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MICROBIOLOGY

Improved detection of gastrointestinal pathogens using generalised sample processing and amplification panels

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Summary

We aimed to streamline the diagnosis of gastrointestinal disease by producing multiplexed real time polymerase chain reaction (PCR) panels employing universal sample processing for DNA and RNA containing pathogens. A total of 487 stored, previously characterised stool samples comprising bacterial, viral, protozoan and *Clostridium difficile* positive samples were tested using four multiplexed real time PCR panels. A further 81 pre-selected clinical samples from a teaching hospital were included to provide an independent validation of assay performance. Improved sensitivity was achieved using the protozoan panels and 16 more mixed infections were observed compared to tests with conventional methods. Using the *C. difficile* panels, 100% sensitivity was achieved when compared to the gold standard of toxigenic culture. In addition, hypervirulent strains including ribotype 027 could be identified directly from primary sample without the need for ribotyping methods. Bacterial and viral panels detecting *Salmonella*, *Shigella*, *Campylobacter*, *Yersinia enterocolitica*, *Listeria monocytogenes*, norovirus groups I and II, rotavirus A, astrovirus, sapovirus, rotavirus B, adenovirus and adenovirus 40/41 performed as well as conventional methods, whilst allowing detection in 3 hours from processing to result. Multiplex real time PCR panels with universal sample preparation allow streamlined, rapid diagnosis of gastrointestinal pathogens whilst extending the characterisation of pathogens present in stool samples from affected patients.

Key words: Automated nucleic acid extraction platforms, gastrointestinal pathogens, multiplex real time PCR, universal sample processing.

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INTRODUCTION

Gastrointestinal disease is a major cause of morbidity and mortality world-wide. In developed countries the mortality due to gastrointestinal infections is lower than in developing countries, but morbidity and economic consequences are high.[1] In developing countries gastrointestinal disease is the second most common cause of morbidity and mortality, causing the death of approximately 2 million children less than 5 years of age each year.[2]

Human viral gastroenteritis is caused by many aetiological agents including most frequently noroviruses, rotaviruses, adenoviruses, astroviruses and sapovirus. More recently, a number of emerging viral causes of gastrointestinal illness have been described. These include enteroviruses, bocaviruses, Saffold cardioviruses, klasseviruses and parechoviruses.[3–8] However, the role of these viruses in the aetiology of gastroenteritis at present remains unclear due to low numbers detected, the presence of the agents in both diseased and control samples or the lack of control cohorts in many studies. Norovirus is the most commonly isolated agent as the cause of acute viral gastroenteritis,[9] and children under five years old are frequently infected with rotavirus. In both developing and developed countries viruses are the most common agents responsible for gastrointestinal disease, accounting for over 60% of cases.[10,11]

According to the Centers for Disease Control and Prevention (USA), data obtained from 46 million persons across 10 participating states in the USA showed 43% of bacterial infections are caused by *Salmonella* species, followed by *Campylobacter* species, which accounted for 33% of infections, *Shigella* species with 17%, *Escherichia coli* with 4.1% and *Yersinia* species which caused 0.9% of infections.[12] In developing countries the distribution of bacterial species is markedly different, with cholera remaining as one of the great epidemic diseases of the tropical world. In addition, higher rates of *Shigella* species, and diarrhoeagenic *Escherichia coli* (DEC)[10] are observed than in developed countries. Other causes of bacterial gastroenteritis are toxigenic strains of *Clostridium difficile* that have emerged worldwide, particularly hypervirulent strains such as polymerase chain reaction (PCR) ribotype 027.[13] These strains cause significant morbidity and mortality, particularly in the elderly, the immunocompromised, and patients on long-term antibiotic therapy.[14]

The protozoa *Giardia intestinalis, Cryptosporidium* species and *Entamoeba histolytica* are considered the most common and important causes of protozoan diarrhoea[15–17] although other species such as *Dientamoeba fragilis* and *Blastocystis hominis* may have a role in gastrointestinal disease. Many of these species of protozoa have a worldwide distribution, but the range of species and their prevalence is much higher in developing areas due to lower levels of sanitation and hygiene.[10] *Cryptosporidum* species for example were detected in <1% of

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samples from patients with gastrointestinal disease in a recent UK survey while a prevelance rate of 5–15% can be seen in children with acute diarrhoea presenting at treatment centres in developing countries.[10,11] The World Health Organization (WHO) estimates that some 50 million people worldwide will suffer from amoebic infection each year resulting in 40,000–100,000 deaths.[18–20]

Traditionally the diagnosis of these agents has been challenging, with different departments providing specialist diagnostics, with a wide range of different diagnostic methods used.[21,22] The time to final diagnosis using conventional culture techniques is 3–5 days for *Salmonella*, *Shigella* and *Yersinia* species and 2–4 days for *Campylobacter*,[23] and using stool microscopy to detect protozoan infections can take 1–2 days. All of these diagnoses are further complicated when different diagnostic methods are used, particularly if they are in different laboratories.

We have previously shown that a novel chemistry[24] universally modifies the nucleic acid genomes of pathogens, allowing improved efficiency of multiplex real time PCR detection and diagnosis of these infections. The difference in DNA sequence for two primers and probes that target either wild-type or 3base (Genetic Signatures, Australia) converted nucleic acid sequences are compared in Fig. 1. In this example, the cytosine bases (C) in the microbial nucleic acid are ultimately converted to thymine (T) via a chemical modification, forming a genome comprised of only three bases (A, G and T). This change in target sequence allows for primers and probes to be designed that have a more similar melting temperature (Tm) to each other as compared to the wild-type nucleic acid sequence. In addition, the 3base conversion results in the genomes of different subtypes becoming more similar to each other, and therefore primers and probes that target 3base sequences contain fewer mismatches (Fig. 2), are more homologous, and are less cross-reactive in assays designed to detect multiple subspecies of pathogens.[24] The increased sequence homology after 3base conversion does not result in reduced specificity as primer length can be increased to compensate, probes may be used in the PCR detection, and such assays can have greater specificity compared with other methods.[24]

In order to streamline the detection of enteric pathogens, we combined this novel chemistry with a simple 15 minute sample preparation method resulting in efficient extraction of nucleic acid from bacterial, protozoan and viral pathogens. Furthermore, we developed four separate multiplexed real time panels that target the detection of the most common causes of gastroenteritis (Table 1). The kits are supplied with an internal positive control, a synthetic target already present in the mastermix, to control for any PCR inhibition. The kits are also supplied with an extraction control, whereby primers and probes in the mastermix target a universal bacterial sequence that should be endogenously present in all stool specimens. As the PCR amplifcation conditions are common across all panels, a single specimen of faecal material can now be screened simultaneously for a broad range of pathogens.

Strain	Sequence
Type-F	ACGCCTCGGAGTACCTGAGCCC
41	ACGCCTCGGAGTATCTGAGTCC
14	ATGCTTCGGAGTACCTGAGTCC
Type-A	ATGCCTCGGAGTACCTGAGCCC
2	ACGCCTCGGAGTACCTGAGCCC
5	ACGCCTCGGAGTACCTGAGCCC
7	ATGCTTCGGAGTACCTGAGTCC
30	ACGCCTCGGAGTACCTGAGCCC
50	ATGCTTCGGAGTACCTGAGTCC
64	ACGCCTCGGAGTACCTGAGCCC
3base probe sequence	ATGTTTTGGAGTATTTGAGTTT

Fig. 2 The genomic sequence of a portion of the real time PCR probe used for the universal detection of adenovirus. As can be seen from the figure the 3base probe sequence is a 100% (22/22 bases) match to all strains.

MATERIALS AND METHODS

3base chemistry

The 3base conversion was performed during the lysis step of the EasyScreen Sample Processing Kits (Genetic Signatures), where the specimen was added directly to a lysis buffer (combined reagents 1 and 2), heated at 95°C for 15 min, followed by a purification of the 3base form of the nucleic acids via automated platforms or manual spin columns. A PCR positive control was available separately, providing synthetic templates for all targets. All targets were already in a 3base form and were provided at a low copy number in order to substantially reduce any potential for contamination.

Whole bacterial and viral standards

The following whole bacterial and viral standards were obtained from Zeptometrix (USA): NATtrol *Clostridium difficile* NAP1 [Cat# NATCdi (NAP1) ERCM], Adenovirus Type 40 (Cat# NAT ADV40-ST), Norovirus GI (Cat# 0810086CF), Norovirus GII (Cat# NATNOVII-ST) and Rotavirus A (Cat# NATROTA-ST). For sensitivity studies using whole viral particles, 10 μL each of Norovirus GII, Adenovirus Type 40 and Rotavirus were processed using an EasyScreen Sample Processing Kit (Manual Extraction) according to the manufacturer's instructions, and eluted in 12 μL of elution solution. The eluate was then reverse-transcribed using iScript reverse transcriptase (Bio-Rad, USA) using random hexamers according to the manufacturer's instruction in a final volume of 20 μL. Serial dilutions were then prepared from this stock and seeded into the PCR reaction. Bacterial DNA was obtained from the American Tissue Culture Collection (ATCC) as listed in Table 2 and used to determine any cross-reactivity of the individual components of the assay.

Sensitivity and specificity experiments

The sensitivity of detection for all microbial targets was determined in three ways. Firstly oligonucleotides were synthesised containing the identical target region sequence of the organism of interest and converted to 3base form using an EasyScreen Sample Processing Kit (Manual Extraction), however given the small size of the oligonucleotides the purification was performed via

Conventional	Sequence	Tm	3base	Sequence	Tm
Primer1	GTACACACCGCCCGTCGCTCCTACC	77°C	GTATATATTGTTTGTTGTTTTTATT		52°C
Primer2	GAAGGAGAAGTCGTAACAAG	56°C	GAAGGAGAAGTTGTAATAAG		50°C
Probe1	TGAATAAAGAGGTGAAATTCTAGG	59°C	TGAATAAAGAGGTGAAATTTTAGG		59°C
Probe2	GAAGGGCCGCGAGCCCCCGCGC	87°C	GAAGGGTTGTGAGTTTTTGTGT		62°C

Fig. 1 Sequences of primers and probes before and after the modification reaction. As can be seen the melting temperatures (Tm) of the 3base primers and probes are now more compatible with each other.