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Comparative evaluation of the new xTAG GPP multiplex assay in the laboratory diagnosis of acute gastroenteritis. Clinical assessment and potential application from a multicentre Italian study

C. Vocale [a,*], S.G. Rimoldi [b], C. Pagani [b], R. Grande [b], F. Pedna [c], M. Arghittu [d], G. Lunghi [d], A. Maraschini [d], M.R. Gismondo [b], M.P. Landini [a,f], E. Torresani [d], F. Topin [e], V. Sambri [c,f]

[a] Unit of Clinical Microbiology, Regional Reference Centre for Microbiological Emergencies, St. Orsola Malpighi University Hospital, Via Massarenti 9, 40138 Bologna, Italy
[b] Laboratory of Clinical Microbiology, Virology and Bioemergency, "L. Sacco" University Hospital, Milan, Italy
[c] Unit of Clinical Microbiology, The Hub Laboratory of the Greater Romagna Area, Pievesestina, Cesena, Italy
[d] Department of Clinical Chemistry and Microbiology, Bacteriology and Virology Units, Fondazione IRCCS Ca' Granda, Ospedale Maggiore Policlinico, Milan, Italy
[e] Luminex Corporation, Oosterhout NB, Netherlands
[f] DIMES, University of Bologna, Bologna, Italy

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SUMMARY

Objective: Gastroenteritis caused by a single pathogen or multiple pathogens remains a major diagnostic challenge for the laboratory. The treatment of diarrhoea is based on microbiological results. Diagnosis is achieved using different laboratory techniques that have variable sensitivity and specificity. xTAG GPP is a new multiplex PCR assay that simultaneously detects 15 different pathogens responsible for diarrhoea. The results of the first multicentre study in Italy to evaluate the potential clinical application of the GPP assay in the laboratory diagnosis of diarrhoea are reported here.
Methods: Faeces specimens ($N = 664$) from hospitalized patients were tested with the GPP assay using a Luminex 200 instrument. All specimens were run using comparator methods following a routine algorithm: culture for bacteria, enzyme immunoassay and PCR for viruses, and microscopy for parasites.
Results: Of the samples tested with the GPP, 53.61% (356/664) gave positive results, as compared to 45.33% by routine testing. Of the positive specimens, 34.55% showed the presence of genomic DNA from multiple pathogens. The Luminex method showed an increase in the percentage of positivity of 8.28%.
Conclusions: The GPP assay can be considered a helpful tool for the detection of gastrointestinal pathogens, with a hands-on time of 5 h; it provides accurate data for the clinical management of hospitalized patients and for epidemiological surveillance.

1. Introduction

Infectious gastroenteritis (GE) is a significant cause of morbidity and mortality worldwide. Diarrhoea, the main clinical symptom of GE, is currently the second leading cause of illness worldwide after the common cold.[1] In 2009, the World Health Organization (WHO) estimated 2 billion cases of severe illness related to GE, with about 1.8 million deaths per year.[2]

The microbiological aetiology of GE may include bacterial, viral, or parasitic pathogens, and the related clinical picture can range from mild to severe and life-threatening cases.[3] The ability to manage severe GE cases in the fastest and best possible way is largely dependent on the prevention of and control measures for the spread of infectious diarrhoeal diseases.

The effective treatment of GE is dependent upon an efficient and rapid diagnostic algorithm that allows the implementation of drug therapy and/or isolation measures to prevent the spread of the infectious agent from the ill patient to healthy individuals. The laboratory diagnosis of GE is currently based on the combined use of different tests, each one with variable sensitivity and specificity

* Corresponding author. Tel.: +39 051 2144316; fax: +39 051 2143076.
E-mail address: cate.vocale@gmail.com (C. Vocale).

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and typically able to identify only a defined number of pathogens at a time.[4–6]

Most clinical microbiology laboratories use conventional diagnostic methods, such as culture, microscopy, and ELISA, to identify GE-related pathogens.[7] This approach involves multiple selective media and reagents, followed by the identification of the microorganism and biochemical testing. When microscopy is used, the correct identification of ova and parasites depends largely upon the experience and skill of the microscopist. ELISA offers generally stable and quality-controlled kits, but typically this technique has long run times,[8] and the overall performance depends on the quality of capture and detection of antibodies.[9] With culture-based protocols, the final results are usually obtained at 3–4 days and the sensitivity may be affected by the limited viability of selected microorganisms during sampling and transportation of the specimen. Taken together, all of the above reported weaknesses in the diagnostic workflow may lead to empiric and often inappropriate and delayed therapy of GE.

Molecular-based methods could streamline the laboratory workflow, improve the diagnostic performance, and consequently enhance clinical management, given the better and more rapid identification of the aetiology of GE cases.[10] A few commercial tests are now available with CE-IVD marking for the detection of enteric pathogens in human stool. In particular, the Luminex xMAP Technology offers a novel platform for high-throughput nucleic acid detection and can be used in a variety of applications.[1] Recently, the use of this technology has enabled the sensitive and specific detection of the major gastrointestinal pathogens.[11]

xTAG GPP (GPP), manufactured by Luminex Corporation (Austin, TX, USA), is a new qualitative bead-based multiplex assay able to simultaneously detect, in a single human stool sample, 15 different pathogens, including those responsible for hospital-acquired infections such as *Clostridium difficile*, food-borne illness agents like Salmonella, and viral paediatric diarrhoea pathogens such as a rotavirus.

The aim of this study was to compare the analytical performance of the GPP assay with the routine tests used for the detection of GE-related pathogens in a population of patients suffering from suspected GE. In addition, the potential clinical application of this multiplex assay in the diagnostic practice and management of diarrhoea was also evaluated.

2. Materials and methods

A total of 664 stool samples were collected between January 1, 2011 and March 31, 2013 from hospitalized symptomatic patients suffering from suspected GE. One sample was received from each patient. All raw stool specimens and stool specimens in Cary–Blair transport medium (Faecal swab; Copan, Brescia, Italy) were collected and submitted for microbiological evaluation at the following four laboratories: St. Orsola Malpighi University Hospital (Bologna), "*L. Sacco*" University Hospital (Milan), Ospedale Maggiore Policlinico (Milan), and the Unit of Microbiology of the Hub Laboratory of the Greater Romagna Area (Pievesestina). All of the specimens were first evaluated using the available routine methods at each individual participating laboratory, as summarized in Table 1.

The stool samples were tested with the GPP test after the routine assays had been performed, using the Luminex 200 instrument; testing was performed in accordance with the manufacturer's instructions. Briefly, the samples were pre-treated as described in the package insert and then underwent automated nucleic acid extraction using the NucliSENS EasyMAG system (BioMérieux, Marcy l'Etoile, France). The GPP assay includes an RNA-based internal control (MS2 bacteriophage), which was spiked into each sample prior to extraction, to control for PCR inhibition and assess each step in the workflow and consequently monitor the entire assay performance.

The RT-PCR reactions and subsequent hybridization step were performed according to the instructions in the GPP manual, by a single trained operator who was blinded to the identity of the specimens and to the results obtained with the standard diagnostic methods. PCR amplification was performed using a Mastercycler gradient thermal cycler (Eppendorf, Hamburg, Germany) using the recommended cycling parameters; negative and positive controls were included in all runs of the GPP assay.

The data were acquired on the Luminex 200 analyzer and data analysis was carried out using TDAS data analysis software.

All clinical specimens were also tested according to the following routine algorithm: conventional culture for the detection of bacteria,[12] enzyme immunoassay (EIA) for *C. difficile* detection (Wampole C Diff Quik Chek EIA (GDH-Q) and Wampole Tox A/B Quik Chek EIA (AB-Q); Alere S.r.l., Italy), Rapid Strip ROTA/ADENO (Meridian Bioscience Inc., Italy) or real-time PCR for virus detection,[13] and microscopic examination for parasite identification (Lugol, Giemsa) (Table 1).[14]

3. Results

A total of 301/664 (45.33%) samples tested positive with the routine tests versus 356/664 (53.61%) using the GPP assay (Figure 1). In the majority of the positive samples, only one target was detected by routine workflow, whereas 123/356 (34.55%) of the GPP-positive specimens were positive for multiple targets (Figure 2). This suggests the presence of genomic sequences from different pathogens simultaneously; these results clearly open the hypothesis of co-infecting germs in one individual sample (Table 2). In particular, with the routine diagnostic procedure, adenovirus, norovirus, and rotavirus were the most frequently co-detected putative pathogens, whereas with the Luminex method, the main co-infections detected were due to *C. difficile*, norovirus, and Cryptosporidium.

In relation to the bacterial panel, the routine tests were not able to identify all of the double infections, unlike the Luminex method, which in 9/74 positive cases for Salmonella showed the simultaneous presence of the genome of this organism together with another pathogenic microorganism (Table 2).

In most cases, the additional target detected was not the one hypothesized by the clinician.

Table 1
Routine methods for the diagnosis of gastroenteritis

Routine	Tests for	Method	Turn-around time
Stool culture	Single or few bacterial pathogens per test (i.e., Shigella, Campylobacter, Salmonella)	Bacterial isolation and drug resistance/ susceptibility test	~72 h
Parasites	Parasitic pathogens	Microscopy	~24 h
ELISA	Viral pathogens and *Clostridium difficile* infection (GDH)	Detection of a single antigen per test	~72 h
Rapid tests	Viral pathogens and *C. difficile* infection (toxin A/B)	Detection of a single virulence factor/toxin per test	~72 h
Real-time PCR	Viral pathogens	Amplification of a specific DNA fragment	~24 h

GDH, glutamate dehydrogenas.

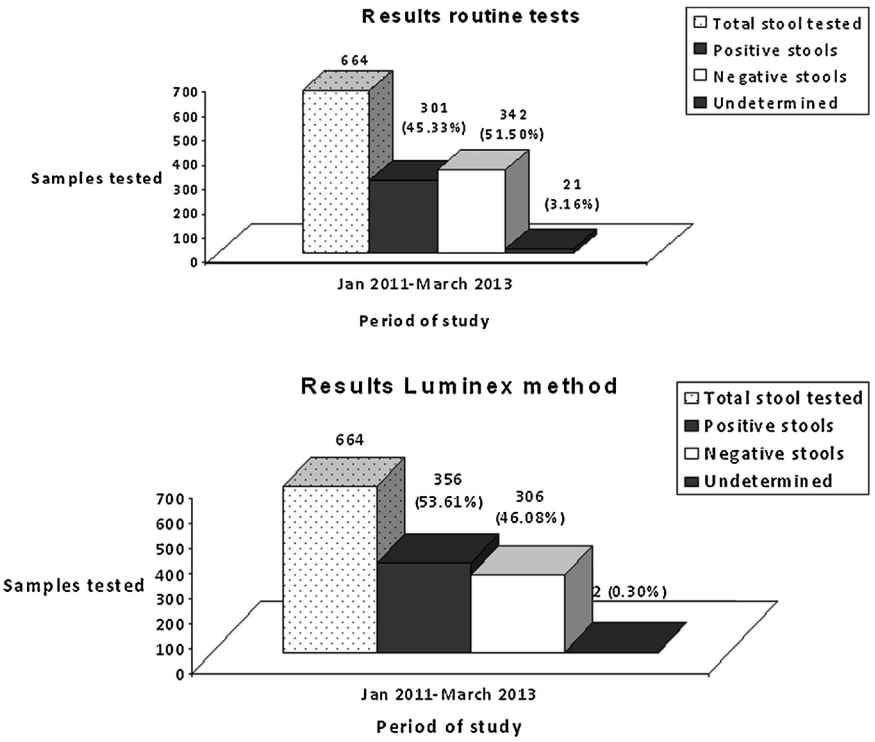

Figure 1. Comparative results between routine tests and Luminex method.

No further investigation was carried out on samples with multiple positive results. The real clinical relevance of the presence of this DNA remains to be clarified, since pathogens such as Salmonella and Giardia may be present in humans as colonizing organisms. The results obtained were comparable across all the centres involved in the study. The percentage of positive samples detected with the Luminex method was superior by 8.28% in respect to the positives found by the standard diagnostic methods (53.61% and 45.33%, respectively).

4. Discussion

This study showed that the xTAG GPP has very good sensitivity and specificity for the detection of a large panel of pathogens related to acute diarrhoea, with the capability of increased detection and improved diagnostic yields from stool specimens as compared to the reference methods used routinely. An added value of this new molecular test is the capability of identifying the presence of pathogens that would remain undetected under the

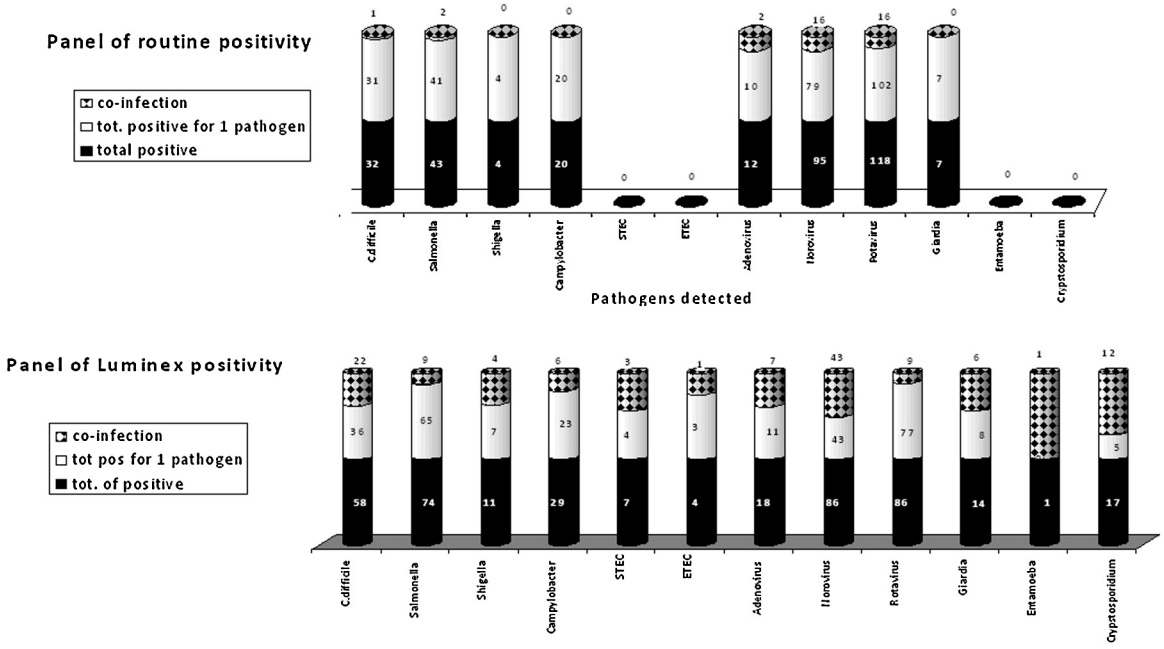

Figure 2. Summary of xTAG GPP positive samples compared with panel of routine positivity.

Table 2
Summary of xTAG GPP results

Target	Total positive	Positive for single pathogen	Co-infection	Other pathogens detected (number)
Bacterial targets				
Clostridium difficile	58	36	22	Norovirus (7), Shigella (1), adenovirus (1), rotavirus (5), Giardia (1), Cryptosporidium (1), Campylobacter (1), norovirus/adenovirus (2), norovirus/rotavirus (2), norovirus/Cryptosporidium (1)
Salmonella	74	65	9	Giardia (1), norovirus (4), adenovirus (1), Campylobacter (1), norovirus/rotavirus (1), Cryptosporidium/Giardia (1)
Shigella	11	7	4	Giardia (2), *C. difficile* (1), norovirus (1)
Campylobacter	29	23	6	Norovirus (2), adenovirus (2), *C. difficile* (1), rotavirus/Cryptosporidium (1)
STEC	7	4	3	Norovirus (1), *E. coli* O157 (1), adenovirus (1)
ETEC	4	3	1	Norovirus (1)
Viral targets				
Adenovirus	18	11	7	Campylobacter (2), *C. difficile* (1), Salmonella (1), rotavirus/STEC (2)
Norovirus	86	43	43	*C. difficile* (8), Giardia (2), Campylobacter (3), Salmonella (4), ETEC (1), rotavirus (9), Cryptosporidium (4), Shigella (1), adenovirus (2), rotavirus/Salmonella (1), *C. difficile*/adenovirus (2), *C. difficile*/Cryptosporidium (1), rotavirus/Cryptosporidium (1), rotavirus/*C. difficile* (2), ETEC/Cryptosporidium (1), Cryptosporidium/*E. histolytica* (1)
Rotavirus	86	77	9	*C. difficile* (5), *E. coli* O157 (1), Cryptosporidium (2), Campylobacter/Cryptosporidium (1)
Parasitic targets				
Giardia	14	8	6	Norovirus (2), Shigella (2), Salmonella (1), Cryptosporidium/Salmonella (1)
Entamoeba histolytica	1	0	1	Norovirus/Cryptosporidium (1)
Cryptosporidium	17	5	12	Norovirus (3), rotavirus (2), *C. difficile* (1), norovirus/*C. difficile* (1), norovirus/rotavirus (1), Campylobacter/rotavirus (1), norovirus/ETEC (1), Giardia/Salmonella (1), norovirus/*E. histolytica* (1)

STEC, shiga toxin-producing *Escherichia coli*; ETEC, enterotoxigenic *Escherichia coli*.

routine diagnostic workflow, and in particular to unravel putative pathogens, even if only by genome detection, that were not among those hypothesized by the treating physician. This is clearly a consequence of the fact that the standard diagnostic procedures are technically based on performing individual tests focused on the most probable pathogens as suggested by patient presentation and as ordered by the physician.

The multiplex molecular-based approach, such as the GPP, is not specifically oriented towards a single microbe, but can simultaneously detect the presence of all of the target analytes included in the panel. Based on this broad range capability to detect multiple genomic targets and the enhanced sensitivity that characterizes diagnostic methods based on nucleic acid amplification, the GPP appears to be a promising tool for the detection and identification of GE-related pathogens. It is able to replace a heterogeneous set of methods commonly seen in laboratories, such as culture, ELISA, and rapid tests, with a single standard assay, above all for undetected pathogens. In addition, as evidenced in this study, the assay provides an improved workflow with a hands-on time of no more than 5 h.

A point for discussion is that molecular-based methods only detect the presence of genomic sequences derived from both living and dead putative pathogens. Thus, the clinical relevance of detecting pathogen-derived DNA in a microbiologically complicated specimen such as stool, and where all the genes derived from the intestinal microbiome could be present, must be considered. Today, the aetiology of acute infectious diarrhoea is generally accepted as monomicrobial, meaning that one pathogen is accepted as responsible for the particular disease episode. The results obtained in this study clearly demonstrate that multiple diarrhoeal pathogens are frequently present simultaneously in the stool of patients suffering from acute GE, and their nucleic acids can be detected and identified using a comprehensive multiplex molecular test, such as the GPP. Further, this suggests the possibility that multiple organisms can contribute to the pathogenesis of this illness.

This hypothesis may also be corroborated by the newly accepted concept of the human microbiome as an enormous microbial community that includes viruses, bacteria, fungi, and likely protozoa.[15] This large community of diverse microorganisms must be regarded as an additional 'organ' that largely contributes to the maintenance of the body homeostasis and the health of each individual. Based on this fact and on the findings of this study, demonstrating the presence of multiple pathogens in the stool of acute GE patients, the hypothesis of diarrhoea as a 'dysmicrobial syndrome' clearly arises. In other words, diarrhoea could be seen as a condition of altered ecological equilibrium among the members of the human gut microbiome. A test like the xTAG GPP that can identify multiple organisms within one specimen could be helpful to determine a more precise aetiology of acute GE, without the need to test the sample several times with different panels (bacterial, viral, and parasitological), reducing, although only 24 samples/day, all the work in the hands of a single operator.

This expanded detection capability is particularly relevant in light of the latest knowledge about the human gut microbiome, whose composition is greatly influenced by several host-associated parameters (antibiotic therapy, nutrition and food consumption, genetic background, etc.), and emphasizes the role of this huge microbial community in the development of enteric diseases.[16–19] The use of multiplex amplified molecular techniques in the field of GE diagnosis could provide additional evidence to the above hypothesis.[20] Although the detection of a nucleic acid sequence does not imply the presence of a viable organism, additional investigations are warranted in order to clarify the putative role of mixed infections in the genesis of acute GE. Detection does not necessarily mean disease, as many pathogens can exist asymptomatically (e.g., *Salmonella spp*) or subclinically (e.g., *C. difficile* non-toxigenic strains) in a colonization-like status.[21,22]

Furthermore, great emphasis should be given to the management of 'true-negatives' resulting from the molecular test, where negativity is truly associated with the absence of the putative pathogenic organism in the sample tested and not due to a limitation of the diagnostic test used, especially considering the improved sensitivity of xTAG GPP.

The most attractive feature of multiplex diagnostic methods for the clinician lies in the wide spectrum of pathogens identified and

the rapidity of results. There are still many interpretative points that must be considered, including an in-depth analysis and clinical correlation of the results, and the overall cost–benefit ratio should be determined before introducing this approach into the routine laboratory workflow for GE. Of course a general increase in laboratory costs would be expected, since a molecular-based technique implies a higher cost than standard methods in terms of reagents and personnel training. On the other hand, the introduction of a sensitive molecular method has been shown in this study to greatly increase the overall performance of the laboratory diagnosis of GE, thus likely allowing a better management of the patient both in terms of a reduction in hospitalization time and the appropriate choice of therapy, thereby reducing overall costs.

Moreover, as shown in this study, the GPP allowed us to identify undiagnosed infections as well as unsuspected co-infections. One last aspect of the findings of this study that should be highlighted is that the use of the GPP allowed a substantial reduction in the personnel involved in the workflow of GE diagnosis. Having performed this study, we emphasize the great promise that this novel molecular approach gives to solving cases of unknown aetiology; it is important to note that the sensitivity is significant not only in those symptomatic cases where the laboratory data confirm the clinical picture, but especially in those cases where the diagnostic suspicion is very strong, but symptoms are unclear.

In conclusion, in this study, the xTAG GPP assay was found to have great potential for improving the diagnosis of GE. Additional larger studies are necessary to further investigate and confirm this possibility.

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Conflict of interest: Francois Topin was employed by Luminex Corporation at the time of the study. The remaining authors declare no conflicts of interest and no competing financial interests.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijid.2015.02.011.

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