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Performance evaluation of gastrointestinal viral ELIte panel multiplex RT-PCR assay for the diagnosis of rotavirus, adenovirus and astrovirus infection

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ABSTRACT

Rotavirus, adenovirus, norovirus and astrovirus are considered to be among the major causes of sporadic cases and outbreaks of acute gastroenteritis globally. Rapid and accurate identification of enteric viruses is still a challenge for the clinical laboratory. Recently, several molecular platforms for the detection of viral enteric pathogens have become available. In this study, the diagnostic accuracy of InGenius Gastrointestinal Viral (GV) Elite Panel, a newly developed one-step multiplex real-time RT-PCR assay simultaneously detecting rotavirus, adenovirus and astrovirus, was evaluated retrospectively analyzing an archival collection of 128 stool samples of children hospitalized with acute gastroenteritis. The overall sensitivity and specificity for the GV assay was 100% and 96.2% for rotavirus, 96.9% and 100% for astrovirus, 100% and 100% for adenovirus, respectively. The InGenius GV assay showed a high concordance with the reference methods and was able to detect all tested genotypes of rotavirus (including G1, G3, G4, G9 and G12P[8] and G2P[4]), adenovirus and astrovirus (AstV-1 and 2). Studies of considerable sample size are required to determine robust Cycle threshold cut-off values to effectively correlate infection to disease. These preliminary results suggest that InGenius GV assay can be recommended as a valuable method for accurate diagnosis of epidemic and sporadic gastroenteritis.

1. Introduction

Acute gastroenteritis (AGE) is one of the most common illnesses in humans worldwide, being responsible for half a million deaths among children aged < 5 years, mostly in developing countries (Troeger et al., 2017). Although in industrialized nations deaths from AGE are rarely seen, gastrointestinal diseases remain an important cause of morbidity in younger children resulting in substantial medical and healthcare expenses, lost productivity, and other costs to society and families (Beckmann et al., 2014). The appropriate treatment and control of infectious gastroenteritis depends on the ability to rapidly detect the wide range of etiologic agents associated with the disease (Buss et al., 2015). Enteric viruses have been recognized as the most significant etiologic agents of AGE in children and four viruses are being considered as clinically relevant: group A rotavirus (RVA), norovirus (NoV), adenovirus 40/41 (AdV) and astrovirus (AstV) (Oude Munnink and Van der Hoek, 2016; Zhang et al., 2015). The high genetic and antigenic variability of these viruses, due to evolutionary mechanisms such as point mutations accumulation, recombination and cross-species transmission and reassortment, poses an unceasing challenge for their detection (Clark and McKendrick, 2004; De Grazia et al., 2013; van Beek et al., 2018; Wilhelmi et al., 2003). Epidemiological studies have relied on sequencing techniques, which allow molecular characterization of viral enteric pathogens but their high cost and complexity of data discourage the adoption of such methods in clinical microbiology laboratories, where accurate, sensitive, easy-to-use and timely efficient diagnostic assays are generally preferred (Chhabra et al., 2017; Corcoran et al., 2014; Khamrin et al., 2011). Historically, the diagnosis of infectious diarrhea has progressively evolved from electron microscopy and culture on cell lines, to viral antigens detection and nucleic acids amplification (RT-PCR). A combination of these tests is often required to distinguish between infectious etiologies that have similar clinical presentations (Chhabra et al., 2017). Recently, several specific multiplex molecular assays for comprehensive (syndromic) gastrointestinal pathogens detection have become commercially available. Multiplex molecular assays have the potential to consolidate laboratory workflow reducing the time to result, improving diagnostic accuracy and allowing to simultaneously detect different pathogens (Binnicker,

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Table 1
Sample panels used for the evaluation of InGenius GV ELITe MGB® assay.

Reference method			InGenius GV				
	N° samples tested	Viral agents detected	Ct value§ (range)	N° GV-positive samples	RVA Ct value (range)	AstV Ct value (range)	AdV Ct value (range)
	15	RVA G1P[8]	14.64-30.93	15	< 15*-17.64	ND	ND
RVA-positive	8	RVA G2P[4]	17.17-34.57	8	< 15*-19.85	ND	ND
	1	RVA G3P[8]	25.51	1	< 15*	ND	ND
	3	RVA G4P[8]	21.99-28.89	3	< 15*	ND	ND
	9	RVA G9P[8]	11.18-26.37	9	< 15*-16.22	ND	ND
	5	RVA G12P[8]	15.5-25.42	5	< 15*	ND	ND
Subtotal	41			41	< 15*-19.85	ND	ND
AstV-positive	20	AstV-1	–	19	ND	< 15*-31.27	ND
	2	AstV-1 + NoV	–	2	ND	< 15*	ND
Subtotal	22			21	ND	< 15*-31.27	ND
AdV-positive	29	type 40/41	–	29	ND	ND	< 15*
Subtotal	29			29	ND	ND	< 15*
RVA-AstV	3	RVA G1P[8] + AstV-1	16.19-30	3	< 15*	< 15*-23.4	ND
co-infections	1	RVA G1P[8] + AstV-1 + NoV	33.2	1	32.81	< 15*	ND
	1	RVA G9P[8] + AstV-1	30	1	< 15*	21.2	ND
	1	RVA G9P[8] + AstV-2	29.9	1	< 15*	< 15*	ND
	2	RVA G12P[8] + AstV-1	21.5-29.2	2	< 15*	28.7-31.2	ND
Subtotal	8			8	< 15*-32.81	< 15*-31.2	ND
AdV-AstV	1	AdV 40/41 + AstV-1	–	1	31.67	< 15*	31.84
co-infection							
	1	AdV 40/41 + AstV-1	–	1	ND	< 15*	23.88
	1	AdV 40/41 + AstV-1 + NoV		1	ND	30.23	< 15*
Subtotal	3			3	ND-31.67	< 15*-30.23	< 15*-31.84
Total positive	103			102			
RVA-AstV-AdV-NoV-	25	–	–	2	31.06-34.4	ND	ND
negative							
Totals	128			104			

Reference methods: NoV (Kageyama et al., 2003); RVA (Pang et al., 2004); AstV (Noel et al., 1995) and AdV (Vikia Rota-Adeno, BioMeieux).
§ Ct value is shown only for RVA reference method.
*input template concentration too high for Ct calculation.
ND:Not Detected.

2015; Gray and Coupland, 2013; Reddington et al., 2014; Zhang et al., 2015). This study aims to evaluate the clinical performance of a newly proposed one-step multiplex real-time reverse transcription PCR (RT-PCR) assay, InGenius Gastrointestinal Viral (GV) Elite Panel (ELITechGroup Molecular Diagnostics, Puteaux, France), able to simultaneously detect RVA, AdV and AstV in clinical samples.

2. Methods

2.1. Study population

A total of 128 samples were included in the study: 103 had a positive test for at least one gastroenteritis-causing virus included in the InGenius GV panel (49 RVA positive, 33 AstV positive and 32 AdV positive, including 8 co-infections RVA-AstV and 3 AdV-AstV) and 25 had a negative test for any diarrhea-causing viruses included in the InGenius GV panel and also for NoV (Table 1). All stool samples used in this study were collected from children (0–14 years of age) hospitalized with AGE at the "G. Di Cristina "Children Hospital of Palermo. Stools were collected within 12 h after admission to the hospital and stored at -20 or −80 °C until processing at the Enteric Viruses Laboratory of the University Hospital of Palermo, Italy (member of the Italian Study Group for Enteric Viruses, ISGEV; http://isgev.net).

2.2. Reference methods

Viral RNA, extracted from 140 µl 10% fecal suspensions using the QIAamp Viral RNA kit according to the manufacturer's instructions (QIAGEN, Hilden, Germany) was retrotranscribed using random primers reverse transcription, as previously described (Iturriza-Gomara et al., 1999). RVA positive samples were detected by a real-time RT-PCR assay with specific primers targeting the NSP3 gene of RVA (Pang et al., 2004). RVA positive samples were G/P genotyped by RT-PCR of VP7 and VP4 genes followed by a hemi-nested multiplex PCR reactions using a mixture of specific primers for each genotype (genotypes G1-G4, G6, G8, and G9- G12 and P[4], P[6], P[8]-P[11] and P[14], for VP7 and VP4 types respectively) (Gentsch et al., 1992; Gouvea et al., 1990; Iturriza-Gomara et al., 2004). AstV genome was detected by conventional RT-PCR (Noel et al., 1995) and confirmed/genotyped by sequence analyses, while AdV antigens were searched by ICT assay (CerTest Rotavirus + Adenovirus and VIKIA Rota-Adeno). To evaluate infections by other enteric viruses, stool samples were also analyzed for the presence of NoV by real-time RT-PCR (Kageyama et al., 2003). These consolidated detection and typing assays are in use at ISGEV laboratories for routine surveillance activities and were considered as reference methods in this study.

2.3. InGenius gastrointestinal viral (GV) elite panel

The archived stool samples were processed according to the manufacturer's protocol on InGenius, a completely automated cassette based sample-to-results solution combining a universal extraction and independently controlled Real-time PCR thermal cycler (ELITechGroup Molecular Diagnostics, Puteaux, France). Briefly, 1 ml of stool samples diluted 1:10 in ultrapure water was vortex mixed to homogenize the mixture (20–30 s) and centrifuged at 13,000 × g (RCF) for 1 min to clarify the sample. Then, 200 µL of the clarified stool supernatant were carefully transferred into a dedicated tube and loaded on the InGenius instrument for testing. Finally, the InGenius instrument was supplied with extraction/amplification Internal Control (IC), the GV ELITe MGB amplification Master mix, and extraction and amplification cassette consumables provided by the manufacturer (ELITechGroup Molecular

Diagnostics, Puteaux, France). In order to validate nucleic acid extraction, reverse transcription and amplification processes, a threshold cycle (Ct) value of the IC ≤ 35 cycles was required. Results interpretation was performed according to the instruction manual of the GV ELITe MGB® assay.

2.4. Statistical analyses

The Cohen's kappa coefficient (k) was used to compare the results of InGenius GV assay to the reference methods, and the *t*-test was used to compare the Ct values obtained. The kappa value was interpreted as follows: < 0.20, poor agreement; 0.21–0.40, fair agreement; 0.41–0.60, moderate agreement; 0.61–0.80, good agreement; and 0.81–1.00, very good agreement (Altman et al., 1991). All analyses were performed with http://vassarstats.net/ and two-tailed p-values less than 0.05 were considered to be statistically significant.

3. Results interpretation

InGenius GV test results were compared to the reference methods and analyzed. A sample was deemed to have concordant results if one enteric virus was detected by both assays. A sample was classified as being discordant if a given virus was detected by only one of the two assays (InGenius or reference ones). All samples with discordant results were confirmed through repeat testing with both assays. The Ct value obtained from real-time RT-PCR amplification assays (InGenius GV and rotavirus RT-PCR) was used as a proxy measure of fecal viral load (low Ct value indicating high fecal viral load).

4. Results

4.1. Evaluation of InGenius GV ELITe MGB® panel sensitivity and specificity

In this study, the performance of a newly proposed commercial multiplex RT-PCR assay detecting RVA, AstV and AdV genome (InGenius GV ELITe panel) was evaluated using an archival collection of 128 stool samples retrieved from children admitted to hospital for acute diarrhea. The results of the InGenius GV assay were compared to the reference methods (Table 2) showing a "very good agreement" revealed by a Cohen's kappa coefficient (k) ≥ 0.95. Overall, 99% of RVA-, AdV- and AstV-positive samples (102/103) were detected by the InGenius GV assay, while a single AstV positive sample remained undetected by the commercial assays. Therefore, the sensitivity of the GV ELITe test was 100% [95% CI: 91–100] for RVA, 100% [95% CI: 86.6–100] for AdV, and 96.9% [95% CI: 82.5-99.8] for AstV. For the majority of the positive samples (89.8% of RVA, 57.6% of AstV and 100% of AdV positive stools) the detector was identified by InGenius GV assay with a "template concentration too high for Ct calculation" (< 15 Ct value) (Table 1). In order to evaluate the analytical sensitivity of the InGenius GV assay the RVA Ct values were compared to the reference real-time RT-PCR. The samples detected as RVA-highly-positive (Ct < 15) by InGenius were significantly more frequent (46 vs 4) with respect to the reference method (chi square 68.75, p-value < 0.01), suggesting a higher analytical sensitivity of the InGenius assay (Table 3). Since for AstV and AdV the reference method was not based on a real-time methodology, for such detectors it was not possible to compare Ct values. The RVA- and AstV-positive samples included in the study had been selected in order to be representative of the heterogeneity of the circulating population of these enteric viruses. The sample collection included RVAs of six different genotypes (G1, G3, G4, G9 and G12P[8]), and two genotypes of AstV (human AstV-1 and -2) (Table 1). All genotypes included in the samples panels tested were efficiently detected by GV ELITe MGB® assay.

Table 2
Comparison of the results obtained by InGenius Gastrointestinal Viral (GV) ELITe Panel Assay with the reference methods, and agreement values.

Virus	Reference methods*/InGenius GV Panel				Total	k
	P/P	P/N	N/P	N/N		
Rotavirus	49	0	3	76	128	0.95
Astrovirus	32	1	0	95	128	0.98
Adenovirus	32	0	0	96	128	1

*RVA (Pang et al., 2004); AstV (Noel et al., 1995) and AdV (Vikia Rota-Adeno, BioMeieux).
P:positive; N negative; k Cohen's kappa coefficient.

Table 3
Evaluation of the analytical sensitivity of the InGenius assay comparing its Ct values in RVA-positive samples to those obtained by the reference method.

RVA-positive samples			
Ct *(Viral Load)*	InGenius	Reference RT-PCR	p
< 15 (*high*)	45	4	< 0.01
15-22.99 (*medium-high*)	3	26	< 0.01
23-35 (*medium-low*)	1	19	< 0.01

The analytical specificity of the InGenius GV ELITe MGB® assay was assessed using a collection of 25 triple-negative samples and including in the analyses the expected negative results of single- and double-positive reference samples. Such samples had also been tested negative for NoV. Therefore, the total expected negative results were 79 for RVA, 95 for AstV and 96 for AdV. Accordingly, the overall specificity for the GV assays was 96.2% [95% CI: 88.5–99] for RVA and 100% for AstV [95% CI: 95.1–100] and AdV [95% CI: 95.2–100]. In fact, in two cases RVA RNA was detected by InGenius GV test in samples of the triple-negative-panel but in both cases the Ct values were high (30 < Ct < 35), being indicative of a low RVA concentration. Overall, InGenius GV ELITe MGB® assay showed a positive predictive value (PPV) and negative predictive value (NPV) of 94% and 100% for RVA; 100% and 99% for AstV and 100% and 100% AdV and the diagnostic accuracy exceeded 0.98.

4.2. Co-infections detected by InGenius GV assay

Among the 103 positive samples tested eleven co-infections were included. In particular, eight RVA-AstV and three AdV-AstV, one each also involving NoV. Moreover, two AstV positive samples were co-infected with NoV. In seven RVA-AstV co-infections, the RVA template concentration detected by InGenius was too high to be calculated (Ct < 15) and in two of these, such high concentration was observed also for AstV; in the remaining five RVA-AstV co-infection samples the AstV Ct values ranged between 21.21–31.23 (average value 25.34). A single sample showed < 15 Ct value for AstV coupled with a RVA Ct of 32.81. Both AdV-AstV co-infections were detected by InGenius, showing < 15 AstV Ct values combined with an AdV Ct of 23.88 and 31.84, respectively. In the latter sample also RVA genome was unexpectedly detected by InGenius with a Ct of 31.67 (Table 1).

5. Discussion

Most clinical laboratories routinely screen stool samples of symptomatic children hospitalized for AGE for RVA and AdV, and ICT represents the most common test used. However, the etiologic role of other enteric viruses such as NoV and AstV in medically-attended childhood gastroenteritis is well documented. Therefore, the use of multiplex panels able to detect several viral pathogens, including NoV and AstV, represents an important progress to fill the diagnostic gap of viral enteritis (Binnicker, 2015; Gray and Coupland, 2013). In this study, we examined the performance of a new commercial multiplex real-time RT-PCR, InGenius Gastrointestinal Viral Elite Panel (GV), able

to simultaneously detect RVA, AstV and AdV in fecal specimens. The retrospective analysis of a 128 stool samples collection showed a substantial agreement between the multiplex GV panel and the reference methods ($k \geq 0.95$) and the overall sensitivity and specificity of InGenius GV was 100% and 96.2% for RVA, 97% and 100% for AstV and 100% and 100% AdV, respectively (Table 2). The results of this study are consistent with the findings of several studies on multiplex gastrointestinal panels' performance (Binnicker, 2015; McAuliffe et al., 2013; Reddington et al., 2014; Zhang et al., 2015). However, the high genetic heterogeneity of enteric viruses and the diversity of circulating viral geno/serotypes has rarely been taken into consideration while testing the performance of diagnostic assay (Corcoran et al., 2014; McAuliffe et al., 2013). For this purpose, in this study 49 sequence-confirmed RVA-positive samples containing strains belonging to six different genotypes (G1, G3, G4, G9 and G12P[8]), 33 AstV-positive samples with strains of types 1 and 2 and 32 AdV-positive samples were included into the sample panels used to evaluate the performance of GV InGenius (Table 1). This samples selection represents a picture of the most common viral types detected in children hospitalized with acute gastroenteritis over the last twenty years in Italy (Banyai et al., 2012; Colomba et al., 2006; De Grazia 2013; De Grazia 2014). All viral genotypes tested were correctly detected by InGenius GV assay. InGenius showed an higher analytical sensitivity when compared to reference methods showing a significantly superior percentage of high positive RVA samples ($Ct < 15$) with respect to the reference method ($p < 0.01$). A high analytical sensitivity might also account for the InGenius capability to detect low viral loads of RVA RNA ($Ct > 30$) in two specimens of the negative panel and in a specimen of the AdV + AstV panel testing negative by the reference RVA Real-time RT-PCR and genotyping tests. Conversely, InGenius failed to detect a single sample selected as AstV positive. Systematic testing of fecal samples with a broad range multiplex PCR test will increase the diagnostic efficiency of clinical laboratories and will probably allow to fill the diagnostic gap for enteric pathogens in AGEs, since underreported viruses such as AstV, are possibly responsible of significant rates of infection. Moreover, the use of multiplex panels simultaneously revealing several pathogens with high sensitivity and specificity might increase the detection of co-infections. In this study, eleven co-infections were included in the samples collection to be tested and all of them were correctly detected by the GV multiplex RT-PCR. Interestingly, in the majority of co-infections (8/11) viral agents were shed in unequal amounts, with one of the virus genomes being greatly prevalent over the other. Genotype combinations of RVAs and AstVs involved in co-infections were selected in order to reflect the prevalent genotypes circulating in the study period (De Grazia et al., 2013); 2014). Co-infections can be acquired from a common source or a new infection can occur while shedding from a previous infection is not still over (Binnicker, 2015; Gray and Coupland, 2013). However, the question remains whether co-infection can increase the severity of enteric disease and systematic detection of co-infections could help clarify this issue. Undeniably, co-infections still represent a challenge for the diagnostic laboratory and multiplex detection methods allowing to calculate viral loads might be a valuable help for guiding therapeutic decision and control measures practice, especially for young children and elderly or fragile patients. Finally, systematic detection of viral loads would be critical to ascertain the role of asymptomatic as well as pre symptomatic and post symptomatic virus shedding in the transmission of food-borne infections while investigating outbreaks (Bosch, 2014).

6. Conclusions

This study, although analyzing a limited number of samples, suggests that InGenius GV assay can be recommended as a valuable method for accurate diagnosis of viral gastroenteritis, being able to detect a wide panel of different virus types and to deliver information on viral concentration in stools in a reduced hands-on and turnaround time (5 min and ~2 h, respectively). Although the best time/effort performance is obtained by the use a dedicated instrumentation (InGenius), the cost for reagents exceeds the double of in-house real-time RT-PCR assays (about 52€/sample vs 20€/sample). Widespread adoption of multiplex PCR diagnostic methods will help to adequately define the prevalence of enteric viruses and their etiologic role in AGE in both single and co-infections. Further studies on larger samplings are needed to fully estimate the clinical importance of rapid multiplex assays in terms of improvement of patients outcome and their cost-effectiveness and to define robust Ct cut-offs allowing to reasonably associate the detection of enteric viruses in stools with patient's illness.

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