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Short communication

The development of a multiplex real-time RT-PCR for the detection of adenovirus, astrovirus, rotavirus and sapovirus from stool samples



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A B S T R A C T

Article history:

Received 11 April 2016

Received in revised form 7 November 2016

Accepted 15 December 2016

Available online 28 December 2016

Keywords:

Viral gastroenteritis

Multiplex PCR

Adenovirus

Astrovirus

Rotavirus

Sapovirus

Viral gastroenteritis is a major health problem with significant morbidity and economic consequences. Viral gastroenteritis is caused by a number of viruses, including norovirus, rotavirus, adenovirus, astrovirus, and sapovirus. Conventional diagnosis is based on direct antigen detection and electron microscopy, however enzyme immunoassay's are insensitive and not available for all relevant pathogens, and electron microscope (EM) is no longer routinely carried out in most laboratories. Most laboratories now offer norovirus real-time PCR testing however the availability of other assays is variable. Commercial methods for the detection of infectious intestinal disease (IID) are available but these can be expensive and are not commonly used. This paper describes the development of a single multiplex assay for the simultaneous detection of adenovirus, astrovirus, rotavirus and sapovirus from stool samples. The multiplex was evaluated by assessing endpoint sensitivity, specificity, panel of clinical samples, quality control (QC) panel and the robustness and reproducibility of the multiplex.

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1. Introduction

Viral gastroenteritis is a major health problem with significant morbidity and economic consequences. Viral gastroenteritis is caused by a number of viruses, including norovirus, rotavirus, adenovirus, astrovirus, and sapovirus.

Norovirus is the most common cause of viral outbreaks and sporadic cases across all age groups and was reported to be responsible for 74% of outbreaks reported in Scotland (Danial et al., 2011). In addition norovirus can cause chronic infection in the immunocompromised patient. There are five genogroups of norovirus of which GI and GII are most associated with infection in humans. Rotavirus was the main cause of severe diarrhoea in children under 2 years old in the UK with some studies finding rotavirus the cause of 20–60% of hospitalisations (Wilhelmi et al., 2003) however since the introduction of the vaccine in 2013 in the UK this has dramatically reduced (Atchison and Hassounah, 2015). Rotavirus infections in adults and the elderly are less common but have been documented and severe and prolonged infections have been documented in immunocom-

promised patients. Astrovirus, sapovirus and adenovirus are also important etiological agents of gastroenteritis. Astroviruses have been reported to be a leading cause of gastroenteritis in the community causing 4–10% of cases, and has been reported to cause cases in all age groups but mainly in those less than two years old (Hye Sook Jeong et al., 2012). Eight human serotypes exist with type one being the most common. Adenovirus is a common cause of infectious intestinal disease (IID) in children with group F types 40 and 41 being the main causes of gastroenteritis however other types can cause IID (Wilhelmi et al., 2003). Adenovirus is endemic and is an uncommon cause of outbreaks and has been reported to be the cause of 1–8% of cases in industrialised countries (Wilhelmi et al., 2003). Sapovirus is a common cause of gastroenteritis in young children, although rare cases have been documented in older ages and outbreaks have occasionally been documented (Robinson et al., 2002). There are five sapovirus genogroups of which GI, GII and GIV cause illness in humans.

Conventional diagnosis of these viruses is based on direct antigen detection (EIA) and electron microscopy, cell culture has limited application however these techniques are not suitable for all pathogens and so most laboratories no longer use these techniques. Enzyme immunoassay's are insensitive and not available for all IID causative pathogens, and EM is no longer routinely carried out in most laboratories. Most laboratories now offer in-house norovirus real-time PCR testing which is considered to be the gold standard due to the lack of commercial molecular and

Abbreviations: PCR, polymerase chain reaction; Ct, cycle threshold; GAS, gastroenteritis; IID, infectious intestinal disease.

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<http://dx.doi.org/10.1016/j.jviromet.2016.12.016>

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poor performance of EIA and other assays. Commercial methods for the detection of IID are available but these can be expensive and are not commonly used. For example [Liu et al. \(2012\)](#) present an in-house assay based on Luminex bead-based technology, however this technology is not available in most laboratories. Also some commercial available real-time panels for gastroenteritis do not have a single multiplex of all four viruses, for example the FTD (Fast-Tract Diagnostics) Viral gastroenteritis panel (Fast-Track Diagnostics Ltd, Malta) consists of a multiplex for adenovirus, astrovirus and rotavirus with a separate sapovirus single test. There are few in-house methods that offer comprehensive testing one example is [van Maarseveen et al. \(2010\)](#) who present two internally controlled multiplexes that screen for the viral causes of IID.

At the West of Scotland Specialist Virology Centre (WoSSVC) two screening algorithms are used for viral gastroenteritis which have been in use since 2004. As more than 90% of gastroenteritis outbreaks are caused by norovirus, like many other labs the testing algorithm for gastroenteritis states that all outbreaks samples are screened for norovirus. At the WoSSVC an in-house developed norovirus multiplex which detects and differentiates GI and GII subtypes as well as an internal control is used. Norovirus negative outbreaks are subsequently tested for adenovirus, astrovirus, rotavirus and sapovirus using a 'GAS' (gastroenteritis) panel which consists of four single tests for adenovirus, astrovirus, rotavirus and sapovirus. Sporadic gastroenteritis cases in children (less than 10 years old) and immunocompromised patients (all ages) are screened for adenovirus, astrovirus, rotavirus and sapovirus and norovirus simultaneously (both panels of assays set up at the same time). At the WoSSVC in order to streamline the molecular service most assays have been multiplexed based on syndrome/sample type ([Bennett et al., 2011, 2013](#); [Gunson and Carman, 2011](#)). This has improved the rapidity, cost, ease of use and throughput of testing ([Gunson et al., 2008](#)).

One of the few un-multiplexed panels in the laboratory are the GAS assays. Previous attempts to multiplex this panel of assays have not been successful due to interactions between components within the assays. The main problems encountered were determined to be due to the astrovirus and rotavirus tests, these assays interacted with other assays in the multiplex causing false negative results. In order to streamline the molecular service a single multiplex assay for the simultaneous detection of adenovirus, astrovirus, rotavirus and sapovirus from stool samples was developed. This 'GAS' multiplex was evaluated by comparison to four single existing validated in-house assays (used since 2004) through assessment of endpoint sensitivity, specificity, a panel of clinical samples, quality control (QC) panels, and the robustness and reproducibility of the multiplex was also determined.

1.1. Materials and methods

1.1.1. Sample preparation and nucleic acid extraction

Stool suspensions were prepared by adding a small volume of stool (approximately 250 μ l) to 1 ml of deionised water, this was then vortexed and centrifuged at 15000 rpm for 15 min. The supernatant was then used for RNA extraction and an in-house grown internal control (IC) was added.

All samples were extracted on the NucliSens EasyMAG (bioMérieux, Hampshire, UK) according to manufacturer instructions. 200 μ l of the sample was extracted and nucleic acid was eluted into a volume of 110 μ l.

1.1.2. Real-time RT-PCR

One-step RT-PCR was performed on 6 μ l of nucleic acid extract with Superscript III Platinum One Step Quantitative RT-PCR System (Thermal Fisher (Invitrogen), UK) on an ABI Prism 7500 SDS real-time platform (Thermal Fisher (Applied Biosystems), UK) in a 15 μ l

reaction volume. The following thermal profile was used: a single cycle of reverse transcription for 10 min at 50 °C, 2 min at 95 °C for DNA polymerase activation followed by 35 amplification cycles of 8 s at 95 °C and 34 s at 60 °C each (annealing-extension step).

1.1.3. Assays used in the multiplex

Single real-time assays for the detection of adenovirus, astrovirus, rotavirus and sapovirus have been carried out routinely for 12 years at the WoSSVC. The assays used are previously published assays by [Heim et al. \(2003\)](#) (adenovirus), [Le Cann et al. \(2004\)](#) (astrovirus), [Pang et al. \(2004\)](#) (rotavirus) and [van Maarseveen et al. \(2010\)](#) (sapovirus) (sequences in [Table 1](#)). These assays have been shown to be sensitive and specific, and to detect all relevant IID causing strains. These assays have been previously validated against conventional PCR methods, and these assays also been accredited by the Clinical Pathology Accreditation (CPA) and the UK Accreditation Service (UKAS). In addition since implementation in 2004 these assays have been assessed by annual/biannual external QC panels to ensure sensitivity and specificity. Previous attempts to multiplex these four assays into one multiplex assay have failed. The main problems encountered were determined to be due to the astrovirus and rotavirus tests, these assays interacted with other assays in the multiplex causing false negative results. Due to interactions between the assays we used alternative astrovirus and rotavirus assays published by a well established research group Eric Claas – [van Maarseveen et al. \(2010\)](#) and attempted to multiplex with the existing adenovirus and sapovirus assays (sequences in [Table 1](#)). Again these assays have been shown to be sensitive and specific, and to detect all relevant IID causing strains. The astrovirus assay was designed by the Claas group and assessed by assessment of analytical sensitivity and specificity and clinical evaluation of 239 clinical samples. The rotavirus assay used by the Claas group is an adapted version of the Pang et al. rotavirus assay which is established in the WoSSVC laboratory, this same probe with adapted primers was used. The multiplex assay was compared to the existing single assays in use at the WoSSVC.

1.1.4. Assessment of the multiplex assay

1.1.4.1. Primer probe optimisation. The primer and probe concentrations for all assays, singleton and multiplex, were individually optimised using in-house protocols ([Gunson et al., 2003](#)), all primers and probes are shown in [Table 1](#). Each probe was labelled with a different fluorescent reporter dye ([Table 1](#)). The sapovirus probe (MGB) and all primers were purchased from Applied Biosystems (Cheshire, UK). The remaining probes were purchased from Eurogentec (Seraing, Belgium). The optimised concentration for each probe was 100 nM and each primer 1000 nM.

1.1.4.2. Endpoint sensitivity of single assays compared to the multiplex assay. The endpoint detection limits of each component in the multiplex were directly compared to the current routine single assays using a dilution series of positive controls for each target, dilutions were tested in duplicate. These were carried out to ensure that multiplexing the assays did not result in a loss of sensitivity at the endpoint of detection and to compare the new published assays (astrovirus and rotavirus) to the established assays.

1.1.4.3. Analytical sensitivity, efficiency and linearity of the multiplex assay. Three standards for each target were tested in triplicate over 12 runs to determine the linearity of the method. For accurate and reproducible quantification, it is ideal to have a standard curve with linearity between -3.1 and -3.6 (an ideal curve is -3.33) and reaction efficiency (R2) near to 100%.

1.1.4.4. Analytical specificity of the multiplex assay. The specificity of the multiplex was further assessed by testing commonly encoun-

Table 1
Primer and probe sequences of assays.

Target	Primers	Probe with reporter dye	Reference
Alternative astrovirus	F-TCT YAT AGA CCG YAT TAT TGG R- TCA AAT TCT ACA TCA TCA CCA A	ROX – CCC CAD CCA TCA TCA TCT TCA TCA	van Maarseveen
Original astrovirus	F-CCG AGT AGG ATC GAG GGT R-GCT TCT GAT TAA ATC AAT TTT AA	FAM -CTT TTC TGT CTC TGT TTA GAT TAT TTT AAT CAC C	Le Cann
adenovirus	F-GCC ACG GTG GGG TTT CTA AAC TT R-GCC CCA GTG GTC TTA CAT GCA CAT C	Atto647 TGC ACC AGA CCC GGG CTC AGG TAC TCC GA	Heim
Alternative rotavirus	F- ACC ATC TWC ACG TRA CCC TC R- CAC ATA ACG CCC CTA TAG CC	HEX - ATG AGC ACA ATA GTT AAA AGC TAA CAC TGT CAA	van Maarseveen (adapted Pang)
Original rotavirus	F- ACC ATC TAC ACA TGA CCC TC R- GGT CAC ATA ACG CCC C	FAM -ATG AGC ACA ATA GTT AAA AGC TAA CAC TGT CAA	Pang
sapovirus	F- CAG GCT CTC GCC ACC TAC R- CCC TCC ATY TCA AAC ACT AWT TT	FAM - TGG TTC ATA GGT GGT RC-MGB	van Maarseveen

F: forward; R: reverse.

tered pathogens: human herpes virus types 6 and 7; erythrovirus (parvovirus) B19; norovirus types GI and GII; enterovirus; parechovirus; cytomegalovirus, Epstein barr virus; measles virus; mumps virus; rubella virus; influenza A, B; influenza A H1N1 (2009); influenza A H1N1 (2009) H275Y; coronaviruses 229E, OC43, NL63, HKU1; parainfluenza types 1–4; rhinovirus; respiratory syncytial virus types A and B; Human metapneumovirus types A and B; *Treponema pallidum* (syphilis); *Pneumocystis jirovecii*; *Mycoplasma pneumoniae*, *Clostridium difficile*, *Salmonella enteritidis*, *Cryptosporidium hominis*, *Cryptosporidium parvum*, and *Cyclospora*. No-template controls (water) were also tested to ensure the assay produced no false positive traces.

1.1.4.5. Assessment of clinical sample panel (diagnostic specificity). A panel of 137 clinical samples were tested through the 'GAS' multiplex and the results were compared to previous results from routine single testing. The panel consisted of 35 adenovirus, 34 astrovirus, 35 sapovirus and 33 rotavirus positives. These samples had a range of Ct values for each pathogen and were received from a range of patient age groups (mainly children and elderly) at the WoSSVC between October 2014 and January 2015 and between January 2016 and October 2016. This panel was assessed to ensure that the clinical sensitivity and specificity was comparable to the existing singleplex assays.

1.1.4.6. Viral gastroenteritis QCMD panel. To assess the sensitivity and specificity of the multiplex a QCMD (Quality Control for Molecular Diagnostics, Scotland) panel was tested (2015 panel) and compared to routine results (single tests). These results were also compared to the results of other laboratories. This panel was extracted on the bioMérieux easyMag platform (bioMérieux, Hampshire, UK).

1.1.4.7. Inter-assay and intra-assay variability. The inter-assay and intra-assay variability of the multiplex was also assessed to determine the repeatability and the reproducibility of the multiplex. A set of three controls (strong, moderate and weak) for each target were tested six times in one assay (intra-assay) and six times over six separate PCR runs (inter-assay). This assesses the whole testing system by including extraction and PCR runs with different users.

1.2. Results

1.2.1. Endpoint sensitivity

Comparing the endpoint detection limit of each component of the multiplex assay to the single assays showed that multiplexing had no detrimental effect on the endpoint detection limit of each component (Table 2). The adenovirus single assay detected the 10^{-3} dilution in 1 out of 2 occasions, the multiplex detected down to the 10^{-4} dilution. When testing the dilution series of astrovirus, the single assay detected the 10^{-3} dilution; the multiplex assay (containing a new astrovirus assays) detected the 10^{-5} dilution in 1 out of 2 occasions, highlighting that the new astrovirus assay has an improved endpoint detection limit in comparison to the established astrovirus assay. The rotavirus single assay and the new rotavirus assay within the multiplex both has an endpoint detection limit of 10^{-5} . When testing the dilution series of sapovirus, the single sapovirus assay detected the 10^{-6} dilution; the multiplex detected the 10^{-5} dilution.

1.2.2. Analytical sensitivity, efficiency and linearity of the multiplex assay

Three standards for each target were tested in triplicate over 12 runs to determine the linearity of the method. The results are summarised in Table 2 with the R² and slope values given. The results suggest that the linearity of the method over 12 runs is consistent; suggesting the quantification component of the assay is robust, all slope values were between -3.1 and -3.6 and reaction efficiency was near to 100% (99–100) for all curves.

1.2.3. Specificity

The specificity of the assay was confirmed by testing a panel of other commonly encounter pathogens and no-template controls and no false positive results were encountered.

1.2.4. Panel of clinical samples

A total of 137 clinical samples were tested retrospectively through the multiplex and the results compared to previous single results (data not shown). All were detected positive by the multiplex. Most samples had a comparable Ct, some Ct values were slightly higher by the multiplex and some had a stronger Ct value than previously tested.

Table 2
Overall performance of the multiplex.

Assessment	adenovirus		astrovirus		rotavirus		sapovirus		
EDL	Single	10 ⁻³ +/-		10 ⁻³		10 ⁻⁵		10 ⁻⁶	
	Multiplex	10 ⁻⁴ +/-		10 ⁻⁵ +/-		10 ⁻⁵		10 ⁻⁵	
	R2	Slope	R2	Slope	R2	Slope	R2	Slope	
Linearity and efficiency ranges	0.99–1.0	-3.1 → 55	0.99	-3.2 → 51	0.99	-3.11 → 27	0.99	-3.1 → 25	
Repeatability and reproducibility		Mean Ct	C _V	Mean Ct	C _V	Mean Ct	C _V	Mean Ct	C _V
Intra-assay	S3	22.3	0.00479	18.14	0.00529	18.87	0.00903	18.51	0.00656
	S2	27.83	0.00829	24.2	0.00788	24.66	0.01525	24.16	0.00612
	S1	33.93	0.01847	34.13	0.01037	34.6	0.00887	30.75	0.01106
Inter-assay	S3	22.08	0.02397	17.9	0.00582	18.88	0.01497	18.17	0.01221
	S2	27.28	0.12473	24.2	0.01727	24.64	0.03103	24.07	0.01248
	S1	34.29	0.01735	32.14	0.14854	31.8	0.02164	30.67	0.01364

EDL: endpoint detection limit; R2: reaction efficiency; C_V: co-efficient of variation.

1.2.5. Viral gastroenteritis QCMD panel

A QCMD panel consisting of nine samples containing norovirus, adenovirus, astrovirus, rotavirus and sapovirus was tested through the multiplex and compared to the single assay results. The results suggest that the multiplex performs with comparable sensitivity to the single assays identifying all expected results at similar Ct values to the singleplex assay (two samples were norovirus positive).

1.2.6. Intra- and intra-assay variability

A set of three standards for each target was tested through the multiplex assay six times within the same PCR run and over six PCR runs to determine the repeatability and reproducibility of the multiplex over a range of Ct values and PCR runs. The results of are summarised in Table 2 with the mean Ct value and C_V values. Based on the low C_V values obtained for each standard the results suggest that the assay is robust even at weaker Ct values.

1.3. Discussion

This paper describes the development and validation of a multiplex real-time PCR assay, which will allow rapid and simultaneous detection of adenovirus, astrovirus, rotavirus and sapovirus in stool samples. Multiplexing the four assays had no effect on the performance of any of the individual components by assessment of dilution panels, no loss in sensitivity was observed, also the addition of new assays for astrovirus and rotavirus lead to no loss in sensitivity, and in fact some assays performed better in the multiplex than the single assay. The multiplex assay was shown to be specific by assessment of quality control panels and a panel of commonly encountered pathogens. In addition the performance of the multiplex was determined by assessment of the analytical sensitivity, linearity and efficiency, clinical sensitivity and specificity of the multiplex.

One limitation of this assay is that the 'GAS' multiplex described here does not contain an internal control however as all samples will also be screened by the norovirus assay, either prior to or simultaneously with the 'GAS' multiplex and so all samples will have been internally controlled and therefore monitored for inhibition. Future work will attempt to incorporate an internal control into the 'GAS' multiplex, however this will be difficult due to potential cross talk issues that may be encountered with the limitations of channels available on the PCR instruments.

Real-time PCR is a sensitive method for the diagnosis of viral causes of IID, and with increasing use of PCR an increase in detection rates has been observed. Multiplexing single real-time PCR assays by sample type/disease syndrome simplifies the routine service;

overall costs are reduced when compared to single testing as rapid diagnosis can reduce unnecessary costs. This is a result of less staff, reagents and machine usage being required for one assay versus four assays, therefore the laboratory can make more adequate use of resources, and reductions in sample turn around times have been achieved by reducing the amount of separate tests needed to be carried out. This ensures more samples can be tested within the working day (Gunson et al., 2008). In additional results for all four pathogens will be available at the same time so users are not drip-fed results.

As stated previously, few in-house multiplex real-time PCR assays for the detection of viral gastroenteritis are presented in the literature. This may be due to the difficulties encountered when attempting to multiplex the assays. van Maarseveen et al. (2010) present two assays for diagnosis of viral gastroenteritis, however they have chosen to multiplex norovirus with the other assays which would not suit the testing algorithm at the WoSSVC. Liu et al. (2012) present an assay based on Luminex bead-based technology, however this technology is not available in most laboratories. Several commercial assays are available (reviewed by Reddington et al. (2014)), however these do not always include all relevant viral causes of IID, and some commercial available real-time panels for gastroenteritis do not have a single multiplex of all four viruses.

Between January and October 2016 our laboratory covering the West of Scotland screened 2420 stool samples for adenovirus, astrovirus, rotavirus and sapovirus of which 325 were positive. Adenovirus was responsible for the majority of the positives detected (52%) followed by sapovirus (20%), astrovirus (16%) and rotavirus (12%). This data highlights the importance of diagnostic screening for these viral pathogens and indicates the need for a multiplex screen that will be both rapid and more cost effective than single assay screening.

2. Conclusions

This paper describes the development and validation of a multiplex real-time PCR assay, which will allow rapid and simultaneous detection of adenovirus, astrovirus, rotavirus and sapovirus in stool samples. Multiplexing by sample type/disease syndrome simplifies the routine service; overall costs are reduced when compared to single testing as rapid diagnosis can reduce unnecessary antibiotic usage and investigations. Reductions in sample turn around times have been achieved by reducing the amount of separate tests needed to be carried out. This ensures more samples can be tested within the working day (Gunson et al., 2008).

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