Universal extraction method for gastrointestinal pathogens

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A universal stool extraction method for recovery of nucleic acids (NAs) from gastrointestinal pathogens was developed to support rapid diagnostics for the London 2012 Olympics. The method involved mechanical disruption (bead beating) of the stools, followed by automated extraction and detection using real-time PCR. This method had been used extensively in the Second Infectious Intestinal Disease Study (IID2) for the isolation of NA from bacteria and parasites (and was effective for the robust recovery of Cryptosporidium spp.) but had not been used for enteric viruses. To ensure this method was universally suitable, panels of samples known to contain target bacteria, viruses or parasites were processed in triplicate using the pre-treatment method routinely used for each target and the new extraction method (bead beating). The extracts were tested using real-time PCR and the cycle threshold values were compared. The results from this study showed that bead beating improved yields for the bacterial and parasitic targets and was suitable for the viral targets. The implementation of this universal method should confer cost- and time-saving benefits and streamline the processes required for the characterization of an array of pathogens from faecal samples.

INTRODUCTION
Outbreaks of gastrointestinal infections are a likely consequence of mass gatherings such as the Olympic and Paralympic Games, and typically place tremendous pressure on the public health infrastructure (Jorm et al., 2003). Consequently, enhanced surveillance and early detection tools are required to enable outbreaks to be rapidly identified and contained.

Automated nucleic acid (NA) extraction followed by real-time PCR is a high-throughput process allowing the detection of pathogen NAs from stool samples. It has been widely adopted in diagnostics; however, the organization of hospital and public health departments often means that the different pathogen groups (bacteria, protozoan parasites and viruses) that may be found in stool samples are investigated by separate disciplines. In order to streamline and simplify the detection of a range of gastrointestinal pathogens that are important from a public health perspective [Campylobacter jejuni and Campylobacter coli, Salmonella, Shigella, Escherichia coli (enteroaggregative Escherichia coli (EAGG), verocytotoxin-producing Escherichia coli (VTEC) and O157), norovirus genogroup I (GI), norovirus GII, rotavirus, adenovirus, astrovirus, sapovirus, Giardia duodenalis (=Giardia lamblia/intestinalis), Cryptosporidium spp. and Entamoeba histolytica], a universal method was sought allowing NAs to be extracted from a stool sample for the

Abbreviations: EAGG, enteroaggregative Escherichia coli; IID2, Second Infectious Intestinal Disease Study; NA, nucleic acid; VTEC, verocytotoxin-producing Escherichia coli.
comprehensive screening of potential sources of diarrhoeal disease.

Extraction of NAs from stools can be challenging and involves the liberation of the NA, protection from degradation, removal of inhibitory substances, concentration of the target NA and recovery in a condition suitable for longevity and future analysis (Espy et al., 2006). A pre-treatment step is commonly required (prior to the extraction procedure) to maximize the liberation of NAs, pellet any debris from stool sample and reduce the amount of PCR inhibitors present.

In the Second Infectious Intestinal Disease Study (IID2), Tam et al. (2012) performed automated extraction and real-time PCR for the detection of a range of gastrointestinal pathogens from faecal samples. The methods have been published elsewhere (O’Brien et al., 2010). Recovery of NA from stools containing Cryptosporidium spp. depended largely on the mechanical lysis and disruption of a stool sample using bead beating on a MagNA Lyser platform (Roche) to liberate the NA, followed by the removal of solid particulate matter (and inhibitors) by centrifugation. Such disruption was needed, as the robust oocyst structure of Cryptosporidium spp. renders the NA difficult to extract (Elwin et al., 2012). Although not necessary, this method was also applied for the isolation and characterization of other targets (Campylobacter spp., Salmonella enterica, Listeria monocytogenes, EAGG, VTEC and G. duodenalis) in order to streamline the IID2 testing algorithm. Mechanical lysis was neither used (nor necessary) for viral targets, as viral NA is readily liberated from a simple stool suspension without pre-treatment.

**METHODS**

To define a single pre-treatment method suitable for all target pathogens [C. jejuni and C. coli, Salmonella, Shigella, Escherichia coli [EAGG, VTEC and O157], norovirus GI, norovirus GII, rotavirus, adenovirus, astrovirus, sapovirus, Giardia duodenalis, Cryptosporidium spp. and Entamoeba histolytica], a variety of pre-treatment methods (herein referred to as A, B and C) were used on a panel of samples. These comprised five stools from the IID2 archive that were known to be positive for rotavirus, Cryptosporidium spp., G. duodenalis, C. jejuni, norovirus (GI and GII) and EAGG. Four of the five samples contained a single pathogen; the remaining sample contained norovirus GI and GII and EAGG. These stools had been tested by real-time PCR shortly after collection (as part of IID2 2007–2010) and then stored at 4°C prior to this testing (~13–17 months later). During long-term storage, they were not subjected to any freeze–thaw cycles.

Methods A and B (currently being used by collaborators for viral and parasitic detection, respectively) involved the disruption of cell membranes through vortexing, boiling the sample and centrifugation. Briefly, method A (routinely used as a pre-treatment for the extraction of viral NA at Barts Health NHS Trust) involved the addition of 200 µl or 200 µg stool to 800 µl PBS to make a suspension. This was then briefly vortexed, heated at 95°C for 10 min, separated by centrifugation (12000 g for 5 min) and the supernatant used for extraction. Pre-treatment method B (used for protozoa) was very similar, with the same quantity of stool added to 400 µl ATL buffer (Qiagen), followed by vortexing, heating and centrifugation as above. The neat supernatants (300 µl) from both of these methods were then extracted on a Qiasymphony automated platform (Qiagen) using the Qiasymphony DNA Midi kit and the ‘complex pathogen 200’ programme.

Method C (as used in IID2) involved physical disruption of the material using 1.4 mm ceramic beads (Roche Diagnostics) and a bead-beating machine (MagNA Lyser; Roche). For this, 200 µl of each stool sample was added to 900 µl lysis buffer (Severn Biotech) and 20 µl isomyl alcohol in a bead-beating tube. The suspension was beaten for 1 min at 3000 r.p.m. and then centrifuged (12000 g for 15 s) to separate out all the particulate matter. The supernatant (250 µl), mixed with PBS in a 1:1 ratio, was then processed on a Qiasymphony as above.

Each of the five samples in the initial testing panel was processed in triplicate using each of the three pre-treatment methods described above. Following extraction on the Qiasymphony platform, they were immediately subjected to real-time PCR analysis (O’Brien et al., 2010) to assess the recovery of the NA. The cycle threshold ($C_t$) values (the cycle at which the PCR product was first detected above a pre-determined fluorescence threshold value) were compared for each pre-treatment method.

Following on from this, additional testing was performed on a panel of 12 viral NA-positive stool samples using pre-treatment methods A and C. The stools originated from the IID2 archive (samples 2, 4 and 6–12), and from the routine laboratory service (Enteric Virus Unit, PHE Microbiology Services, London) (samples 1, 3, 5), and included two samples each for the six viruses (norovirus GI, norovirus GII, rotavirus, adenovirus, astrovirus and sapovirus).

Finally, validation experiments were conducted on a wide variety of pathogens to compare different detection methods to extraction using the pre-treatment method C and the Qiasymphony. For each pathogen group, method C was compared with the routinely used method of detection. For viruses, this involved extracting a panel of 62 viral NA-positive stools from the IID2 archive in duplicate with methods A and C and then comparing the $C_t$ values. This panel was made up of stools (collected between 2007 and 2010, as described above) that were positive for adenovirus (ten stools), astrovirus (ten), sapovirus (four), norovirus GI (eight), norovirus GII (11) and rotavirus (nine), and ten stools with a dual viral infection.

Bacterial validation was performed on 33 PCR-positive O157 Escherichia coli stools that were culture positive and had recently been extracted using the Instagene extraction from broth method (Bio-Rad).

For the protozoan parasites, method C was compared with microscopy using a panel of: (i) 55 Cryptosporidium spp. stools (that had been collected over a 2-month period and stored at 4°C), (ii) 28 Giardia spp. stools (collected over a 2-year period and stored at 4°C) and (iii) 13 Entamoeba histolytica stools (collected over a 5-year period, frozen at −20°C and freeze–thawed approximately five times). Pre-treatment method C was also tested on a panel of 50 Cryptosporidium spp. stools, originally tested via salt flotation, boiling and extraction using a QIamp DNA Mini kit (Qiagen).

**RESULTS**

The initial experiment, where five NA-positive stools were processed in triplicate using all pre-treatment methods, suggested that: (i) the NA could not be recovered for rotavirus or Giardia duodenalis for any of the
pre-treatments, (ii) the pre-treatment method used had minimal impact on the recovery of C. jejuni and EAGG NA, (iii) norovirus NA was detectable after the bead-beating process and (iv) the recovery of Cryptosporidium spp. NA was improved by the bead-beating (method A gave a $C_T$ of 36.17 compared with 27.58 with method C).

As Cryptosporidium spp. NA was not detectable after pre-treatment with method B, this method was not tested further. Method A (although inferior to the others for the isolation of norovirus NA in the IID2 mixed-stool sample) was investigated further, as this was the pre-treatment method used successfully for a range of other pathogens in IID2. Method C was also selected for further testing based on the improvement in recovery of Cryptosporidium spp. NA. Whilst it appeared that neither of the methods was suitable for the recovery of rotavirus or Giardia duodenalis NA, the impact of long-term storage on the samples was unknown and hence further testing was performed using fresh samples in order to rule out degradation of the NA.

The additional testing performed using methods A and C on a viral panel of 12 samples showed that norovirus NA could be detected after pre-treatment with methods A and C, as well as adenovirus, astrovirus and sapovirus NA, although across the panel there were four samples where viral NA could not be detected with method A (Table 1). Rotavirus NA could also be detected with little difference between the methods (sample 5), but the $C_T$ value was higher than that obtained during the initial IID2 testing (data not shown).

**Validation**

Sixty-two stool samples containing a variety of viral pathogens (see Methods) were extracted using methods A and C (bead beating), and the resultant $C_T$ values compared (Table 2). For 59 samples (across all viruses tested), $C_T$ values obtained with bead beating were in agreement or within 3 $C_T$ values (±1 log$_{10}$) of those obtained using method A. Values differed by 3–6 $C_T$ values for one sample (sapovirus), with method A giving the lower $C_T$. Furthermore, two samples (one rotavirus and one astrovirus) were undetectable by bead beating but gave $C_T$ values of 38 and 31, respectively, using method A. Although this may suggest inferiority of the bead-beating method, it must be noted that, for eight of the nine rotavirus-positive and nine of the ten astrovirus-positive stool samples, $C_T$ values obtained with bead beating agreed (or were within 3 $C_T$) with those of method A.

Validation for the bacterial pathogens involved the dual extraction of a panel of 33 O157 VTEC culture-positive stool samples using method C and the Instagene extraction from broth method (see Methods). Both extracts per

### Table 1. Additional testing of a viral panel with pre-treatments A and C

<table>
<thead>
<tr>
<th>Virus (target gene)</th>
<th>Sample</th>
<th>Pre-treatment</th>
<th>$C_T$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Norovirus GI (Cog1)</td>
<td>1</td>
<td>A</td>
<td>21.65</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C</td>
<td>23.01</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>A</td>
<td>29.51</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C</td>
<td>28.55</td>
</tr>
<tr>
<td>Norovirus GII (Cog2)</td>
<td>3</td>
<td>A</td>
<td>32.46</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C</td>
<td>28.95</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>A</td>
<td>RND</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C</td>
<td>35.35</td>
</tr>
<tr>
<td>Rotavirus (vp6)</td>
<td>5</td>
<td>A</td>
<td>32.00</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C</td>
<td>33.97</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>A</td>
<td>RND</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C</td>
<td>29.14</td>
</tr>
<tr>
<td>Adenovirus (40/41 long-fibre gene)</td>
<td>7</td>
<td>A</td>
<td>13.17</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C</td>
<td>13.57</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>A</td>
<td>DND</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C</td>
<td>12.00</td>
</tr>
<tr>
<td>Astrovirus (capsid protein precursor gene)</td>
<td>9</td>
<td>A</td>
<td>27.98</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C</td>
<td>13.59</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>A</td>
<td>RND</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C</td>
<td>20.94</td>
</tr>
<tr>
<td>Sapovirus (ORF1 pol–capsid junction)</td>
<td>11</td>
<td>A</td>
<td>36.25</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C</td>
<td>26.34</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>A</td>
<td>21.53</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C</td>
<td>36.03</td>
</tr>
</tbody>
</table>
Table 2. Details of the validation experiments

<table>
<thead>
<tr>
<th>Pathogen group</th>
<th>Panel size (n)</th>
<th>Comparison method</th>
<th>How does method C (bead-beating) compare?</th>
</tr>
</thead>
<tbody>
<tr>
<td>Viruses</td>
<td>62 positive stools (from the IID2 archive)*</td>
<td>Pre-treatment method A</td>
<td>59/62: Results in agreement or within 3 CT values</td>
</tr>
<tr>
<td></td>
<td>33 VTEC O157</td>
<td>Instagene extraction from broth</td>
<td>1/62: CT values different by 3–6 CT values</td>
</tr>
<tr>
<td>Protozoan</td>
<td>96</td>
<td>Microscopy†</td>
<td>24/33: Results in agreement or within 3 CT values</td>
</tr>
<tr>
<td>parasites</td>
<td></td>
<td></td>
<td>7/33: Detected using bead beating methodology only</td>
</tr>
<tr>
<td>Cryptosporidium</td>
<td>50</td>
<td>Salt flotation followed by boiling and extraction using a QIAamp DNA Mini kit</td>
<td>2/62: Detected using method A only</td>
</tr>
<tr>
<td>spp. only</td>
<td></td>
<td></td>
<td>Bead beating and PCR detected: 54/55 microscopically-positive Cryptosporidium samples, 28/28 microscopically-positive Giardia samples, 12/13 microscopically-positive Entamoeba histolytica samples</td>
</tr>
</tbody>
</table>

*The panel contained stools positive for a single virus or mixtures of viruses (co-infections): adenovirus (ten stools), astrovirus (ten), sapovirus (four), norovirus GI (eight), norovirus GII (11), rotavirus (nine) and mixed stools (ten).
†Analysis of ParaSep concentrated stool samples stained with Ziehl Neelsen (for Cryptosporidium spp.) or iodine (for Giardia spp. and Entamoeba histolytica).

Finally, validation work was performed for the protozoan parasites comparing bead beating with either microscopy or extraction following salt flotation (Elwin et al., 2012). Bead beating/PCR detected 54/55 Cryptosporidium spp., 28/28 Giardia spp. and 12/13 Entamoeba histolytica microscopy-positive samples (Table 2). Bead beating also compared favourably with salt flotation for Cryptosporidium spp., with 50 stool samples positive by both methods with a mean difference of 2.29 in CT values. Method C (bead beating) yielded the higher CT values and this was statistically significant (P<0.05), although encouragingly the lower yields did not affect the positivity or diagnostic sensitivity when compared with those obtained from individualized extractions.

DISCUSSION

The universal bead-beating pre-treatment method performed well for the recovery of NA from the target bacteria [C. jejuni and C. coli, Salmonella, Shigella, Escherichia coli (EAGG, VTEC and O157)] and protozoan parasites (G. duodenalis, Cryptosporidium spp. and Entamoeba histolytica) when compared with extraction using 'tailed' pathogen-specific methods established in our laboratory (Boom et al., 1990; McLauchlin et al., 1999). This pre-treatment method is also acceptable for the isolation of viral NA from norovirus GI and GII, adenovirus, astrovirus and sapovirus in comparison with our current extraction protocols (Lee et al., 2011). The initial results for rotavirus suggested that bead beating may not be appropriate for the recovery of NA. However, it is important to emphasize that none of the pre-treatment methods (in the initial experiment) resulted in the detection of rotavirus NA. Hence, it is likely that the rotavirus NA had degraded during the long-term storage at 4 °C, rendering it undetectable. Rotavirus NA was detectable following bead beating of a fresh, routine service sample (number 5) and also with sample 6 (an IID2 sample), where there was improved recovery with bead beating compared with samples that had not undergone bead beating. Furthermore, in the validation work, there was agreement in CT values for nine of the ten rotavirus-positive samples tested by both method A and bead beating (method C). However, validation work using further fresh material would be advisable for rotavirus.

Although the CT values for many of the targets were higher for the universal extraction method C (representing lower yields of NA) when compared with individualized pathogen-specific methods (Boom et al., 1990; Lee et al., 2011; McLauchlin et al., 1999), it is worth noting that neither the positivity nor the diagnostic sensitivity was affected. Furthermore, this method has significant time-saving advantages over the routinely used diagnostic gold standards, for example, culture for bacterial pathogens and microscopy for parasites (Chalmers & Katzer, 2013).
In conclusion, a universal stool extraction method has been developed that enables the simultaneous recovery of NA from all relevant organism groups that cause gastrointestinal infections (bacteria, protozoan parasites and viruses). Incorporation of this procedure to allow the simultaneous processing of stool samples for the detection of all these pathogens would streamline processing (Chalmers & Katzer, 2013) and produce a significant time and cost saving compared with procedures where replicate NA extractions are performed by multiple disciplines.

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REFERENCES


