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Protocols

Diagnosing viral gastro-enteritis using the fully automated sample-in, result-out STARlet All in one system (AIOS)

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ABSTRACT

The STARlet All-In-One system is a modular platform that integrates the complete molecular diagnostic workflow from nucleic acid extraction of clinical samples to PCR set-up and amplification. The platform was evaluated in comparison with laboratory developed tests (LDT) on fecal samples from patients with suspected viral gastro-enteritis. In a retrospective study, 72 positive samples were analysed, including all pathogens detected by the Seegene Allplex™ GI-virus assay, adenovirus, astrovirus, norovirus GI and GII, sapovirus, and rotavirus. Concordant results were obtained for 69 samples (96 %). Three discordant results were observed, one norovirus GII positive that gave an invalid result in the AIOS and two samples that were negative in the AIOS. One adenovirus positive that was subtyped as a genotype 2 virus, which is not associated with gastro-enteritis, and a sapovirus. In the prospective part of the study, 661 fecal samples were included. A total of 61 positive samples were detected, of which 60 were also detected by the AIOS. One norovirus GII positive sample (C_T 35.2) was tested negative in the AIOS. Two additional sapovirus positive samples, C_T 37 and 38, were detected by the AIOS but not by the LDT. The STARlet All-In-One platforms results in an automated molecular workflow with reduced hands-on time and enables running assays during out of office hours. Application of the Seegene Allplex™ GI-virus assay showed excellent concordance to the current diagnostic LDT. In a prospective comparison, only three discordant results were observed, all with C_T values over 35 and therefore unlikely of clinical relevance.

1. Introduction

Introduction of the polymerase chain reaction (PCR) in the diagnostic laboratory (Ou et al., 1988; Shibata et al., 1988) and more specifically the introduction of real-time PCR (Higuchi et al., 1993) has revolutionized diagnostic microbiology. In search of the causative agent in patients suffering from a microbiological infection, real-time PCR has become an important tool. The last couple of years, further automation of the molecular diagnostic workflow has greatly improved the reliability and reproducibility of the results. Current state-of-the art molecular diagnostics relies on fully automated, sample-in, result-out platforms for the molecular workflow, minimizing hands-on time. Most large diagnostic companies now market such medium- to high-throughput platforms (Chernesky et al., 2014; Cobb et al., 2017; Mourik et al., 2022, 2023; Wessels et al., 2023) The portfolio provided by these companies for the automated platforms are all very similar and driven by high throughput parameters such as sexually transmitted diseases (*Chlamydia trachomatis, Neisseria gonorrhoea, Mycoplasma genitalium* and *Trichomonas vaginalis*), blood-borne viruses as Human Immunodeficiency Virus, Hepatitis B and C virus, and transplant associated viral load monitoring for cytomegalovirus (CMV), Epstein Barr virus (EBV) and BK virus (BKV). In addition, for respiratory viruses such as influenzavirus (Flu), SARS coronavirus-2 (SARS-CoV-2) and respiratory syncytial virus (RSV), assays are available as well. However, the diagnostic portfolio in the majority of laboratories contains many more viruses, bacteria, parasites and fungi. For some of these, the number of tests per annum is limited and therefore unlikely to become available on the medium- to high- throughput platforms mentioned above. Another complication for Europe is that as of May 2022 the 2017/746, in vitro diagnostic regulation (IVDR) has become effective. This legislation will affect the way laboratory developed tests (LDT) can be used in the diagnostic laboratory (Bank et al., 2020; Vanstapel et al., 2023).

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Therefore, there is a commercial niche for companies marketing medium-throughput platforms in combination with diagnostic assays compliant to IVDR, exemplified by companies like Elitech and Seegene. Here, a multicentre evaluation of the Seegene Allplex™ GI-virus assay is described, tested on the new STARlet all-in-one system (AIOS), a fully integrated and automated molecular workflow, and compared to established LDTs for viral gastro-enteritis.

2. Methods

2.1. Clinical samples

The Allplex™ GI-virus on the AIOS system was evaluated at two different sites, the Leiden University Medical Center (LUMC) and the Meander Medical Center (MMC) Amersfoort, both in the Netherlands. Initially, the MMC used a set of 72 samples, collected from October 2021 to November 2022, previously found positive for pathogens associated with viral diarrhoea, for a retrospective evaluation of the assay on the AIOS. All targets present in the Allplex™ GI-virus assay were included. As the MMC did not routinely test for sapoviruses and astroviruses, positive samples for that evaluation were obtained from the Star-shl laboratory in Rotterdam. Subsequently, samples submitted for diagnosis of viral diarrhea were prospectively tested using the routine diagnostic procedures at both sites and compared to the Allplex™ GI-virus test on the AIOS system. A total of 422 samples submitted to the MMC from March 31 to September 16, 2023 and 239 samples submitted to the LUMC from June 13 to August 10, 2023 were included for this prospective performance study.

2.2. Nucleic Acid extraction stool samples

Semi-automated nucleic acid extraction, all according to the instructions of the manufacturer, was performed for the routine real-time PCR assays. At the MMC, feces were suspended in a 33–50 % w/v concentration in 600 µl ACL lysisbuffer (Qiagen). After thoroughly mixing the suspension, the specimens were centrifuged for 2 min at 13000 rpm. Viral nucleic acids were extracted from the supernatant using the DSP virus/pathogen mini kit on the QiaSymphony (Qiagen, Hilden, Germany). At the LUMC an aliquot of feces was transferred to a 2 ml tube containing stool transport and recovery (STAR) buffer (Roche) and precellys beads (Bertin technologies). After short bead-beating and centrifugation, 200 µl of supernatant was used for total nucleic acid (RNA and DNA) extraction using the DNA and viral NA small volume kit on the MagNApure 96 (Roche, Almere, The Netherlands).

2.3. Real-time PCR for viral gastroenteritis

The reference test for the study is a lab-developed viral gastro-enteritis assay as previously described (van Maarseveen et al., 2010), although the MMC used an assay based on another publication (Kageyama et al., 2003) for norovirus GI detection. Extracted nucleic acids from stool samples were subjected to multiplex real-time PCR as described although the MMC used Quantifast RT mastermix (Qiagen) and the ABI7500 (Applied Biosystems) as real-time PCR platform. A genetically distinct sapovirus genogroup V was identified several years after implementation of the LDT and could not be detected by this PCR (Hergens et al., 2017; Usuku and Kumazaki, 2014). Therefore, a real-time PCR specifically directed at identification of this genogroup was added to the LUMC multiplex PCR (van Maarseveen et al., 2010), using primers and a probe adjusted from a published assay (Chan et al., 2006) as listed in Table 1.

2.4. Allplex™ GI-virus assay on the AIOS

The Seegene all-in-one system (AIOS) system (Fig. 1) combines nucleic acid extraction using the STARMag 96×4 universal Cartridge kit on the STARlet system (SeeGene, South Korea) with assay set-up and subsequent amplification on integrated CFX96 real-time PCR machines. The platform uses integrated software for the molecular workflow and can be bidirectionally coupled to laboratory information systems. Operation of the AIOS was performed according to the manufacturers' procedure using the Allplex™ GI-virus assay, targeting norovirus (genogroup I and II), rotavirus, adenovirus, sapovirus, including sapovirus GV, and astrovirus. Clinical samples for the study were collected and prepared in line with the manufacturer's instructions using either eNAT preservation medium tubes (LUMC, Copan, Italy) or All-TM media (MMC, SG medical, South Korea). The samples were mounted to the AIOS system on a sample-carrier and the run was started according to the manufacturer's instructions. After extraction and amplification, results were analysed using the Seegene Run Manager and compared to the LDT results.

Table 1
Primers and probes for sapovirus GV detection.

Name	Sequence
1776SapoGVs	GYTAYAACAGCTGGTACATWGG
1949SapoGV-TQ-TexasRed	CAGAAATGCCCRCTACCAATGAA
1771SapoGVas	RCCCTCCATYTCAAACACTA

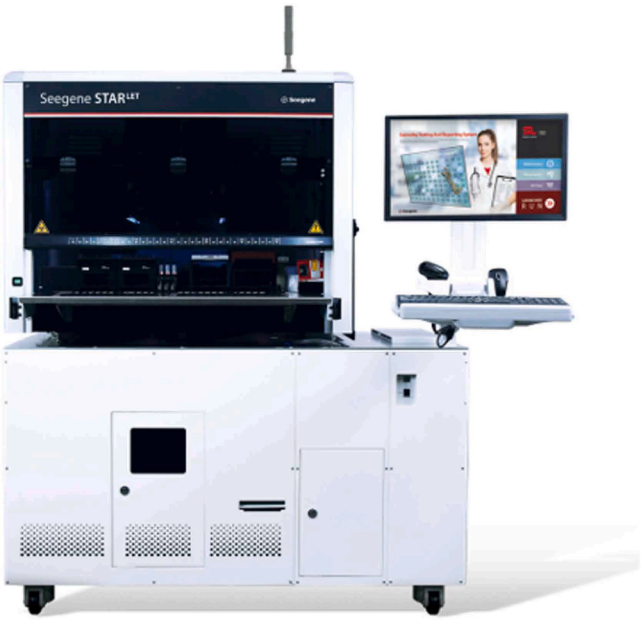

Fig. 1. The STARlet All In One System (AIOS). Detailed information: https://www.seegene.com/instruments/seegene_starlet_aios.

3. Results

3.1. Retrospective evaluation

A selection of 72 stored positive samples comprising all targets of the Allplex™ assay were used for a retrospective evaluation of the assay. A total of 69 samples showed concordance with the LDT, although a difference in C_T values was observed for rotavirus with significantly lower values in the Allplex™ (Table 2). In two samples detection of a double infection was confirmed by Allplex™, one with astrovirus/norovirus GII and one with adenovirus/ sapovirus. One adenovirus positive sample (C_T 29) repeatedly tested negative in Allplex™. Genotyping of the virus revealed that this was not a subgroup F (40/41) virus, which are associated with gastro-enteritis, but a C2 genotype. Another sample was positive for sapovirus with C_T 31 and retesting in duplicate confirmed

Table 2
Retrospective results (MMC).

Samples: N=72		Allplex AIOS results			
Positive in LDT for	N=	Positive	Negative	Invalid	Comment
rotavirus	19	19	0	0	C_T 6–16 higher in LDT
norovirus GII	19	18	0	1	C_T 37, no sample for retest
adenovirus	8	7	1	0	False-positive result in LDT (HAdVC2)
norovirus GI	6	6	0	0	
sapovirus	10	9	1	0	C_T 31, duplicate retest 1/2 positive for sapo
astrovirus	10	10	0	0	

this result as a true positive. The mean difference in C_T value between the original sapovirus result and the results as found on the STARlet-AIOS was 1.8 (range −1.0 – 4.1), in which in 8/9 samples the C_T value was lower using the STARlet-AIOS. One sample with an LDT result of norovirus GII result of C_T 37 was invalid (no IC) and could not be retested due to insufficient sample. No material was left for differentiation of GV viruses from these sapovirus samples.

3.2. Prospective evaluation

For the prospective study, combined from both sites, a total of 661 samples were tested. Four invalid Allplex™ results were observed in the study, two at both sides. Invalid was defined as an inconclusive result that remained inconclusive after retesting. These samples were negative in the LDT, leaving a total of 657 samples with results for both assays. For the analysis of the performance of the Allplex™, the results from both sites were combined as shown in Table 3.

Overall, 10 positive rotavirus samples, 19 norovirus GII, 2 adenovirus, 18 norovirus GI, 10 sapoviruses and 595 negative samples showed concordant results between the LDT and Allplex™ in the initial prospective run. No astroviruses were detected in the study period. Two norovirus positive LDT results were not detected by the Allplex™, one was invalid and became positive after retesting, the other sample, with C_T value 35.2 was positive with C_T 33.8 when retested in Allplex™. Retesting of invalid results is part of the diagnostic workflow, so one false-negative, hence discrepant, result remained as this sample would not have been retested in daily practice. Only one sapovirus GV positive sample was detected at the LUMC in the prospective part of the study. At the MMC, the AIOS detected 11 sapovirus positive samples. As sapovirus real-time PCR is not part of routine diagnostic viral GE PCR at the MMC, the sapovirus assay from the LUMC was retrospectively applied to these samples for proper comparison. One sample was not available for retesting and two (of 11) Allplex™ sapovirus positive samples became negative in the LDT (Allplex™ C_T values of 37.3 and 38.4). The other nine Allplex™ sapovirus positives were confirmed with the LDT. Six of those were sapovirus genogroup I-IV, whereas the other three tested positive for GV sapovirus.

Table 3
Prospective results.

Samples: N=657	**LDT**	**ALLPLEX**	
LDT results	**Positive**	**Positive**	Comments
rotavirus	10	10	100 % concordance
norovirus GII	21	20	1 negative, 1 invalid but pos after retest
adenovirus	2	2	100 % concordance
norovirus GI	18	18	100 % concordance
sapovirus*	10	12	2 not confirmed with LUMC LDT
astrovirus	0	0	Not detected in study period
negative	596	595	

* 11MMC Allplex™ positives retested by LUMC sapovirus assay

Calculated sensitivity, specificity, positive predictive values (PPV), negative predicted values (NPV) and accuracy of the Allplex™ targets as compared to the LDT are shown in Table 4.

4. Discussion

In the current comparison of the Allplex™ GI-virus assay on the automated AIOS system to an established viral gastro-enteritis workflow performed under the scope of the NEN-EN ISO-15189 (2012) guideline for medical laboratories, highly similar results were obtained for all targets. The same assay has been included in an evaluation of the QiaSTAT-Dx gastro-intestinal panel which also provided highly concordant results (Boers et al., 2020). During the current prospective study period, unfortunately, no astroviruses were detected, which may be due to timing, as classical astroviruses usually circulate during the winter season (Bosch et al., 2014). Since astrovirus positive specimens were included in the retrospective part of the evaluation, correct performance of this target was shown. Sapovirus was not a part of the diagnostic workflow at the MMC and therefore only the LUMC part of the study, with only one positive sapovirus sample, was truly prospective. However, as 12 positive samples were detected by Allplex™ GI-virus assay at the MMC, we did test 11 of those samples using the LUMC sapovirus assay. Unfortunately, no material was left of the 12th positive sample. Nine of the 11 sapovirus positives could be confirmed using the LDT. Together with the LUMC positive, 10 positive sapovirus were detected with the LDT of which four (40 %) were genotype V viruses. This is of interest as not all commercially available GE assays are able to detect this genotype, that has been described as causal agent of outbreaks (Usuku and Kumazaki, 2014). Two Allplex™ sapovirus positive samples with C_T values of over 37 remained negative in the LDT. As these were retested from stored, frozen samples, the additional freeze-thaw step may have affected the results. However, in comparative studies like this one, samples with low viral loads (high C_T values) may or may not be detected by the comparator assay due to the stochastic effect in PCR amplification (Lalam, 2006). Clinical relevance of high C_T positive findings is a regular topic of discussion (Wishaupt et al., 2017) and also of in diagnosing gastro-enteritis (Kang et al., 2004). The Allplex™ rotavirus positives had significantly higher C_T values as compared to the LDT, but did not result in discrepant results. In a recent study on the performance of the Allplex™ GI-virus assay, a threshold of C_T 35 has been proposed for reporting results (Massa et al., 2023). In most syndromic cases of viral diarrhoea, very high viral loads and thus low C_T values are observed and therefore it is unlikely that ignoring results with $C_T > 35$ will result in clinically relevant underdiagnosis or mismanagement in infection prevention strategies (Bonacorsi et al., 2021; Shioda et al., 2017). One other discrepant result- was observed with an adenovirus LDT positive sample (C_T 29) that was repeatedly negative in the Allplex™ assay. Subtyping of this adenovirus revealed a C2 genotype, which may indicate that the specificity of the Allplex™ assay for detection of subgroup F (40/41) adenoviruses, as acknowledged cause of gastro-enteritis, is better than our LDT. The results of the study show that a number of invalid results was obtained, which is not uncommon in testing fecal samples. An invalid internal control results in

Table 4
Sensitivity, specificity, PPV, NPV and accuracy per target for Allplex™ compared to the LDT.

	rotavirus	noroGII	adenovirus	noroGI	sapovirus
Sensitivity	100 %	95,5 %	100 %	100 %	100 %
Specificity	100 %	100 %	100 %	100 %	99,7 %
PPV	100 %	100 %	100 %	100 %	83,3 %
NPV	100 %	99,8 %	100 %	100 %	100,0 %
Accuracy	100 %	99,8	100 %	100 %	99,7 %

retesting the sample, usually also including a 1:10 dilution of the nucleic acid extract. At the MMC the initial test resulted in four invalid results in the LDT and 17 invalid results in the AIOS. After retesting, one LDT sample remained invalid and so did not provide a diagnostic result. For the AIOS, 17 of the samples gave valid results after retesting. The difference in initial invalid results between the two sites may be attributed to the different stool media used or differences in the amount of stool sample used. This may be solved by further optimization of the pre-analytical process. An important quality aspect of diagnostic molecular methods is participation in external quality assessment panels. At the MMC, the Allplex™ GI-virus assay was used to analyse the Qnostics Gastroenteritis viral evaluation panel 01 (Qnostics, Glasgow, UK) and all six panel members were correctly identified (data not shown). The STARlet AIOS platform appeared to be a robust system, also when processing fecal specimens which are generally seen as a highly variable specimen type. Our experiences comply with earlier reporting, evaluating the platform by testing viral respiratory pathogens (Brouwer et al., 2024). Also for the current study, the MMC calculated the hands-on-time (HOT) per specimen and mean turnaround time of the results (TAT) after monitoring for three days. Equal to the previous study, the HOT was shorter when using the STARlet AIOS (1 minute per specimen) compared to LDT (3.7 minutes per specimen). The mean TAT of the STARlet AIOS (2.5 hours) is slightly lower than the mean TAT of the LDT (3 hours). In conclusion, the Allplex™ GI-virus assay shows excellent performance for detection of viruses causing gastro-enteritis. The discrepant results all were samples with high C_T values for which the clinical relevance is questionable. The viral gastroenteritis assay itself has been evaluated previously (Massa et al., 2023; Hirvonen, 2019; Ligero-López et al., 2023; Sciandra et al., 2020). Here, the feasibility has been shown of running the assay on the fully integrated AIOS platform, that automates the complete molecular workflow from sample-in to result-out. After starting the run, the complete workflow is automatically performed, which results in a reduction of hands on time and enables an overnight run of the assay. In combination with the portfolio of diagnostic assays provided, the AIOS may be considered an interesting candidate for further automation of molecular diagnostics.

Author agreement statement

We the undersigned declare that this manuscript is original, has not been published before and is not currently being considered for publication elsewhere.

We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. We further confirm that the order of authors listed in the manuscript has been approved by all of us.

We understand that the Corresponding Author is the sole contact for the Editorial process. He/she is responsible for communicating with the other authors about progress, submissions of revisions and final approval of proofs

CRediT authorship contribution statement

Paul Smits: Writing – review & editing, Resources, Investigation, Funding acquisition, Data curation, Conceptualization. **Roel Nijhuis:** Writing – review & editing, Validation, Resources, Project administration, Formal analysis, Conceptualization. **Fabienne Verhees:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation. **Anja Heijne-Tol:** Writing – review & editing, Software, Methodology, Investigation, Data curation. **Luuk Sterk:** Writing – review & editing, Investigation, Formal analysis, Data curation. **Anne Russcher:** Writing – review & editing, Validation, Supervision, Investigation, Data curation, Conceptualization. **Eric C.J. Claas:** Writing – original draft, Validation, Supervision, Project administration, Methodology, Investigation, Formal analysis, Conceptualization. **Youssef Rezek:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper

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