Circulation of classic and recombinant human astroviruses detected in South Africa: 2009 to 2014

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Highlights

- More recombinant than classic strains were detected.
- Astrovirus surveillance in non-human sources will identify reservoirs for recombination.
- Challenges in classifying recombinant strains exist in the current characterisation system.
- The Vesikari severity scale must be used with caution when rating the severity of astrovirus-associated diarrhoea.
- •Genotype HAstV-5 requires further in-depth investigation.

Abstract

Background: Astroviruses (AstVs) are associated with diarrhoeal and extra-intestinal infections in human, animal and avian species. A prevalence of 7% was reported in selected regions in SA while AstVs detected from clinical stool specimens were almost identical phylogenetically to strains identified in environmental and water samples. This study investigated the molecular diversity of astroviruses circulating between 2009 and 2014 in South Africa (SA).

Methods: Astroviruses detected in stool specimens collected from hospitalised children were investigated retrospectively. Astroviruses were characterised using type-specific RT-PCR, partial nucleotide sequence analyses in ORF1 and ORF2 and whole genome sequencing. Different genotypes were compared with clinical features to investigate genotype-related associations. The Vesikari severity scale (VSS) was evaluated for scoring astrovirus diarrhoeal infections.

Results: Of 405 astroviruses detected, 49.9 % (202/405) were characterised into 32 genotypes comprising 66.3 % (134/202) putative-recombinants and 33.7 % (68/202) classic strains. No trends by year of collection, age or site were observed. Whole genome analysis in eight strains revealed that genotypes assigned by partial nucleotide sequence analyses to five astroviruses were incorrect. Bivariate analyses showed there were no significant associations between genotypes and clinical symptoms or severity of infection. A comparison of Vesikari parameters with astrovirus-positive proxy values demonstrated that Vesikari scores for duration of diarrhoea and admission temperatures would result in a milder infection rating in astrovirus-positive cases.

Conclusions: Diverse genotypes co-circulated with putative-recombinants predominating. Astrovirus classification was complicated by the lack of a consistent characterisation system and reliable reference database. The VSS should be used cautiously to rate astrovirus diarrhoea. While surveillance in communities and out-patient clinics must be continued, screening for human astroviruses in alternate hosts is needed to determine the reservoir species.

1. Background

Human astroviruses (HAstVs) were identified in 1975 by electron microscopy of stool specimens collected from infants with diarrhoea and vomiting [1,2]. Since then, HAstVs have been associated with diarrhoeal, respiratory and neurological infections [[3], [4], [5]]. Infections in animals usually present as gastroenteritis while extra-intestinal manifestations including damage to the liver, kidney or immune system are described in avian species [6]. Cross-species spillover events and possible zoonotic transmissions of AstVs have been reported [5,[7], [8], [9], [10]].

The HAstV genome is a positive-stranded RNA of approximately 6.8 kilobases (kb), organised into three open reading frames (ORFs); ORF1a, ORF1b and ORF2 [11]. Regions ORF1a and ORF1b encode non-structural proteins and ORF2 encodes the capsid protein. The most conserved region is ORF1b while ORF2 is most divergent. The ORF1b-ORF2 junction represents a recombination "hot spot" [12].
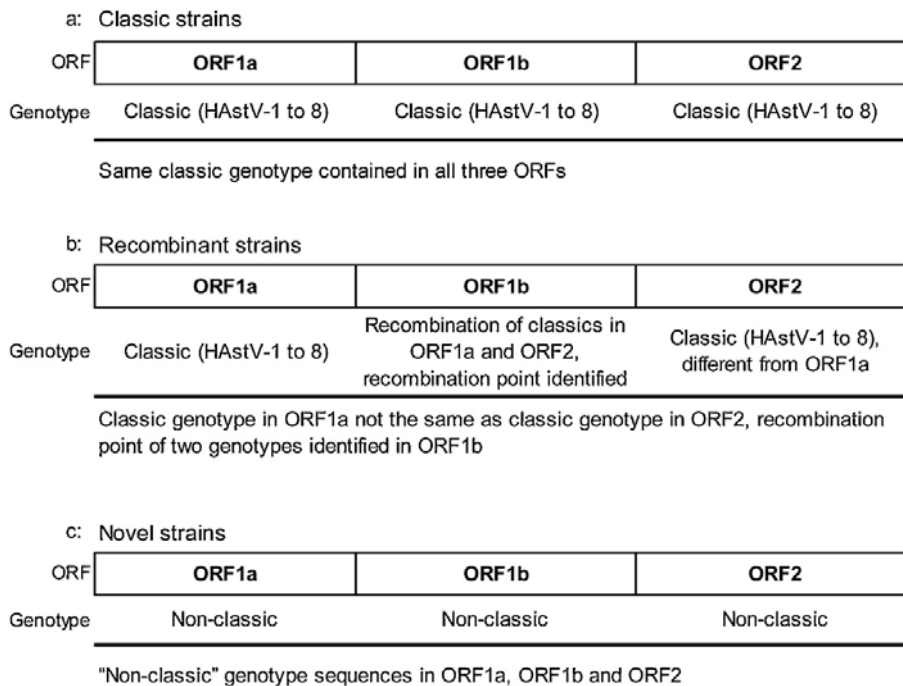

Fig. 1. Schematic representation of the AstV genome and current rationale for characterisation of classic (1a), recombinant (1b) and novel strains (1c).

The variability in characterisation associated with the AstV genome is depicted in Fig. 1 (not drawn to scale). Human AstVs are characterised into classic (HAstV-1 to HAstV-8), novel (MLB, HMOC, VA) and recombinant strains [[13], [14], [15], [16]]. Classic HAstVs display the same genotype in all three ORFs (Fig. 1a) while novel strains contain nucleotide (nt) sequences that are both unique and highly similar to HAstVs (Fig. 1c) [[13], [14], [15], [16]]. Recombinants contain the identities of two classic strains in a single genome with a distinct point of recombination (Fig. 1b) [[17], [18], [19], [20]].

The emergence of recombinant strains is hypothesised to result from infections with two classic HAstVs in a single cell [5], but evidence of dual infections in humans is lacking. Where mixed AstV infections have been reported, novel strains were involved [21]. Studies in bats [10,22] and non-human primates (NHP) [9] for human AstV strains showed that only NHPs [9] harbored these types. Single infections of both classic and recombinant strains were identified in NHPs [9] suggesting that recombination could be occurring undetected in similar synanthropic environments [5,9]. A reservoir host for HAstVs is unknown and tracing the evolution of recombinants and spillover diversity between species is needed [5].

While early surveys showed HAstV-1 was predominant [12,16,23], a decrease in circulation of classic genotypes with the replacement by novel or recombinant strains has been observed worldwide [12]. A study in South Africa (SA) demonstrated that HAstVs detected in clinical stool specimens were almost identical phylogenetically to strains identified in environmental water samples [24]. Further, HAstV-2, HAstV-4 and HAstV-7 were only detected in water samples suggesting that patients infected with these genotypes experienced a milder diarrhoea and therefore did not seek medical attention [24]. The first HAstV-8 strain reported in Southern Africa was identified in SA [25], and confirmed by antigenic and genetic methods [25].

It has been suggested that the severity of HAstV infections is genotype related [13,24]. Whether recombinant and novel strains are associated with more severe infections has not been established [16]. Using a 15-point community-based diarrhoeal severity scale, the Etiology, Risk Factors, and Interactions of Enteric Infection and Malnutrition and the Consequences for Child Health and Development (MAL-ED) study showed that HAstV-diarrhoea severity was equivalent to or higher than other enteropathogens, except rotavirus (RV) [26,27].

Long-term molecular surveillance of AstVs is necessary to identify associations between the genetic variability of circulating strains and changes in patterns of infections or epidemiology [28]. This study investigated the molecular epidemiology of HAstVs and established long-term molecular surveillance.

2. Objectives

To investigate the diversity of HAstVs circulating between 2009 and 2014 among paediatric patients hospitalised for gastroenteritis in selected regions of SA.

3. Study design

3.1. Specimen collection and astrovirus detection

Astroviruses detected in a previous published surveillance study [29] were characterised retrospectively. Stool specimens were collected from April 2009 to May 2014 from patients enrolled in the Rotavirus Sentinel Surveillance program (RSSP) [30]. The RSSP comprised six hospitals in four SA provinces. Informed consent was obtained during enrollment while demographic and clinical data was collected by medical record reviews. Specimens were stored at 4 °C for one year and thereafter moved to an ultralow freezer (- 70 °C) for long term storage.

For astrovirus detection, nucleic acid was extracted from 160 μL of clarified stool suspension using the QIAamp Viral RNA Mini QIAcube Kit (Qiagen Inc., Valencia, CA) on the Qiacube instrument (Qiagen), according to the manufacturers' instructions. The Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics GmbH, Mannheim, Germany) was used to reverse transcribe 10 μl nucleic extract into complementary deoxyribonucleic acid (cDNA), incorporating random primers (Roche). Astroviruses were detected by real-time PCR amplification of 5 μL cDNA in the LightCycler ® ProbeMaster kit (Roche) using primers and probe mix as previously described [31].

3.2. Astrovirus characterisation

Astroviruses were characterised using type-specific RT-PCRs and both partial and whole genome sequence (WGS) analyses. Regions ORF1a and ORF2 were investigated by partial nt sequence analysis for genotype identity.

3.2.1. Type-specific RT-PCR (ORF2)

The capsid region in ORF2 was genotyped using type-specific RT-PCR assays [24,32] with the following modification; the SuperScript® IV (SSIV) First-Strand cDNA synthesis reaction kit (Invitrogen, Life Technologies, Lithuania) was used to prepare complementary DNA (cDNA) according to manufacturer's instructions.

3.2.2. Nucleotide sequence analysis of RT-PCR amplicons (ORF2 and ORF1a)

Genotypes determined by type-specific RT-PCR were confirmed in ORF2 by nt sequence analysis of amplicons generated using primer set Mon2R/prBeg [24,33]. The ORF1a region was characterised by nt sequence analysis of amplicons made using primer set Mon348R/Mon340 F [24,34].

Amplicons were sequenced directly using the Big Dye Terminator v3.1 kit (Applied Biosystems, Thermo Fisher Scientific, Austin, TX) and ABI 3500 Genetic Analyser (Applied Biosystems). Sequence data were verified using BioEdit Sequence Alignment Editor (V.7.0.9.0) software [35]. Nucleotide sequences were compared with references available on the National Center for Biotechnology Information (NCBI) database using Basic Local Alignment Search Tool (BLAST) to identify genotypes.

Astroviruses detected at Ct values <20 cycles during initial screening and containing genotypes of interest were selected for nt sequence analysis by WGS. However, only eight strains were successfully characterised (unpublished data).

3.3. Astrovirus infections: genotypes and clinical features

3.3.1. Association between genotypes and severity of infection

Where available, demographic and clinical information was compared to investigate genotype-related associations. The Vesikari severity scale (VSS) [36] was used to rate the severity of diarrhoeal disease in HAstV-positive cases [29]. Vesikari scores were compared between different strains to identify correlations between genotypes and severity of infection.

Clinical symptoms and factors comprising the Vesikari scale i.e. duration of diarrhoea and number of diarrhoeal episodes per day, duration of vomiting and number of vomiting episodes per day, admission temperature and level of dehydration, were compared by bivariate analyses with different genotypes to test for significant associations.

3.3.2. Evaluation of the Vesikari scale to score astrovirus diarrhoeal infections

The reliability of the VSS for scoring HAstV-diarrhoea was evaluated. Medians and inter-quartile ranges (IQR) were determined for Vesikari factors in HAstV-positive cases and used as proxy measures for comparison with VSS parameters. For all severity score analyses, HAstVs present as single or co-infections were compared as different categories [29].

The equivalence between statistical values calculated in HAstV-positive cases and the VSS parameters was assumed as follows:

1) 25 % IQR was considered equivalent to parameter 1;
2) 50 % median was considered equivalent to parameter 2;
3) 75 % IQR was considered equivalent to parameter 3

3.4. Statistical analyses

All analyses were performed with STATA v11 (StataCorp LP, College Station, TX). The associations between AstVs and factors were analysed using $\chi2$ and Wilcoxon rank-sum tests. Statistical significance was considered at $p < 0.05$. Specimens with missing information were excluded in the bivariate analysis.

4. Results

4.1. Positive rates and genotype distribution

From 405 HAstVs detected in stool specimens collected between 2009 and 2014 [29], 49.9 % (202/405) were characterised in ORF1a and ORF2. The remaining 50.1 % (203/405) of specimens were excluded as 46.9 % (190/405) could not be re-amplified and 3.2 % (13/405) did not have complete nt sequence analyses in ORF1a (n = 11) or ORF2 (n = 2).

Thirty two genotypes were identified ranging from 20.3 % (41/202; T8/T1) to 0.5 % (1/202; multiple strains, n = 10; Table 1). Putative-recombinants comprised 66.3 % (134/202) and

classic HAstVs were present in 33.7 % (68/202) of the total strains characterised. Whole **genome sequence analyses of eight AstVs showed that genotypes assigned in five strains by partial nt sequence analyses were incorrect (data not shown).**

Table 1. Distribution of astrovirus genotypes identified (N = 202).

Classic strains	n = 68/202	% (n/N)
HAstV-1	31	15.3
HAstV-2	5	2.5
HAstV-3	3	1.5
HAstV-4	2	1.0
HAstV-5	18	8.9
HAstV-6	2	1.0
HAstV-8	7	3.5
Putative recombinants (ORF1a/ORF2)	n = 134/202	% (n/N)
T8/T1	41	20.3
T8/T4	12	5.9
KS106210/T3	10	5.0
KS106207/T2	9	4.5
T8/B55	9	4.5
KS106209/T3	7	3.5
T1/T3	6	3.0
USA-OB2038B/T3	6	3.0
KS106209/T2	5	2.5
T1/T8	4	2.0
T2/T3	4	2.0
T1/B55	3	1.5
T1/T4	3	1.5
V1182/T8	3	1.5
T8/T3	2	1.0
KS106209/T4; KS106210/T4; T1/T2; T1/T5; T2/T5; T4/T3; T4/T8; T5/T3; T8/T2; T8/T5	1 each	0.5

4.2. Genotype distribution and year of detection

The prevalence of selected genotypes identified between 2009 and 2014 is shown in Fig. 2. No trends in seasonality were observed when the positive rate of genotypes were analysed by month. The prevalence of recombinant strain T8/T1 decreased from 2009 (41.7 %) to 2013 (19.2 %), while HAstV-1 prevalence increased from 2010 (7.3 %) to 2014 (30.8 %). Genotypes HAstV-1, T8/T1 and T8/T4 were present throughout the study period. Strains T8/T1 and T8/T4 co-circulated from 2009 to 2013 while HAstV-5, the second most commonly detected classic strain, circulated between 2010 and 2013.
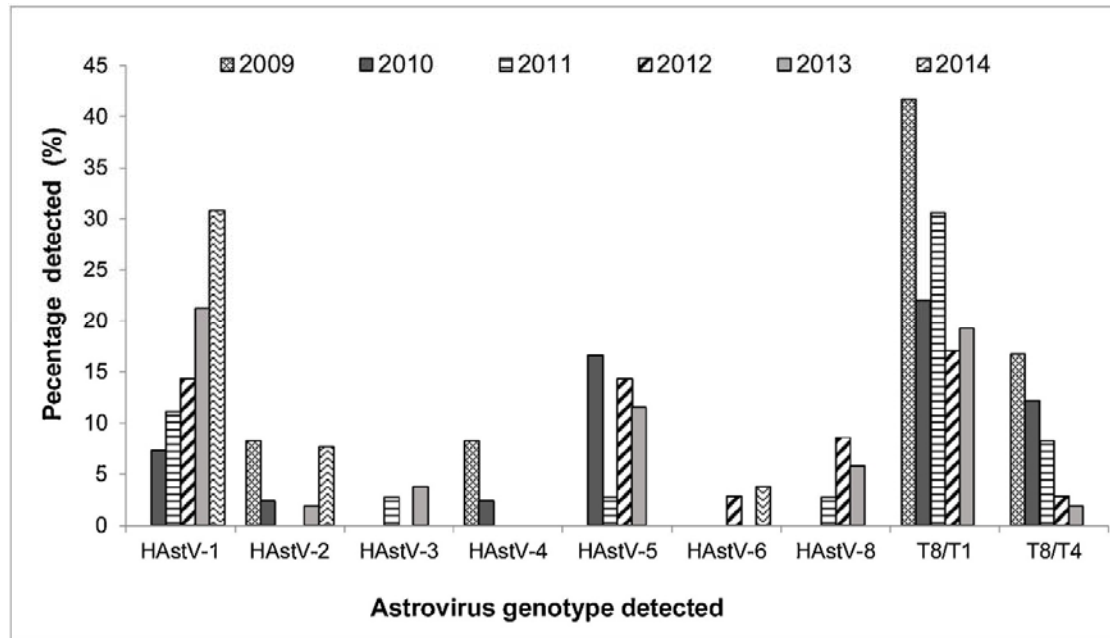
Fig. 2. Percentage of selected genotypes detected over the study period.

The largest genotype diversity was observed in 2013. Genotype 2 was detected in two-year intervals, 2009–2010 and 2013–2014. Genotypes HAstV-3 and HAstV-6 were present over a three-year period with a year's absence in-between i.e. HAstV-3 (2011 and 2013) and HAstV-6 (2012 and 2014). Limited periods of circulation were observed with HAstV-4 (2009 and 2010) and HAstV-8 (2011–2013).

4.3. Genotype distribution per site

The predominant classic strain HAstV-1, was present in all sites except site 2 while the second most common classic genotype, HAstV-5 was detected in all sites. Recombinant strains were predominant in all sites with strain T8/T1 detected in all locations (Supplementary Table 1). The largest strain diversities were observed in sites 1, 2 and 6. Recombinant strain USA-OB2038B/T3 was only detected in sites 6 (n = 1) and 7 (n = 5).

4.4. Genotype distribution and patient age

Strain T8/T1 was predominant in patients aged ≤18 months (22.7 %; 39/172) but was not significantly associated with any age category (Supplementary Table 2). Genotypes HAstV-1 (16.7 %), HAstV-5 (16.7 %) and HAstV-8 (30.0 %) were detected most frequently in patients aged ≥19 months (n = 22). Strains HAstV-5 and T8/T1 was detected in all age groups investigated.

4.5. Astrovirus infections: genotypes and clinical features

4.5.1. Associations between genotypes and clinical features

Bivariate analyses of genotypes and selected symptoms did not show significant associations (data not shown). The median Ct values in single (Ct = 14.03) and mixed (Ct = 21.53) infections were compared. Astroviruses present as single infections were detected at lower Ct

values as compared to mixed infections but earlier detection by RT-PCR was not significantly associated with single infections (p = 0.20).

4.5.2. Evaluation of using the Vesikari scale to rate astrovirus infection severity

Evaluation of single and mixed AstV infections showed there were no distinct differences in disease severity between the two groups when measured using the VSS (data not shown). For most symptoms evaluated, the VSS parameter values and HAstV-positive proxy values were similar (Table 2).

Table 2. Vesikari parameters compared with values calculated for HAstV-positive cases.

Symptom	Scoring system	Parameter 1	Parameter 2	Parameter 3
	Vesikari	25 % IQR	Median	75 % IQR
	This study			
Maximum diarrhoea (per day)	**Vesikari**	1 - 3	4 - 5	≥ 6
	This study	1 - 4	5	≥ 6
Diarrhoea duration (days)	**Vesikari**	1 - 4	5	≥ 6
	This study	1−2*	3*	$\geq 4*$
Maximum vomit(per day)	**Vesikari**	1	2 - 4	≥ 5
	This study	1−2	3	≥ 4
Vomit duration(days)	**Vesikari**	1	2	≥ 3
	This study	1	2	≥ 3
Admission temperature (ºC)	**Vesikari**	37.1 - 38.4	38.5 - 38.9	≥ 39.0
	This study	35.8–36.9*	37.0–37.9*	≥ 38.0
Dehydration (%)	**Vesikari**	NA	1−5	≥ 6
	This study	NA	1−5	≥ 6

The following were observed for diarrhoea duration and admission temperature (*):

1) the total duration of diarrhoea in HAstV-positive cases comprised parameter 1 of the VSS
2) the median value for admission temperatures in HAstV-positive cases comprised parameter 1 of the VSS

5. Discussion

This study highlights the changing molecular epidemiology of HAstVs in SA and reveals a complex co-circulation of multiple genotypes in the same community. Putative recombinants were detected predominantly using these methods. Genotypes were assigned by partial nt sequence analyses and recombination points were not confirmed in most strains. However, where WGS data was available, recombinant strains were identified by recognizing the points of recombination on the genome (publication in progress). In the remaining putative recombinants, genotypes were assigned in the different ORFs by comparisons with published reference strains. Further, phylogenetic trees were constructed to establish similarities and groupings between the SA strains and with the references (publication in progress). These comparisons validated the assignment of genotypes during the characterisation process.

Inadequate long term storage of stool specimens resulted in sub-optimal quality of material for retrospective investigation, as was experienced with attempts for WGS analysis of specimens that were initially detected with higher viral loads. Overall, 50 % positive HAstVs detected could not be characterised further. A future perspective will be to store stool specimens in a suitable buffer that protects the integrity of the nucleic acids. Despite single infections being detected earlier in the assay, the sensitivity for AstV detection was not dependent on whether the virus was present in a single or mixed infection. This may be due to the low number of single AstV infections available for this comparison.

Challenges in the characterisation system used in this study emphasize the need for a systematic and regulated database for AstV classification by nt sequence comparisons. Regarding strains T8/T1 and T8/T4, analysis of the epidemiologic data suggested that these two genotypes could be the same and capsid identity assigned by comparisons with Genbank may be incorrect. Astrovirus strain submissions to reference nt databases need methodical structuring. The call for standardised AstV classification and nomenclature for both human [37] and non-human strains [9] has been stated previously.

Strain epidemiology data suggests that recombinants circulate widely. However, evidence linking recombination events to human hosts is lacking. The emergence of novel cross-species strains and recombination of classic HAstVs may be taking place undetected in non-human sources [5,9]. This study recommends surveillance for human classic strains in non-human species, similarly to studies in Egypt [10], Bangladesh and Cambodia [9]. Astroviruses detected in bats from SA contained nt sequences belonging to bat species only [38]. Extended surveillance beyond human sources is needed to determine AstV reservoirs. This study supports the "One Health" approach to identify links between human and animal health and the environment [22].

The annual distribution of AstVs revealed that T8/T1 was predominant until 2013, when HAstV-1 emerged as the most frequently detected strain. The change in HAstV strain prevalence has not been described before. Detailed molecular analyses will resolve if this event was evolution of a single genotype or indeed replacement between two strains. A comparison of data collected for T8/T1 and HAstV-1 raised an alert that T8/T1 could possibly be an incorrectly characterised HAstV-1 strain. However, this theory was not be tested due to poor quality of stored specimen.

The largest and most diverse proportion of HAstVs were collected from site 1. Site 1 is located at the Chris Hani Baragwanath Academic Hospital (CHBAH), the third largest hospital in the world [39]. This public hospital serves a vast/wide population and epidemiology at site 1 may highlight trends of HAstV prevalence and evolution in SA. The large diversity of strains observed in 2013 could be due to wider surveillance with the increased number of rotavirus cases observed during this year [40]. The largest proportion of AstVs was collected in 2013.

While the majority of AstVs were detected in the ≤ 6 month age group, a mix of classic and recombinant genotypes were present in all age group categories. This distribution is in agreement with reports that describe a lack of heterotypic immunity from past AstV infections [41] and the absence of cross-protection between genotypes [17]. Yet, the existence of protective immunity in children with asymptomatic infections has been suggested [27]. Vaccine development strategies require valency formulations and vaccination

age recommendations. This study recommends further investigations of AstV diversity in asymptomatic children, a shortcoming in these analyses.

An unexpected finding in this study was genotype HAstV-5 emerging as a strain warranting further in-depth investigation. Genotype HAstV-5 was the second most commonly detected classic strain, was present in all sites and in all age groups. Further, of the 22 AstVs strains that contained either a genotype HAstV-5 protease or capsid gene, the majority were characterised as classic HAstV-5 strains (Table 1). Genotype HAstV-5 was reported as the predominant strain circulating in southwestern Nigeria based on genetic analysis of ORF2 [42]. Whether these results attest to the stability or persistence of genotype HAstV-5 needs further clarification and supporting proof. Certainly, such information regarding HAstV-5 will be relevant for valency formulations if a multivalent vaccine is considered.

The association between severity of diarrhoea and AstV genotypes could not be determined. Firstly, the VSS may have been unsuitable to measure severity of infection. The VSS score for admission temperature and duration of HAstV-diarrhoea would result in a milder rating compared with a severe hospitalised event, whether AstVs were present as a single or mixed co-infections. Secondly, AstV-positive cases with mild diarrhoea must be included in the test of genotypes and severity of infection. Investigations which include children in the community and patients presenting at day clinics must be carried out to establish associations between AstV genotypes with diarrhoeal disease severity, as well as to develop a HAstV-diarrhoea severity scoring scale.

The MAL-ED study acknowledged the contribution of HAstVs to diarrhoea and recommended development of a Rotavirus-Astrovirus combination vaccine [27]. It is debatable whether the low clinical impact of AstV infection in healthy persons prioritizes prevention and vaccine-development interest. However, the association of AstVs with extraintestinal diseases and growing evidence for AstV zoonosis raises concern.

This study recommends ongoing surveillance for AstVs in human and non-human hosts. Strains detected in persons working closely or interacting with animals and poultry must be fully characterised to establish species of origin. In addition, screening asymptomatic and case-controls from the community is advised to further understand which types are circulating in the community. Further guidelines for AstV-specific surveillance may be drafted after consultation and conversations with other experts in this field.

An overhauling of the existing AstV classification system is suggested and a characterisation system that integrates genotypes identified from both the serine protease and capsid regions is proposed. This is similar to the schemes implemented for characterisation of rotaviruses [43] and noroviruses [44]. This classification tool must also consider the nomenclature system formulated by Martella et al., [37] which enables assignment of existing AstV species.

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Ethics

CRediT authorship contribution statement

Sandrama Nadan: Conceptualization, Investigation, Methodology, Data curation, Formal analysis, Writing - original draft, Writing - review & editing, Project administration, Funding acquisition. **Maureen B. Taylor:** Conceptualization, Supervision, Writing - review & editing. **Nicola A. Page:** Conceptualization, Supervision, Writing - review & editing, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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