Rationale of using multiplex polymerase chain reaction (PCR) panels for etiological diagnosis of infective diarrhea in the tropics

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Infectious diarrhea causes significant morbidity and mortality [1, 2]. According to the Centre for Disease Control (CDC) report, it is responsible for one in nine deaths among children globally making it second most common cause of death among children under 5 years of age [3]. Infective diarrhea can be caused by multiple pathogens, most commonly due to viruses, protozoan parasites, and bacteria. It is estimated that etiology remains unidentified in 80% of patients with acute diarrhea [4]. The infective diarrhea can present as acute diarrhea, dysentery (loose stools with blood), persistent, or chronic diarrhea including malabsorption syndrome [5, 6].

The diagnosis of infective causes can be made by microscopic examination of stool samples, detection of coproantigens, culture, and molecular methods such as polymerase chain reaction (PCR). Microscopic examination of stool sample is the simplest and cheapest method of determining the infective etiology. The liquid samples must be processed within 30 min of collection [7]. The results of microscopy are subject to various limitations such as observer's skill, need for multiple samples, use of proper staining techniques, false positive (such as non-pathogenic *Entamoeba*, which may be mistaken for *Entamoeba histolytica*), and false-negative results. Concentration methods such as formol-ether technique followed by special staining such as modified Kinyoun and Trichrome staining techniques enhance the detection of pathogens such as *Cryptosporidium*, *Cyclospora*, *Isospora*, and *Microsporidium*, respectively, which are easily missed otherwise. A study observed that for stool examination, microscopy has a low sensitivity of 50.7% though the specificity was as high as 100% compared to PCR [8].

Culture-based methods have some advantages including identification of the species and antimicrobial susceptibility determination. The limitations of the culture include low sensitivity, need for specialized microbiology laboratory, difficulty in isolating non-bacterial agents and the need for fresh samples. The yield of culture in diarrheal samples lies between 1.5% and 54% [9–12]. The other limitations of culture methods include the need of a long period of incubation and variable conditions, which make it a cumbersome activity. The yield of culture is affected by many pre-analytical factors such as previous intake of antibiotics. It is recommended that stool for culture should be obtained at least 2 weeks after the previous antibiotic intake [13]. Detection of antigen in the stool is done for rapid diagnosis of various pathogens including bacteria, viruses and parasites. The enzyme immunoassay (EIA) is available for antigen detection in stool for *Helicobacter pylori*, *Campylobacter* but have a low predictive value [14, 15]. Antigen detection using EIA format helps in discriminating pathogenic from non-pathogenic amebae such as *Entamoeba histolytica* from *E. dispar*, by detecting Gal/GalNAc lectin. The sensitivity is around 100 times less than PCR. This has to be done, however, on fresh or frozen samples only, which may pose difficulty while sample collection and transportation in some circumstances [16]. The specificity is also reported to be less due to cross-reaction with other species. Antigen detection using monoclonal antibodies in direct fluorescent assay are available for parasites such as *Cryptosporidium* and *Giardia* [17]. The assays are affected by antigenic variations in different strains [14]. Also, a negative result may be due to epitope degradation or antigen elution especially in ELISA for *Giardia* as it detects free or soluble antigen only [18]. The molecular methods overcome most of the above limitations of the microscopy, culture-based methods and are highly sensitive and specific and require shorter turn-around time. The first step of the molecular method is nucleic acid extraction, which is important as stool is a rich mixture of various inhibitors of amplification.

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The PCR method amplifying a single target by use of single set of primers is called singleplex PCR. The end-product obtained as a result of amplification can be detected by gel electrophoresis or fluorescent reporter molecules. Real-time PCR collects data continuously while reaction is proceeding so that quantification of nucleic acids can be done. Real-time PCR thus can differentiate between live and dead pathogen also.

Multiplex PCR assays detect multiple targets in a single reaction (Fig. 1). The targets are differentiated on the basis of melting characteristics of amplicons or size differences in gel electrophoresis [4]. There are several assays using multiplexing, which are available commercially. The FDA agency has cleared three such panels, namely xTag Gastrointestinal Pathogens Panel (GPP) (Luminex, Austin, Texas, USA), FilmArray Gastrointestinal Panel (BioFire Diagnostics, Salt Lake City, Utah, USA), and Verigene Enteric Pathogens test (Nanosphere, Chicago, Illinois, USA). The xTag GPP assay (Luminex) detects 14 targets (6 bacteria, i.e. *Campylobacter* spp., *Salmonella* spp., *Vibrio* spp., enterotoxigenic *E. coli* (ETEC), *E. coli* O157, and enteroinvasive *E. coli* (EIEC)/*Shigella*, 2 bacterial toxins (*C. difficile* toxin A/B and Shiga toxin sxt1/sxt2), 3 viruses namely adenovirus 40/41, NoV genogroups I and II (GI/GII)

and rotavirus A and 3 parasites, i.e. *Cryptosporidium*, *E. histolytica*, and *G. lamblia*). The Verigene EP assay detects 9 targets, which include five bacteria, two bacterial toxins, and two viruses from stool in Cary-Blair medium. The FilmArray panel detects 22 targets which include 11 bacteria (*Campylobacter* spp., *Salmonella* spp., *Vibrio* spp., *Vibrio cholerae*, enterotoxigenic *Escherichia coli* or ETEC, enteroaggregative *E. coli* or EAEC, Enteroinvasive *E. coli* or EIEC/*Shigella*, Enteropathogenic *E. coli* or EPEC, *E. coli* O157, *Plesiomonas shigelloides* and *Yersinia enterocolitica*), two toxins (*C. difficile* toxin A/B and Shiga toxin sxt1/sxt2), five viruses (adenovirus 40/41, NoV GI/GII, rotavirus A, sapovirus and astrovirus) and four parasites (*Cryptosporidium*, *E. histolytica*, *G. lamblia*, and *Cyclospora cayetanensis*). Due to its low sensitivity as compared to culture, *Aeromonas* spp. is not included in this panel [19]. Other multiplex assays which have been used are Becton Dickinson's BD MAX enteric bacterial panel (BD, Sparks, Maryland, USA), which detects *C. jejuni* and *C. coli*, *Salmonella* spp., *Shigella* spp./enteroinvasive *E. coli* (EIEC), and the *stx1* and *stx2* genes and Seeplex Diarrhea-VACE (Seegene, Seoul, South Korea), fast track diagnostics (FTD) bacterial viral, parasitic panel (fast track diagnostics) which are not cleared by FDA yet [15].

Fig. 1 Target detection by amplification curve analysis showing different curves for different pathogens. dNTP deoxyribonucleotide triphosphate, DNA deoxyribonucleic acid, PCR polymerase chain reaction

The Luminex assay is an open system, which increases the risk of contamination and has a 5-h turn-around time for 24 samples. In FilmArray Gastrointestinal Panel, there is an automated system in which sample processing, amplification and analysis is done in a single closed system. The sample volume needed for the assay is very small (up to 200 µl) and the turn-around time is approximately 1 h per sample. Thus, FilmArray works best for low throughput laboratories in contrast to Luminex assay, which is more suitable for laboratories with high sample load. It is recommended that 200 µl of Cary-Blair stool be tested on the FilmArray, while the Luminex assay uses test 100 µl of raw stool; hence, the sample used in FilmArray PCR (Cary-Blair stool) is more dilute than that used in Luminex assay, which is raw stools [19–23].

It was observed in a study that both the FilmArray and Luminex panels displayed high sensitivity (> 90%) for the most of the analytes except *Aeromonas* sp. (23.8%) by FilmArray and *Yersinia enterocolitica* (48.1%) by the Luminex assay. In another study, the FilmArray panel was reported to be 100% sensitive for 12/22 targets and ≥ 94.5% for others (except *Vibrio* spp., *V. cholerae*, or *E. histolytica* as these were not detected) when assessed with respect to comparator methods. The specificity was found to be ≥ 97.1% for all the targets [1, 20].

A few studies have evaluated xTag GPP assay (Luminex) and FilmArray panels in immunocompromised patients [24, 25]. A negative result from conventional techniques exposes these patients to more invasive procedures such as colonoscopy. In a retrospective analysis of kidney transplant recipients, it was found that use of multiplex panel detected pathogens in 55.5% of nine patients who had to undergo colonoscopy after conventional work up failed to detect an etiology. Also, it was shown that multiplex panel detected a target in 46.2% of 13 diarrheal episodes, which were initially thought to be due to use of immunosuppressive drugs [24].

In this issue of the *Journal*, a study reported use of a multiplex PCR assay, the FilmArray GI Panel in patients with diarrhea [26]. In this study, 50% of 106 patients were positive by panel in contrast to 17.6% by conventional comparator methods. Among patients in whom an etiology was detected both by the panel and the comparator methods, clinical concordance was better with conventional techniques. Conventional techniques detected pathogens not included in the FilmArray GI panel in 21.1% patients. The study highlights various aspects of multiplexing such as greater detection of co-infections, pathogen identification in chronic diarrhea, detecting different diarrheagenic *E. coli* and viral agents. There are many advantages of using such a system, which covers most of the common pathogens associated with diarrhea. The panels overcome the limitations of culture-based methods. The pathogens which are not routinely tested in laboratories such as viruses and bacteria like *Yersinia* and *Campylobacter* can also be detected. These systems are easy to work with,

requires little training, have short turn-around time, provide better results. Short turn-around time also helps in deciding the treatment of choice. In a study, it was observed that the median time from collection of samples till result of the tests was 3.3 h for the FilmArray GI panel in comparison to 45.4 h for culture (p -value < 0.0001) [23]. A negative result helps in ruling out an infectious cause of illness avoiding unnecessary use of antibiotics. Antibiotic discontinuation rate was reported to be 33% in patients for whom these drugs were started before panel results in comparison to 5.4% after stool culture results (p -value = 0.0014) [27].

This is especially important in immunocompromised patients in whom negative results would mean non-infectious causes such as immunosuppressive drugs or graft-versus-host disease. Rapidity of the results allows execution of timely measures to prevent the nosocomial spread of infection. Rand et al. studied stool samples by FilmArray, which were earlier tested negative for *C. difficile* and/or Rotavirus by conventional methods. In this study, FilmArray detected at least one pathogen in 22% of patients, out of which 42% had an hospital-acquired infection and 60% of these patients were never placed in isolation [28]. Halligan et al. showed that negative results by Luminex panel led to identification of unnecessary isolation in 42% patients [29].

There are some limitations to multiplex assays. Firstly, there is a considerable increase in cost over routine diagnostic techniques. In addition, there is no isolate available for antimicrobial susceptibility testing, as these panels detect dead organisms also. A positive result for two pathogens may not be reliable clinically especially in patients from developing countries where asymptomatic colonization and shedding are common. Moreover, use of these panels results in an increased detection of *C. difficile*, which may not always be associated with clinical illness [19].

Thus, a thoughtful use of these multiplex panels can help in early diagnosis and management while avoiding unnecessary treatment. This would help in controlling the emergence of resistant strains. An early diagnosis is also important in immunocompromised patients such as transplant recipients to initiate early treatment. In epidemics, these panels may be used to guide preventive strategies. The use of multiplex PCRs may reduce the time between sample collection and reporting of the results, which may help to correctly classify the cause of diarrhea and implement appropriate therapy. A negative result could reduce antibiotic use, preventing resistance development.

Compliance with ethical standards

Conflict of interest UG and NT declare that they have no conflict of interest.

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