samples. No routine methods were available in our laboratory for the diagnosis of infections caused by non-O157 strains of enterohemorrhagic *E. coli* (EHEC), enteroinvasive *E. coli* (EIEC), enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC) or enteroaggregative *E. coli* (EAEC). Screening for detection of *Clostridium difficile* toxins was performed on diarrheic samples in patients more than one year old using the rapid dual-device enzyme immunoassay test, C. diff Quik Chek Complete (Inverness Medical Innovations, Inc., Princeton, NJ, USA). Toxigenic *C. difficile* in GDH-positive and toxin A/B-negative samples was confirmed by the GeneXpert® System (Cepheid, Sunnyvale, CA, USA).

2.3. Allplex-GI bacterial panel testing

This assay uses the novel analytical MuDT (Multiple Detection Temperature) technique which is able to detect multiple targets in a single fluorescence channel without melting curve analysis. The TOCE (Tagging Oligonucleotide Cleavage and Extension) technique is employed to design oligonucleotides to detect the DNA target (Lee et al., 2014). The first panel of the Allplex GI-Bacteria Assay (AGI-BI) detects: *Campylobacter* spp., *Clostridium difficile* toxin B, *Salmonella* spp., EIEC/*Shigella* spp., *Vibrio* spp., *Yersinia enterocolitica* and *Aeromonas* spp. The second AGI-BII panel detects: hypervirulent *Clostridium difficile* (*tcdC*Δ117 mutant), *E. coli* O157, EHEC (stx1/*2*), EPEC (*eaeA*), ETEC (*lt/st*) and EAEC (*aggR*). Allplex GI-Bacterial Assay testing was performed according to the manufacturer's instructions. Briefly, after picking the stool with a swab, it was suspended in 1 ml of ASL buffer, pulse vortexed for 1 min, then incubated at room temperature for 10 min, and centrifuged at full speed for two min. Extraction was performed in 400 µl of supernatant using the MagCore® Compact Automated Nucleic Acid Extractor (RBC Bioscience, Taipei, Taiwan).

Using the CFX96TM real-time PCR system (Bio Rad® Laboratories, Richmond, CA), multiplex real-time PCR was performed on 5 µl of bacterial DNA extracted from each sample in the GI-Bacterial assay, under the following cycling conditions: 20 min at 50 °C for 1 cycle; 15 min at 95 °C for 1 cycle; 10 s at 95 °C, 1 min at 60 °C and 30 s at 72 °C for 45 cycles; 10 s at 95 °C, 44 more times. Seegene Viewer Software (Seegene Inc. Seoul, Korea) was used for detection and data analysis. Working with batches of 18 samples, the total turnaround time for this assay was 5 h. A result was considered positive when the PCR cycle-threshold (C_t) curve was < 40, negative when the C_t was > 45, and indeterminate when the C_t was between 40 and 45.

2.4. Analysis of discrepant results

All results positive by the RM but negative by the Allplex GI-Bacteria Assay were considered as false negatives of the multiplex PCR. A different multiplex PCR method, the FTD Bacterial gastroenteritis (FTD-BG) kit (Fast-Track Diagnostics®, Junglinster Luxembourg) was used to analyze all results negative by the RM but positive by Allplex GI-Bacteria Assay (McAuliffe et al., 2013; Zhang et al., 2015). The FTD-BG assay detects: *Salmonella* spp., *Shigella* spp., *Y. enterocolitica*, *C. difficile* toxin, *Campylobacter coli/jejuni/lari*, and EHEC. An Allplex-GI result was considered a true positive when it agreed with the result of the comparator method. Because *Aeromonas* spp. was not included in the FTD-BG panel, the culture was used as the reference method.

2.5. Statistical analysis

Statistical analysis was performed using SPSS version 15.0 (IBM Corp., Armonk, NY, USA) for frequencies. Sensitivity and specificity were calculated using GraphPad QuickCals. 95% confidence intervals were calculated by the modified Wald method.

3. Results

The median age of the patients was 46 years (IQR, 14 to 68) and 58.4% were male. The routine method detected 109 pathogens (27.7%) and the Allplex-GI, 261 (66.2%). Of the 5122 targets tested by multiplex PCR (13 bacteria/394 samples), an indeterminate result was obtained for 15 (0.3%) (5 *Campylobacter* spp., 7 *Salmonella* spp., 2 *Y. enterocolitica* and 1 *Aeromonas* spp) and these were not included in the analysis. The microorganisms detected by the two methods are shown in Table 1.

An analysis of discordant results showed that the Allplex-GI molecular assay detected 44 pathogens that the RM did not detect, which included 23 *Campylobacter* spp., 11 *Salmonella* spp., 3 *Y. enterocolitica*, 2 EIEC/*Shigella* spp., 2 *E. coli* 0157, 2*C. difficile* and 1 *Aeromonas* spp. Five cases were not detected by the molecular method but were by the RM, including 3 *Aeromonas* spp., 1 *Salmonella* spp. and 1 *Y. enterocolitica*. After discrepancies had been resolved with the third method, multiplex PCR FTD Bacterial gastroenteritis (FTD-BG), the percentage sensitivity and specificity of Allplex-GI for all targets were > 95%, except for *Aeromonas* spp., which were 81% and 99%, respectively (Table 2). We found no false-positive (FP) or false-negative (FN) results for *Campylobacter* spp., *C. difficile* toxin B, hypervirulent *C. difficile* toxin B and *E. coli* O157 and detected only 4 FPs (1 EIEC/*Shigella*, 2 *Y. enterocolitica*, and 1 *Aeromonas* spp) and 4 FNs (1 *Salmonella* spp. and 3 *Aeromonas* spp). It was not possible to calculate sensitivity and specificity for diarrheagenic *E. coli* pathotypes, except for *E. coli* O157, due to the lack of a comparator method.

Among the positive samples, the routine method and Allplex-GI assay detected co-infection/colonization in 1 (0.2%) and 59 (15.0%) samples, respectively. Forty three samples (10.9%) had two pathogens, 13 (3.3%) had 3 three pathogens, 2 (0.5%) had four and 1 (0.2%) had