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PCR assays for detection of human astroviruses: *In silico* evaluation and design, and *in vitro* application to samples collected from patients in the Netherlands

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ABSTRACT

Background: Human astroviruses (HAstV) comprise three phylogenetically compact and non-adjacent groups of species including classical HAstV (HAstV-C) and the novel ones (HAstV-VA/HMO and HAstV-MLB). Of these, HAstV-C is known to be responsible for gastroenteritis while the novel HAstV are associated with cases of neurological disorders. Accurate detection of all known variants by (real-time) PCR is challenging because of the high intra- and intergroup genetic divergence of HAstV.

Objectives: To evaluate published HAstV PCR assays *in silico*, design *de novo* real-time PCR assays that can detect and discriminate three groups of HAstV, and apply those to patient samples to analyse the prevalence of HAstV in stool and cerebrospinal fluid (CSF) specimens.

Study design: In silico evaluation of published PCR assays and design of real-time PCR assays for detection of different subsets of HAstV was conducted within a common computational framework that used all astrovirus full genome sequences from GenBank. The newly designed real-time PCR assays were evaluated *in vitro* and applied to faecal samples (collected in January–May 2016) and cerebrospinal fluid specimens (2010–2016) from patients in the Netherlands.

Results: Quantitative *in silico* evaluation of published PCRs is provided. The newly designed real-time PCR assays can reliably assign all available HAstV genome sequences to one of the three phylogenetic groups *in silico*, and differentiate among HAstV-specific controls *in vitro*. A total of 556 samples were tested using these PCR assays. Fourteen fecal samples (2.5%) tested positive for HAstV, 3 of which could be identified as the novel HAstV-MLB variants. No novel HAstV were found in CSF specimens.

Conclusion: Newly designed real-time PCR assays with improved detection of all known HAstV allowed the first-time identification of novel astroviruses from stool samples in the Netherlands.

1. Background

Following the discovery of astrovirus in human stool [1], diverse astroviruses were described in humans and other hosts. They were classified in the family *Astroviridae* that is composed of two genera, *Mamastrovirus* and *Avastrovirus* [2]. Human astroviruses (HAstV) belong to the genus *Mamastrovirus* that includes four established species: *Mamastrovirus 1* (MAstV1; classic HAstV or HAstV-C, types 1–8), *Mamastrovirus 6* (MAstV6; known as HAstV-MLB, types 1–3), and closely related *Mamastrovirus 8* and *Mamastrovirus 9* (MAstV8 and MAstV9; both known as HAstV-VA/HMO, types 1–5 and A–C, respectively), and HAstV-BF34 a prototype of a putative separate species [3,4]. Collectively, HAstV-MLB and HAstV-VA/HMO groups are often referred to as novel HAstV.

Studies on prevalence and pathogenicity have shown HAstV-C to be a causative agent of viral gastroenteritis, mainly affecting children,

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elderly, and immunocompromised patients [5]. Also, HAstV-MLB and HAstV-VA/HMO have been identified in faecal samples obtained from patients with gastroenteritis [6–8]. However, these novel astroviruses have originally been described in association with severe neurological infections and reported to be the causative agent of encephalitis/encephalopathy and meningitis in immunocompromised patients [9–14]. Classical and novel HAstV have been monitored in different countries, but their epidemiology remains poorly understood, particularly for the novel HAstV (for reviews see references [2,15]).

Detection of HAstV-C and novel HAstV has been assisted by (real-time) PCR assays developed in different laboratories [6,16–24], some of which were included in diagnostic platforms for viral gastroenteritis.

2. Objectives

Our aim was to monitor the presence of HAstV in stool samples submitted for diagnosis of viral gastroenteritis and CSF samples from patients with encephalitis/meningitis of unknown aetiology. We employed the in-house bioinformatics platform (VEB-tool) to evaluate accuracy of detecting HAstV by published (real-time) PCR assays and to design *de novo* real-time PCR assays for discrimination between HAstV-C, HAstV-MLB and HAstV-VA/HMO.

3. Study design

3.1. Genome sequences, multiple sequence alignments, and phylogeny of astroviruses

A total of 147 complete genome sequences of viruses of the *Astroviridae* family were retrieved from NCBI GenBank using HAYGENS tool (version 1.0, http://veb.lumc.nl/HAYGENS; Sidorov, I.A. et al., manuscript in preparation) and downloaded into VirAliS platform [25]. One hundred and four complete genome sequences from the VRL division of GenBank with acceptable quality of sequencing (number of non-ACTG symbols is less than 0.1% of the genome length and number of calculated frame shifts in the coding part of genomes is not more than 1) were selected for subsequent analysis. Multiple sequence codon-based alignment (MSA) of these genomes was prepared in the VirAliS platform as described elsewhere [26] using ClustalW [27], MUSCLE [28], HMMER [29], and MAFFT software [30]. Phylogenetic relationships between these viruses were reconstructed by FastTree [31] (version 2.1.8), using MSA of three non-structural proteins (3CL, VPg, and RdRp), of which the most variable columns were removed by the BAGG tool of VirAliS.

3.2. In silico evaluation of existing and newly designed PCR assays

Published (real-time) PCR assays for the detection of astroviruses in diagnostic settings were collected from literature and evaluated *in silico* (Table 2). Briefly, the ability of an assay to detect a subgroup of HAstV ("targets") and other astroviruses ("non-targets") was regarded as sensitivity (SN) and selectivity (SL), respectively. They were calculated taking in account sequence weights [32]. Probability of detection of each virus was estimated based on probability of annealing of each PCR oligo (primer or probe) to the viral DNA template using the nearest-neighbour approach by calculating melting temperature (Tm) for all oligo-template interactions. Location of the annealing sites for each oligo-template interaction was defined by sites with the largest Tm. If the PCR product length was not in the specified range (70–200 nucleotides for all newly designed PCRs in this study and corresponding values for published PCRs), the probability of detection for this template was considered to be 0. When Tm values for both probe and corresponding primer was close or Tm of the probe was at least 6 °C higher than Tm of the corresponding primer, the probability of detection was multiplied by 0.5, and 1.0, respectively; linear interpolation was used to adjust the detection probability for other values of Tm difference. PCR conditions such as primer/probe and template concentration, PCR annealing temperature and salts concentration were also taken into account.

3.3. In silico real-time PCR design

PCRs were designed *in silico* for selected subsets of HAstV and a procedure that is summarized below. Briefly, the MSA of complete genomes of a HAstV subset was analysed, and all conserved continuous alignment regions (sets of no-gap columns) with length ranging between 18 and 28 nucleotides were considered as PCR oligo candidates. Each PCR oligo candidate was tested for annealing with DNA copy of genome sequences of the entire astrovirus-wide dataset using a set of constraints imposed on several parameters for primers and probes including: GC content, 30–80%; maximum number of consecutive identical nucleotides, 4; and maximum degeneracy, 50. After selecting PCR oligo candidates, all possible combinations providing a PCR product of length ranging between 70 and 200 nucleotides were considered as PCR candidates and evaluated. For each candidate, PCR annealing temperature value (Ta) was optimized in the range of 45–95 °C by maximizing PCR SN and SL values. After optimization of Ta, PCR candidates were sorted by quality ((SN + SL)/2). Next, PCR candidates having the same value of quality were sorted by average PCR oligo degeneracy (ascending order; the lower degeneracy, the more preferable the candidate), and calculated Ta value (descending order; the higher the Ta the lower the probability of nonspecific oligo annealing). The PCR candidate at the top of the list was considered as the best and used for *in vitro* virus detection.

3.4. Control samples

In vitro evaluation of the HAstV real-time PCR assays designed in this study was performed using a range of templates: RNA extracted from viral cultures of HAstV-C types 1–8, plasmids of HAstV-MLB types 1–3 and HAstV-VA types 3–5, and, RNA from clinical samples infected with HAstV-VA types 1 and 2 as well as HAstV-BF34, which were kindly provided by the original authors [4,8,11]. In addition, a panel consisting of 7 viral, 9 bacterial and 10 parasitical agents (supplementary Table S1) were tested to determine the specificity of the assays.

3.5. Clinical samples

Remnant nucleic acids extracted from stool samples submitted for diagnosing of viral gastroenteritis to the Department of Medical Microbiology at the Leiden University Medical Center (LUMC) during the period January–May 2016 were included in this study. This timeframe reflects the period when HAstV-C are predominantly circulating, as registered by the Dutch National Institute for Public Health and the Environment (http://www.rivm.nl/Onderwerpen/V/Virologische_weekstaten/Rapportages/Open_rapportages_virologische_weekstaten). In addition, CSF specimens from patients with suspected meningitis or encephalitis, based on clinical characteristics, were tested as well. No selection was made based on age or other patient-characteristics and only specimens with no proven aetiology (*i.e.* tested negative for all the diagnostics performed) were included. Of all available CSF samples received from 2010 to 2016, RNA was extracted and tested by real-time PCR.

3.6. Viral RNA isolation

Remnant nucleic acids used in this study were extracted by adding approximately 0.15 g of stool to 1 ml S.T.A.R.-buffer (Roche Diagnostics, Almere, the Netherlands) and precellys beads (Bertin Technology, Rockville, USA), with subsequent vortexing at 2200 rpm for 5 min. After 5 min incubation at room temperature, centrifugation (1 min at 18,407 *g*) was performed. From both the supernatant of the

Table 1
Real-time PCR primers and probes used in this study.

PCR name (PCR product location and size); PCR oligo name and polarity	Sequence (5'-3')	Concentration
Monoplex PCR #1		
VEB-HAstV-C (orf1b, 191bp)		
HAstV-Cs	TAGTdTGyGCCGAyCCyA	900 nM
HAstV-Cas	GGyCCArTCrAAyTCAAT	900 nM
HAstV-C-TQ-FAMas	CkCCyTCCATwGGTGACCAyC	400 nM
Multiplex PCR #2		
VEB-HAstV-MLB (putative capsid protein, 100 bp)		
HAstV-MLBs	TTCCTAATmGGAATCGCCGTCGTA	700 nM
HAstV-MLBas	GATCCAACGGTGCCrAGTGTTGC	700 nM
HAstV-MLB-TQ-TXRs	ACAACTGGGCCTAArCCTGCGGTGTC	400 nM
VEB-HAstV-VA/HMO (orf1b/putative capsid protein, 200 bp)		
HAstV-VA/HMOs	CTCCTTTGCTAyCGCATmCT	500 nM
HAstV-VA/HMOas	GCTGGrGCTGyyTACCAG	500 nM
HAstV-VA/HMO-TQ-YAKs	ATGCTGGATmrrCTTTGGAGGGGmGG	600 nM
Equine Arteritis Virus (orf1a, 133 bp)		
EAVs	CATCTCTTGCTTTGCTCCTTAG	200 nM
EAVas	AGCCGCACCTTCACATT	200 nM
EAV-TQ-CY5as	CGCTGTCAGAACAACATTATTGCCCAC	250 nM

nM: nanomols/l, s: sense, as: antisense.
Degenerate positions in oligo sequences: d = G or A or T; y = T or C; r = G or A; k = G or T; w = A or T, m = A or C.

stool sample and CSF samples (no pre-treatment), RNA was extracted using the MagnaPure96 system (Roche Diagnostics) with an input of 200 µl and an eluate volume of 100 µl. To every extraction, an aliquot of equine arteritis virus (EAV) was added and used as an internal extraction and inhibition control [24].

3.7. Real-time PCR

For routine diagnosis of viral gastroenteritis in our setting, we use the diagnostic multiplex real-time PCR assays that enables detection of HAstV-C, sapovirus, norovirus GI, norovirus GII, adenovirus and rotavirus [24]. For the purpose of this study it is labelled as diagn-HAstV.

For the current study, we used two real-time PCR assays designed to recognize respective HAstV groups: monoplex VEB-HAstV-C and multiplex VEB-HAstV-VA/HMO and VEB-HAstV-MLB, with the latter also including EAV PCR. Both assays were tested in a CFX96 detection system (Bio-Rad, Veenendaal, the Netherlands), with a 25 µl reaction mixture consisting of 6.25 µl of 4* TaqMan Fast Virus 1-step Mastermix (Thermofisher Scientific, Landsmeer, the Netherlands), oligonucleotides as listed in Table 1, and 10 µl of eluate. The cycling conditions consisted of a reverse-transcriptase step of 5 min at 50 °C, a denaturation step of 2 s at 95 °C and subsequently 45 cycles of 15 s at 95 °C, 10 s at 55 °C and 50 s at 60 °C. In every run, a positive amplification control was tested containing either HAstV-C (RNA of a positive sample), VA-3 and MLB-1 (both plasmids).

3.8. Nucleotide sequence analysis

To determine the subtypes of the novel HAstV detected in this study, the obtained PCR fragment was subjected to nucleotide sequence analysis by the dideoxy chain termination method, using 5µM of the corresponding real-time PCR primers.

4. Results

4.1. In silico evaluation and design of HAstV PCR assays

Human astroviruses form three monophyletic non-adjacent groups in a tree of the *Astroviridae* (highlighted as HAstV-C, HAstV-VA/HMO and HAstV-MLB in Figure S1). Different subsets of these groups were targeted by a range of PCR assays designed in our group [24] and other studies [6,17–23], while a pan-astrovirus assay was also described [16,33] (Fig. 1). We sought to evaluate these PCR assays in *silico* against a genome database of astroviruses available on 21.04.2015 that did not include HAstV-C type 7, using the original computational procedure (see M&M). One genome was excluded from *in silico* evaluation (GenBank, AB308374.1, HAstV-C) due to its low sequencing quality that may lead to underestimation of SN for some PCRs.

All subset-specific PCRs recognized genome sequences of its target subset available at the time of PCR design, although recognition of new variants may be less certain (Tables S2–S4). This uneven performance was evident, when these PCRs were evaluated *in silico* against all available genome sequences of the respective HAstV (sub)group (Table 2). For the entire HAstV-C group (30 sequences), SN and SL of seven published PCRs, developed to detect HAstV-C only, varied between 62.47% and 90.28%, and 90.98% and 100%, respectively (Table 2). The diagn-HAstV assay showed the highest SN and SL (90.28%/100%) but also demonstrated suboptimal detection of certain types of HAstV-C (1, 2, 4 and 6), and, as expected, did not recognize novel astroviruses (Fig. 2, Table S2). For HAstV-VA/HMO and HAstV-MLB groups (both of nine sequences), SN of PCRs designed for detection of viruses from these groups varied between 43.97% and 99.99%, 79.29% and 99.98%, respectively (Tables S3 and S4). None of these PCRs sufficiently recognized BF34 subtype that is basal to the VA/HMO clade. Due to substantial intra-group genetic diversities, the best performed PCRs, which were designed by Cordey et al. [6], were composed of several PCRs with low oligo degeneracy each directed against a specific virus. SN of this PCR for HAstV-C, HAstV-VA/HMO, and HAstV-MLB were 69.15%, 83.66% and 97.71%, respectively (evaluated as multiplex PCRs for VA/HMO and MLB groups). In contrast to other assays, Kapoor et al. [16] designed a PCR to recognize all HAstV, using four sets of oligos with the degeneracy that ranges from 8 to 64. The SN for three HAstV groups was estimated to be 92.49% (HAstV-C), 68.84% (HAstV-VA/HMO), and 80.10% (HAstV-MLB) (Table 2).

This evaluation indicated that further advancement is required to ensure that all known HAstV could be reliably recognized by real-time PCR. We then found that designing a pan-astrovirus real-time PCR assay with a single set of primers/probe was not possible due to the considerable divergence of their genomes. In contrast, we identified between 20,000 and 200,000 candidate real-time PCR sets for sensitive and selective recognition of HAstV-C, HAstV-VA/HMO and HAstV-MLB groups, using alignments of 31, 9 and 9 complete genomes, respectively. The best PCR sets with degeneracy of oligos ranging from 2 to 24 were selected to produce three group-specific assays whose sensitivity and selectivity were > 99% (Newly designed qPCRs, Table 2). At least one degenerate oligo of each of these PCR sets overlapped with an oligo or its complement of published PCRs, but the entire designed sets were unique (Fig. 1).

4.2. In vitro evaluation of HAstV real-time PCRs

When tested *in vitro*, both diagn-HAstV and VEB-HAstV-C assays detected HAstV-C type 1–8 isolates, as expected. Notably, VEB-HAstV-C showed higher sensitivity compared to diagn-HAstV assay by recognizing HAstV-C types 2, 6 and 7 with lower Cq-values (Table 3). Both real-time PCR assays for the novel HAstV (VEB-HAstV-VA/HMO and VEB-HAstV-MLB) enabled correct amplification and detection of

Table 2
Results of *in silico* evaluation of the existing and newly designed PCR assays for detection of target viral groups of HAstV using dataset collected on April 21st, 2015.

PCR (reference)	HAstV target viral subgroup(s)	PCR oligos degeneracy value	Sensitivity/selectivity (%) for detection of HAstV[a]		
			HAstV-C	HAstV-VA/HMO	HAstV-MLB
Published PCRs					
Diagn-HAstV [24]	C	4/3/1	**90.28/100**	0/78.94	0/78.92
Kapoor et al. [16][b]	C, VA/HMO, MLB	64/32/8/16/8	**92.49/34.70**	**68.84/57.61**	**80.10/13.44**
Cordey et al. [6]	C	2/1/4	**69.15/97.94**	0/82.41	0/82.39
	VA/HMO[c]	2/1/2;1/1/1;1/1/1;1/1/1	1.11/88.64	**83.66/97.81**	0.02/90.12
	MLB[c]	2/2/1;1/1/1;1/1/1	9.00/70.82	7.02/73.65	**97.71/97.48**
Holtz et al. [17]	MLB2	1/1/1	0/97.84	0/98.17	**24.87/100**[d]
Jiang et al. [18][g]	C	1/1/1	**62.47/99.63**	0/84.84	0/84.82
Noel et al. [19]	C	1/1	**66.30/98.75**	0/83.72	0/83.72
Svraka et al. [21]	C	1/2/1	**64.82/95.76**	0/81.50	0/81.49
Sakon et al. (22)[f]	C	1/1	**85.00/90.98**	0/73.83	0/73.82
Smits et al. [23]	VA1	1/1	0/94.63	**42.40/100**[d]	0/95.42
Finkbeiner et al. [20][e]	C	1/1	**67.13/98.72**	0/83.51	0/83.51
	VA1	1/1	0/94.52	**43.41**/100[d]	0/95.33
	MLB1	1/1	0/100	0/94.94	**58.47/100**[d]
Newly designed qPCRs (this study)					
VEB-HAstV-C	C	24/16/16	**99.03/99.23**	0/76.30	0/76.29
VEB-HAstV-VA/HMO	VA/HMO	4/16/8	0/100	**99.91/99.98**	0/91.08
VEB-HAstV-MLB	MLB	2/2/2	0/100	0/91.97	**99.99/100**

[a] values of sensitivity and selectivity for the viral group that includes or included in PCR target group are shown in bold.
[b] evaluated as multiplex PCR with 5 published oligos.
[c] PCRs for this target groups of astroviruses were evaluated as multiplex of 4 or 3 PCRs designed in [6] for detection of VA1/HMO-C/SG/PS/UK1, VA2/HMO-A, VA3/HMO-B, and VA4 or MLB1, MLB2 (also evaluated separately, presented in(17)), and MLB2/3, respectively.
[d] target viruses for this PCR comprise a subgroup of the viral group for which selectivity is evaluated. Detailed analysis of the sensitivity for all shown target groups for these PCRs in comparison with newly designed ones is represented in Fig. 2.
[e] only the second specific step of the two-step PCRs designed for detection of the corresponding targets in this study was used for evaluation.
[f] AC230/AC1′ primer combination was used for evaluation.
[g] Astrovirus specific PCR was evaluated separately from other non-Astrovirus PCRs.

the plasmid materials containing HAstV-VA types 3–5 and HAstV-MLB types 1–3 as well as the extracted RNA containing HAstV-VA types 1 and 2 and HAstV-BF34. Efficiency was assessed by testing a 10-fold dilution series in duplicate for HAstV-C (type 1 only), HAstV-VA types 3–5 and HAstV-MLB types 1–3 and also the limit of detection was assessed (Table 4).

All micro-organisms of the specificity panel tested negative in the newly designed assays.

4.3. Monitoring the presence of astroviruses in clinical samples

A total of 556 faecal samples were collected from 461 different patients and tested for the presence of HAstV. In three samples inconclusive results were obtained due to inhibition of the PCR (IC out of range). In 14 stool samples obtained from 13 patients, HAstV were detected with a range in Cq-values of 13.9–37.3. Co-infections, two with norovirus genogroup II, one with rotavirus and one with adenovirus were demonstrated in four samples. HAstV-C were detected in 11 out of 14 astrovirus positive samples, of which 10 were also detected by the diagn-HAstV assay that missed a sample with a Cq-value of 33.6. Conversely, one specimen which remained negative by VEB-HAstV-C was found positive by the diagn-HAstV assay (Cq-value 35.7). Further sequence genotyping of the HAstV-C discrepancies was not performed due to the high Cq-values. The remaining three HAstV detected belonged to HAstV-MLB and were obtained from two different patients. Nucleotide sequence analysis revealed the closest match to HAstV-MLB type 1 (MLB1) and type 2 (MLB2), respectively. No HAstV-VA/HMO was detected in the faecal samples.

Two samples containing MLB1 were obtained from an immunocompromised child in January 2016 (Cq-value 22.3, submitted because of watery diarrhoea) and March 2016 (Cq-value 21.1, obtained because of increasing periods of diarrhoea). The latter showed a coinfection with adenovirus (Cq-value 29.3). The MLB2 (Cq-value 31.6) containing stool sample was obtained from an immunocompetent child suffering from chronic diarrhoea with a rotavirus co-infection (Cq-value 10).

A total of 142 CSF samples obtained from 136 patients were analysed with the VEB-HAstV-VA/HMO and VEB-HAstV-MLB multiplex PCR. Fifty of the patients were suspected for encephalitis. None of the tested specimens were positive for HAstV-VA/HMO or HAstV-MLB.

5. Discussion

Continuing discovery of new HAstV presents a perpetual challenge to diagnostics of these viruses. To this end, we here introduce three group-specific real-time PCR assays, which is the fewest feasible number for covering the entire genetic diversity of HAstV. Their SN and SL approached 100% *in silico*, both using the original and most recent (as of 21.01.2017, data not shown) datasets of complete genome sequences of HAstV. These assays use degenerate oligos directed to genome regions conserved in diverse viruses, so they may retain ability to recognize also variants of these three groups that are yet to be described.

We designed all three oligo sets without regard to published PCRs. Yet, they overlap with others in the region around of the RdRp-Capsid junction of approximately 1700 nucleotides (Fig. 1). This observation reveals a link between the new and established PCRs that adds to their credibility. We observed good correlation between results of our *in silico* evaluation and those reported *in vitro* by the original authors of the published PCR assays for comparable virus datasets as well as in our comparison of VEB-HAstV-C *versus* diagn-HAstV. However, when the published assays were evaluated *in silico* against the entire genome sequence database, they demonstrated suboptimal SN and were outperformed by the VEB-HAstV assays. Their SL was predominantly poor (Table 2), since this parameter was not sufficiently optimized in PCR design. On the other hand, established PCRs, especially designed by Cordey et al., can detect and discriminate many HAstV, while VEB-HAstV PCRs can assign HAstV only to a set of types [6].

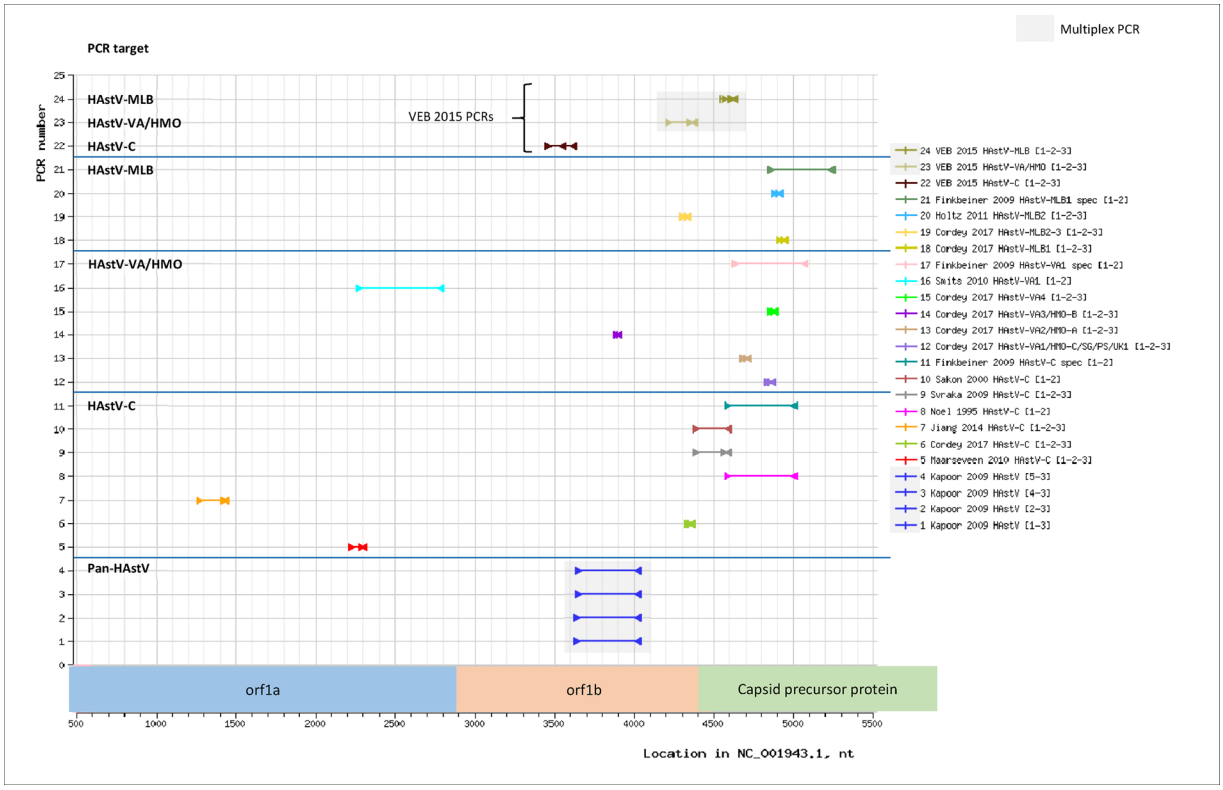

Fig. 1. Genome location of the annealing sites of HAstV PCR oligos. The genomic location of oligos, as mapped to sequence NC_001943.1, was determined for the viruses detected by the corresponding assay with the highest probability evaluated *in silico*. All assays were clustered in five groups, specified at the left and separated by horizontal blue lines. Arrowheads show the polarity of the corresponding primer/probe at the 3′end of the oligos; 5′ends of the oligos are shown with the vertical bars; solid lines between vertical bars depict the PCR product oligos. Functional organization of HAstV genome is indicated at the bottom. At the right, key to assays and theirs numbering are provided. Combination of oligo ranks for each PCR are shown in brackets after PCR name. Multiplex PCRs (Kapoor 2009, and VEB-HAstV-MLB, VEB-VA/HMO) are highlighted with gray background. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

The prevalence of HAstV in fecal samples found in this study was 2.5% (14/556), which is comparable to that reported by a recent study [6] (3.1%) and higher than found by us in preceding years in the same time-period (laboratory information system data: 0.7%–2.2%). The detection of HAstV-MLB1 and HAstV-MLB2, not targeted previously, could explain this increase. While in our testing they accounted for ~22% of all HAstV, their share in the Cordey et al. study was 50%, and included VA2 type not present in our samples.

In the present study, all faecal specimens have been tested using both the diagn-HAstV and the newly designed assays. They agreed on 10 positives and each additionally recognized an extra sample, both with high Cq-values. These results were in agreement with the high quality of these assays according to *in silico* evaluation. The false negative result obtained by the diagn-HAstV assay might be due to HAstV-C type 2, 6 or 7, but further typing has not been performed and therefore no definitive conclusion is possible.

Here, the first report of HAstV-MLB1 and HAstV-MLB2 types in the Netherlands has been presented. Two MLB1-containing samples were of high viral load and collected from the same patient over 3 months. Although adenovirus (Cq-value 29.3) was identified in the later sample as well, the higher HAstV-MLB1 load (Cq-value 21.1) pointed toward HAstV as being most likely the main aetiological agent. Since novel HAstV have also been detected in other specimens as urine and serum/plasma [9,14], available plasma and urine samples of this patient were tested but no HAstV-MLB was detected. The HAstV-MLB2 containing stool also contained rotavirus (low Cq-value) which is a more likely explanation of the gastroenteritis. No further samples of this patient were available.

In several case reports, novel HAstV has been associated with encephalitis or meningitis in patients. Therefore, we tested CSF samples from patients with neurological symptoms without proven aetiology, but no novel HAstV was detected in the 142 samples tested in this study.

In conclusion, we present *in silico* evaluation of several published HAstV PCR assays against 104 full genome sequences, representing the entire known genetic diversity of HAstV, using a common computational framework. Its results are in good agreement with the published *in vitro* evaluations by the original authors. We designed and evaluated *in vitro* three new real-time PCR assays for improved detection of the respective known phylogenetic groups of HAstV and their discrimination. These new PCRs assisted a survey of HAstV in patient specimens that included the first-time identification of novel astroviruses from stool samples in the Netherlands. We expect these assays to facilitate detection of HAstV by other researchers.

Author contributions

Conceived and designed the experiments: RN, IS, EC, AEG. Performed the *in silico* experiments: IS, AAG. Performed the *in vitro* experiments: RN, PC. Collected and analyzed the *in silico* data: IS, AAG,

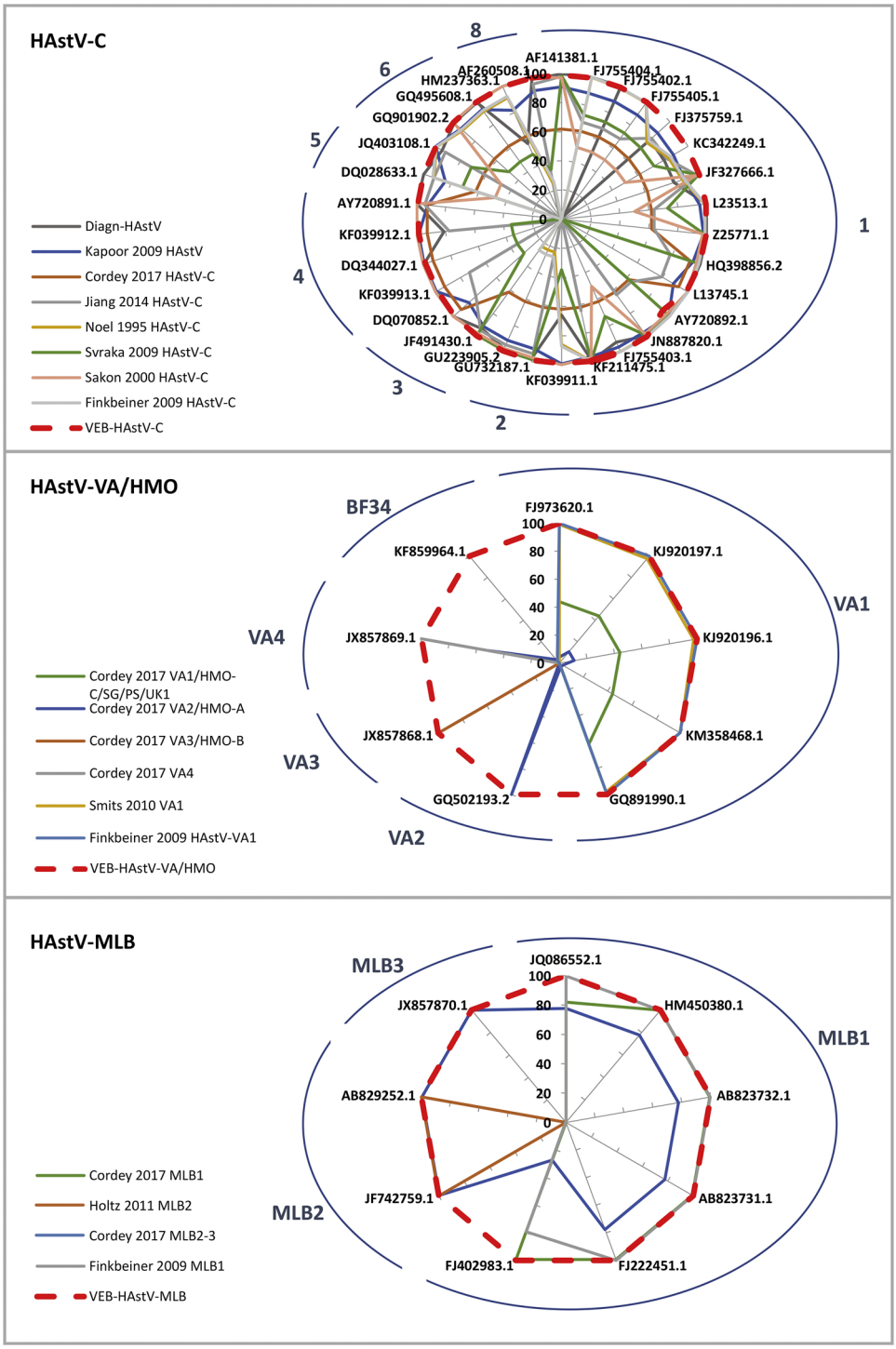

Fig. 2. Radar diagrams of probability of detection (0–100%) of HAstV for PCRs presented in Table 2. Detection is shown for three groups of HAstV: HAstV-C, HAstV-VA/HMO, and HAstV-MLB. Viruses are represented by GenBank accession number and version. PCRs are named according to the first author and year of the publication followed by the PCR target. Groups of sequences belonging to each genotype are shown with blue arcs. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

AEG. Collected and analyzed the *in vitro* data: RN, PC, EW, JV, EC. Wrote the manuscript: RN, IS, EC, AEG. Reviewed and approved the manuscript: all authors.

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Conflict of interest

None of the authors have conflicts of interest to declare.

Table 3
Cq-values for detection of HAstV-C type 1–8 by VEB-HAstV-C and diagn-HAstV PCR assays.

HAstV-C type	VEB-HAstV-C	diagn-HAstV	dCq
1	27.0	26.0	−1.0
2	29.6	32.1	2.5
3	24.4	23.0	−1.4
4	24.9	24.3	−0.6
5	19.2	18.9	−0.3
6	20.4	24.5	4.1
7	18.8	25.8	7.0
8	21.3	21.4	0.1

dCq: delta Cq, difference in Cq-values between VEB-HAstV-C and diagn-HAstV assays.

Table 4
Efficiency and limits of detection of HAstV real-time PCR assays.

PCR	HAstV type	Efficiency	R^2	Slope	LoD (copies/PCR)
VEB-HAstV-C	HAstV-C type 1	101.1%	0.991	−3.296	nd
VEB-HAstV-VA/HMO	VA3	93.3%	0.996	−3.494	10.37
	VA4	94.4%	0.996	−3.463	12.79
	VA5	112.4%	0.999	−3.056	14.43
VEB-HAstV-MLB	MLB1	107.4%	0.994	−3.157	144.9
	MLB2	106.6%	0.988	−3.173	7.57
	MLB3	96.2%	0.992	−3.416	7.62

LoD: limit of detection, nd: not determined.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jcv.2018.09.007.

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