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Brief Original Article

Application of Luminex Gastrointestinal Pathogen Panel to human stool samples from Côte d'Ivoire

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Abstract

Introduction: Gastrointestinal infections caused by viruses, bacteria, and parasites are endemic in most developing countries due to inadequate provision of safe water supplies, sanitation, and hygiene. To investigate the enteric pathogens infecting people living in Côte d'Ivoire, the Luminex Gastrointestinal Pathogen Panel (xTAG GPP) assay was used to analyze 34 human fecal samples. This study represents the first application of this technology to samples from a sub-Saharan African country.

Methodology: Thirty-four stool samples from asymptomatic and symptomatic patients, 1–15 years of age, were analyzed by xTAG GPP. The Luminex assay represents a qualitative bead-based multiplexed molecular diagnostic test able to identify concurrently 15 enteric pathogens, including bacteria, viruses, and parasites.

Results: Overall, 22 out of 34 (64.7%) fecal specimens were detected to be positive by xTAG GPP. Sixteen were from asymptomatic subjects, and 10 patients (45.4%) showed co-infections. *G. duodenalis* was detected in 15 patients, in both mono- and co-infections, representing the most frequent pathogen, followed by enterotoxigenic *Escherichia coli* (ETEC) LT/ST. Four norovirus isolates were also detected and assigned to genogroups I and II.

Conclusions: Considering the burden of enteric infections in developing countries, particularly among children, and the high rate of co-infections in asymptomatic subjects, this study shows the need for diagnostic tools such as xTAG GPP to improve diagnosis and treatment of these infections in endemic areas.

Key words: xTAG GPP; Luminex; enteric pathogen; gastroenteritis; Côte d'Ivoire.

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Introduction

Enteric infections are a major cause of morbidity and mortality in the developing world [1]. They are caused by a wide range of viruses, bacteria, and parasites, which are linked by the common fecal-oral transmission route and diarrheal disease. The highest burden of infections is concentrated in areas characterized by poor water, sanitation, and hygiene (WASH), and affects mostly children and young adults [2-4]. In sub-Saharan Africa, the mortality rate due to acute diarrhea varies from 1.9% in Gambia and 9% in Côte d'Ivoire, up to 37% in Nigeria [5-6]. Furthermore, previous data from endemic areas have shown that, in addition to symptomatic gastroenteritis, enteric pathogens can be detected in apparently asymptomatic individuals. However, the etiology and the impact of these silent infections on the health of impoverished people are still uncertain. There are reports suggesting that constant exposure to fecal-oral contamination and sustained episodes of self-limited intestinal infections represent the main factors involved in the development of a subclinical condition known as environmental enteropathy (EE), which commonly affects people in developing countries. EE is characterized by small-intestinal inflammation, reduced absorptive capacity, and increased intestinal permeability. Consequently, asymptomatic enteric infections could represent not only a crucial source of new infections, but they also contribute to worsening the state of malnutrition and making people more susceptible to infectious diseases [2,7].

Overall, more than 30 pathogenic microorganisms are responsible for gastrointestinal infections, and differential diagnosis is a challenge in developed

countries. For this reason, several diagnostic tests must be combined to avoid overlooking important pathogens. So far, most of the research efforts have been focused on single enteric pathogen detection. Recently, the xTAG Gastrointestinal Pathogen Panel (xTAG GPP), a novel bead-based multiplexed molecular diagnostic test designed to simultaneously detect and identify the most common human enteric pathogens, was developed (Luminex Molecular Diagnostics, Toronto, Canada). The assay is able to identify concurrently 15 intestinal pathogens among viruses, bacteria, and parasites. In the present investigation, we used the xTAG GPP assay as a diagnostic tool on stool samples collected from patients living in the south of Côte d´Ivoire.

Methodology

Patients and sample collection

The study was based on 34 patients randomly and voluntary sampled in June 2013 and in January 2014 in three different localities (Bonoua, Kimoukro, and Assouinde) in the Sud-Comoé region of Côte d'Ivoire. The stool samples collected in 2013 were obtained from both asymptomatic and symptomatic subjects suffering from diarrhea, abdominal pains, and fever. The second group of samples, collected in 2014, was obtained from asymptomatic subjects (Table 1). All other relevant clinical information, including other diseases and therapy, were also noted. One fecal sample was collected in a sterile stool container from each patient and delivered to the clinical laboratory of the Centre don Orione in Bonoua within 12 hours.

Table 1. Patients characteristics and enteric pathogens detected by Luminex xTAG GPP assay

Patient ID	Age (years) / gender	Date of sampling	Place of sampling	Symptoms	xTAG GPP results
1	8/F	June 2013	Kimoukro	Diarrhea	Negative
2	5/F	June 2013	Kimoukro	Abdominal pain	ETEC, *Shigella*
3	8/F	June 2013	Kimoukro	Abdominal pain	*E. coli* 0157, *G. duodenalis*
4	6/M	June 2013	Bonoua	Abdominal pain	*Campylobacter*, ETEC, *G. duodenalis*
5	10/F	June 2013	Bonoua	Abdominal pain	*E. coli* 0157, ETEC, *G. duodenalis*
6	5/F	June 2013	Bonoua	Abdominal pain	*Campylobacter*, ETEC, *G. duodenalis*
7	3/M	June 2013	Bonoua	NS	*G. duodenalis*
8	1/F	June 2013	Bonoua	Fever	Negative
9	9/M	June 2013	Bonoua	NS	Negative
10	12/F	June 2013	Assouindé	Diarrhea	Norovirus GI, *G. duodenalis*
11	12/M	June 2013	Assouindé	Diarrhea	Negative
12	9/F	June 2013	Bonoua	NS	ETEC, STEC, *G. duodenalis*
13	8/M	January 2014	Bonoua	NS	*E. histolytica*
14	10/M	January 2014	Bonoua	NS	Negative
15	3/M	January 2014	Bonoua	NS	*G. duodenalis*
16	9/M	January 2014	Bonoua	NS	Negative
17	14/M	January 2014	Bonoua	NS	Norovirus GII
18	7/M	January 2014	Kimoukro	NS	Negative
19	5/F	January 2014	Assouindé	NS	Negative
20	15/F	January 2014	Assouindé	NS	*G. duodenalis*
21	15/M	January 2014	Assouindé	NS	*G. duodenalis*
22	11/F	January 2014	Assouindé	NS	*Campylobacter*, *G. duodenalis*
23	11/F	January 2014	Assouindé	NS	Norovirus GI
24	4/F	January 2014	Assouindé	NS	Negative
25	8/M	January 2014	Assouindé	NS	*G. duodenalis*
26	2/M	January 2014	Kimoukro	NS	ETEC, *G. duodenalis*
27	1/F	January 2014	Kimoukro	NS	*G. duodenalis*
28	15/F	January 2014	Kimoukro	NS	Negative
29	9/M	January 2014	Kimoukro	NS	*Campylobacter*, *E. coli* 0157, ETEC, STEC, *G. duodenalis*
30	3/M	January 2014	Bonoua	NS	Negative
31	1/F	January 2014	Bonoua	NS	ETEC
32	2/F	January 2014	Bonoua	NS	*G. duodenalis*
33	14/M	January 2014	Bonoua	NS	Norovirus GII
34	4/F	January 2014	Bonoua	NS	Negative

NS: no symptoms; ETEC: enterotoxigenic *E. coli*; STEC: Shiga-like-toxin-producing *E. coli*

After registration, the samples were immediately frozen at -20°C until transport in ice packs to the Institute of Virology of the University of Cologne, where they were kept at -80°C. The medical committee of Don Orione Centre provided approval and oversight of the study. Informed verbal consent was obtained from the parents or the guardians of children, and a local dialect interpreter was used to explain the study's aims, procedures, and significance when necessary. All investigations and protocols followed the principles of the Helsinki Declaration.

Sample preparation and nucleic acid extraction

One hundred milligrams of each stool sample were suspended in 1 mL of phosphate-buffered saline (PBS). The total nucleic acids were extracted from 700 µL of the stool suspension using the automated platform VERSANT kPCR Molecular System and the VERSANT Sample Preparation 1.0 Reagents Kit (Siemens Healthcare Diagnostics, Erlangen, Germany) into an elution volume of 100 µL, according to the manufacturer's instructions.

xTAG GPP assay

Fifteen human enteric pathogens were simultaneously tested by the xTAG GPP. This assay is a bead-based multiplexed molecular diagnostic test, which concurrently detects and identifies from a single stool specimen adenovirus subtypes 40/41, norovirus genogroups I and II (GI/GII), group A rotavirus, *Campylobacter* spp., *Clostridium difficile* toxin A/B, *Escherichia coli* O157, enterotoxigenic *Escherichia coli* (ETEC) LT/ST, *Salmonella* spp., Shiga-like-toxin-producing *E. coli* (STEC) stx1/stx2, *Shigella* spp., *Vibrio cholerae*, *Yersinia enterocolitica*, *Cryptosporidium hominis*, *C. parvum*, *Entamoeba histolytica*, and *Giardia duodenalis*.

After extraction and purification of the total nucleic acids from each fecal specimen, a multiplex reverse transcription polymerase chain reaction (RT-PCR) reaction using 10 µL of eluate was performed using target-specific tagged primers and biotin-labeled primers. Each target results in PCR amplicons ranging from 58 to 293 bp. After sonication, 20 µL of the xTAG bead mix were aliquoted for each sample into a 96-well microtiter plate, and 5 µL of appropriate RT-PCR product were added. The xTAG 0.22 streptavidin, R-phycoerythrin conjugate (SAPE) was diluted with xTAG reporter buffer, and 75 µL of the reporter solution were added into each well. The RT-PCR and hybridization reactions were performed using Biometra T3 Thermocycler (Biometra GmbH, Göttingen, Germany). Following hybridization, the median fluorescence intensity (MFI) was generated for each xTAG bead population using the Luminex 100/200 instrument pre-heated to 45°C. The data were analyzed using the xTAG Data Analysis Software for the GPP (TDAS) to establish the presence or absence of bacterial, viral, or parasitic targets and the internal control in each sample.

Detection of norovirus by real-time PCR

In order to confirm the preservation of RNA during the storage and the shipping of stool samples, 5 µL of each eluate were analyzed by real-time PCR for the detection of norovirus GI/GII using the commercially available RealStar Norovirus RT-PCR Kit 2.0 (Altona Diagnostics, Hamburg, Germany) according to the manufacturer's instructions.

Results

A total of 34 stool samples were examined from young patients (18 females and 16 males) between 1 and 15 years of age (average 7.6; median 8). Overall,

Table 2. Number of mono- and co-infections for each detected pathogen

Detected pathogen	No. of infected patients	Mono-infections	Co-infections
Norovirus GI	2	1	1
Norovirus GII	2	2	0
E. coli 0157	3	0	3
ETEC	8	1	7
STEC	2	0	2
Campylobacter spp.	4	0	4
Shigella spp.	1	0	1
G. duodenalis	16	7	9
E. histolytica	1	1	0

ETEC: enterotoxigenic *E. coli*; STEC: Shiga-like-toxin-producing *E. coli*

22 of 34 (64.7%) samples were detected positive by xTAG GPP (Table 1); 16 of 22 were from asymptomatic patients. Four norovirus isolates were detected and assigned to genogroups I and II, as confirmed also by real-time PCR. Among bacteria, *E. coli* 0157 (3/22), ETEC (8/22), STEC (2/22), *Campylobacter* spp. (4/22), and *Shigella* spp. (1/22) were identified. Concerning enteric protozoa, 15 samples were tested positive for *G. duodenalis*, whereas *E. histolytica* was detected in only one sample. No patient resulted positive for *C. hominis* or *C. parvum.*

Of 22 positive children, 12 (54.6%) presented a mono-infection: 3 with norovirus GI/GII, 1 with ETEC, 7 with *G. duodenalis*, and 1 with *E. histolytica*. A total of 10 patients (45.4%) showed co-infections. In particular, 5/10 and 4/10 samples were detected positive for two and three different pathogens, respectively. A co-infection with four bacteria and one parasite species was revealed in a nine-year-old boy. All bacterial isolates were detected as co-infections, while only one patient was revealed to have a mono-infection by ETEC. No co-infections with both bacteria and viruses were identified (Tables 1 and 2).

Discussion

Gastroenteritis is recognized as one of the most important public health issues in developing countries, where low socio-economic level and favorable climatic conditions enable environmental contamination by pathogen microorganisms. In particular, the risk of contracting diarrheal disease is currently fivefold higher in sub-Saharan Africa than it is in industrialized countries. Diarrhea is often more severe or fatal in children suffering from malnutrition. In developing countries, the frequent combination of recurrent diarrhea and malnutrition can lead to a vicious cycle with dire consequences, especially for children under five years of age [10]. Moreover, laboratory diagnosis of infectious diseases still represents a challenge for most of the healthcare centers in low-income regions such as sub-Saharan Africa and, consequently, the appropriate therapeutic choice is frequently based only on clinical symptoms [11]. Most of the studies on enteric pathogens in sub-Saharan Africa over the last 20 years have focused on children under five years of age. Rotavirus, *Cryptosporidium* spp., *Shigella* spp., enterotoxigenic *E. coli* (ETEC), *Campylobacter jejuni*, and *Vibrio cholerae* have been reported to be the main etiological agents of pediatric diarrhea [12-14]. Fisher *et al*. (2010) systematically reviewed the etiology of diarrhea in children older than five years of age, adolescents, and adults worldwide. They found that ETEC and *V. cholerae* were the most frequently isolated pathogens among hospitalized patients in low- and middle-income countries [15]. Recent data from Senegal showed that bacteria and parasites are equally frequent in all age groups, whereas viral infections are significantly more frequent in children under five years of age and during the dry season [16]. Lamberti *et al*. (2014) underlined the impact of *Shigella* spp. and ETEC on morbidity and mortality among older children, adolescents, and adults in South Asia and Africa [17].

The Luminex xTAG GPP is the first commercially available assay able to simultaneously detect viruses, bacteria, and parasites on total nucleic acid isolated from a single stool specimen [18]. The present study represents the first application of xTAG GPP to human stool samples collected in Côte d'Ivoire. Overall, the results obtained using the Luminex panel assay are in agreement with the data from studies focused on single pathogens or classes of microorganisms in sub-Saharan Africa [19-21]. Regarding viruses, only norovirus GI/GII was detected, and three of the four positive patients were asymptomatic. Huynen *et al*. (2013) reported a similar prevalence of norovirus in both symptomatic and asymptomatic patients in Burkina Faso [22]. Enteric infections among apparently asymptomatic individuals represent a serious issue in endemic areas [23-25]. This unrecognized source of infections and the low level of hygiene and sanitation constitute a hazardous combination, which contributes to the endemic transmission of EE [7]. Regarding bacteria, ETEC, STEC, *E. coli* 0157, *Campylobacter* spp., and *Shigella* spp. were identified, as previously reported in African countries [12,17,26]. Regarding parasites, whereas the high rate of *G. duodenalis* confirms our previous data from Tanzania and Côte d'Ivoire, no patients were detected positive for *C. hominis* and *C. parvum* [27-28]. This might be due to the low number of samples tested but also because the xTAG GPP is not able to identify *C. meleagridis*, a species frequently reported in humans in Africa [29]. Noteworthy was the molecular detection of *E. histolytica*, considering that the cysts are morphologically indistinguishable from those of the non-pathogenic *E. dispar* and *E. moshkovskii* and the high prevalence of *E. dispar* in the area of study [30]. Finally, the present study evidenced the issue of co-infections also in apparently healthy individuals.

Conclusions

In the context of the surveillance of diarrheal disease in sub-Saharan Africa, our results show evidence that the application of xTAG GPP might significantly improve the available knowledge about the etiology of symptomatic and asymptomatic enteric mono- and co-infections. Considering the impact of gastroenteritis in developing countries, new efforts from the international scientific community, including the application of innovative molecular technology, should be encouraged in order to improve diagnosis and treatment of intestinal infections in endemic areas.

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