Use of a Heminested Reverse Transcriptase PCR Assay for Detection of Astrovirus in Environmental Swabs from an Outbreak of Gastroenteritis in a Pediatric Primary Immunodeficiency Unit

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An outbreak of astrovirus gastroenteritis occurred in the Primary Immunodeficiency Unit at Newcastle General Hospital in March 2004. Environmental swabbing of the unit was undertaken after the outbreak, with multiple sites swabbed pre- and postcleaning. Astroviruses were detected in four environmental swabs and from two patient fecal samples using heminested reverse transcriptase PCR. An astrovirus genotype 3 strain was identified in both environmental swabs and fecal specimens and was the strain identified as being responsible for the outbreak. Environmental transmission of the virus was thought to have occurred by contamination of a syringe pump outside the laminar-flow curtain of a patient who was admitted with astrovirus gastroenteritis. This was subsequently transmitted to a cubicle next door and to a television/games console in a parents' room in the ward. Environmental monitoring of surfaces/equipment, using PCR assays for gastroenteric viruses in hospital situations where infection can give rise to serious clinical complications, may have a role in controlling and monitoring cleaning and the subsequent prevention of nosocomial transmission of gastroenteritis.

Human astroviruses are members of the *Mamastrovirus* genus of the family *Astroviridae*, according to the International Committee on Taxonomy of Viruses. Human astroviruses (HAsVs) are single-stranded positive-sense RNA viruses that are closely related to caliciviruses and picornaviruses. Currently there are eight serotypes (1 to 8), which correlate with genotypes determined through sequencing of the ORF2 region of the genome (34).

Astroviruses have been increasingly identified as important agents of acute gastroenteritis in children (7, 14, 23, 31, 32) and the elderly (11). Outbreaks of astrovirus gastroenteritis have been reported for young adults, including military recruits (2), students, teachers (28), and children (25). Astrovirus and other gastroenteric virus infections can occur in immunocompromised children (5, 30), such as those undergoing bone marrow transplantation (6, 37), those with severe combined immunodeficiency (4), those with T-cell immunodeficiency in cartilage hair hypoplasia (10, 36), or those with human immunodeficiency virus/AIDS (18).

Traditionally, astroviruses have been diagnosed by electron microscopy (19, 20, 26), more recently replaced in many lab-

oratories by in-house enzyme immunoassays (15, 26, 27) and more recently commercial enzyme immunoassays(22).

Several groups have developed type-specific reverse-transcriptase PCR (RT-PCR) assays for HAsVs with type-specific primers (21, 29). Others have developed primers in ORF1b (1) or the 5′ end of ORF2 (Mon269/Mon270) (27) to detect HAsVs.

Little information has been published on transmission of astroviruses in hospitals through environmental contamination of surfaces, furniture, or hospital and medical equipment.

Studies have been conducted using swabs to test for environmental contamination: with noroviruses by using a nested RT-PCR (3, 13) in hotels and hospitals and with rotaviruses by using an RT-PCR (33, 35) in hospital pediatric and neonatal units.

This report describes the application of a heminested RT-PCR for the detection of astrovirus RNA in environmental swabs collected from surfaces in the Primary Immunodeficiency Unit at Newcastle General Hospital during an astrovirus outbreak.

MATERIALS AND METHODS

Clinical and outbreak details. Patient 1 was referred on 2 February 2004 to the unit with a diagnosis of immunodeficiency polyendocrinopathy, enteropathy, X-linked syndrome and had presented with persistent diarrhea starting at a few days old. Extensive investigation including gut biopsy had not revealed any viral infection, and gut biopsy appearances were consistent with a diagnosis of autoimmune enteropathy. The child received an umbilical cord blood hemopoietic stem cell transplant at the age of 10 months with engraftment by day 27 post-

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TABLE 1. Environmental swabs examined and astrovirus PCR and genotyping data

| Sample | Site | Single-round PCR on swabs | | Heminested PCR on swabs | | Strain designation | Astrovirus genotype |
		Preclean (03/24/04)	Postclean (04/18/04)	Preclean	Postclean		
1	Staff toilet door handle	−	−	−	−		
2	Staff toilet light switch	−	−	−	−		
3	Telephone, outside cubicles 3 and 4	−	−	−	−		
4	Toys (jigsaw and bricks)	−	NS[a]	−	NS		
5	Microwave	−	−	−	−		
6	Cubicle 4, outside flow-syringe pumps	−	−	+	−	NewcastleGH-S1/2004/UK	HAsV-3
7	Cubicle 3, outside flow-syringe pumps	−	−	+	−	NewcastleGH-S2/2004/UK	HAsV-3
8	Parents' phone	−	−	−	−		
9	Parents' room door handle	−	−	−	−		
10	Television and games console	−	−	+	−	NewcastleGH-S3/2004/UK	HAsV-3
11	Parents' toilet door handle	−	−	−	−		
12	Parents' toilet taps	−	−	+	−	NewcastleGH-S4/2004/UK	HAsV-1

[a] NS, no sample.

transplant (PT). The volume and frequency of diarrhea had diminished by day 11 PT, and stools were formed. By day 44 PT, the stool volume and frequency had increased, and stools had become more watery. Astrovirus was detected for the first time in the stools on day 59 PT (15 March 2004) by electron microscopy.

Patient 3 was referred on 19 December 2003 with a late diagnosis of T cell/B cell severe combined immunodeficiency at the age of 8 months. A number of infections were detected at the time of referral to the unit, including superficial *Staphylococcus aureus* skin abscesses, *Pneumocystis carinii* pneumonia, and astrovirus diarrhea. The patient underwent a phenotypically identical whole marrow bone marrow transplant but continued to excrete astrovirus throughout the transplant period. Clinical details of patients 2 and 4 are not included, since astrovirus was not detected in their stool specimens and they were asymptomatic.

Preliminary virological examination. Fecal specimens from four of eight patients in the unit during the outbreak were sent to the Leeds Laboratory, Health Protection Agency (HPA) Yorkshire and Humber, for virological testing by electron microscopy, and a diagnosis of astrovirus gastroenteritis was confirmed for two patients.

Environmental sampling. To confirm if astrovirus was present on environmental surfaces within the Primary Immunodeficiency Unit at Newcastle General Hospital, various swabs were taken from the most likely sites of fecal contamination (Table 1). These were taken precleaning on 24 March 2004 and postcleaning on 18 April 2004 (cleaning occurred on 2 April 2004). All equipment is cleaned on a weekly basis with Cutan detergent wipes (DEB Ltd., Belper, United Kingdom), all surfaces with cubicles were damp dusted with detergent wipes and dried with paper towels, and floors were washed with detergent twice daily.

The swabs were dipped in 0.1 M phosphate-buffered saline, pH 7.2, and applied to various surfaces (Table 1; Fig. 1). The swabs were broken, placed into 1.5-ml screw-cap tubes, and sent to the Enteric, Respiratory and Neurological Virus Laboratory, Specialist Reference and Microbiology Division, HPA Colindale, for virological examination. Several fecal samples from patients involved in the outbreak were also sent for comparative studies. A fecal sample from patient 1 (F1) was collected on 24 March 2004 and from patient 3 (F3) on 25 March 2004. Follow-up fecal samples were collected on 15 June for patients 1 (F1b) and 3 (F3b); (Table 2). No fecal specimens prior to the outbreak were available from symptomatic cases. Patients 1 and 3 were in cubicles 4 and 3, respectively (Fig. 1).

RNA extraction of fecal and swab samples and reverse transcription assay. Fecal specimens were prepared as 10% fecal suspensions, and total RNA was extracted from 100 μl using the guanidinium thiocynate (GTC)-silica method (12).

For swab specimens, 1 ml of GTC solution (L6 buffer; Severn Biotech Ltd., Kidderminster, United Kingdom) was added to each swab in a 2-ml screw-cap tube and incubated at room temperature for 1 h, and the supernatant was

transferred to a new tube to which 20 μl of silica matrix (Severn Biotech Ltd.) was added. This was followed by washing with L2 buffer (Severn Biotech Ltd.). Thereafter, the GTC-silica method was followed (12). RNA was eluted in 40 μl of nuclease-free water (Promega, Southampton, United Kingdom). cDNA was prepared using random priming as previously described (9).

Astrovirus PCR primers. Primers for the single-round PCR assay were Mon269 and Mon270 (27) and are located between nucleotides (nt) 4574 and 5022 in the ORF2 capsid gene of human astrovirus type 1 (HAsV-1). A forward primer internal to Mon269 and designated Mon269N (5′-GAC CAA AAC CTG CAA TAT GTC A-3′) between nt 4599 and 4620 (5 nt upstream from the end of Mon269) was designed from a conserved region by means of sequence analysis of all eight genotypes of human astrovirus.

Astrovirus single-round and heminested PCR assays. Single-round astrovirus primers were Mon269 and Mon270 (27) and the heminested second round PCR used primers Mon269N and Mon270. Both assays were performed on all swabs and fecal specimens (Tables 1 and 2). cDNA (5 μl) was added to a single round, 45-μl PCR mix containing 10 mM Tris, pH 8.0, 50 mM HCl, 2.5 mM MgCl$_2$, 1 mM of each deoxynucleoside triphosphate (Invitrogen), 20 pmol of Mon269 primer and 20 pmol of Mon270 primer, and 1 unit of *Taq* DNA polymerase (Invitrogen). Products were amplified using the following conditions: 95°C for 2 min and then 40 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min, followed by 1 cycle of 72°C for 5 min, and then a hold at 15°C. The heminested PCR involved adding 1 μl of first-round PCR product to a 49-μl PCR mix containing 10 mM Tris, pH 8.0, 50 mM HCl, 2.5 mM MgCl$_2$, 1 mM of each deoxynucleoside triphosphate (Invitrogen), 20 pmol of Mon269N primer and 20 pmol of Mon270 primer, and 1 unit of *Taq* DNA polymerase (Invitrogen). Standard control measures and unidirectional workflow were used for all nested PCRs, with the second-round PCR setup performed in a PCR workstation with UV decontamination. Water controls were used in each assay as negative controls.

PCR amplicons of 449 bp for Mon269 and Mon270 and 424 bp for Mon269N and Mon270 were detected by gel electrophoresis in 2% agarose gels (MP agarose; Roche Molecular Biochemicals, Lewes, United Kingdom), stained with ethidium bromide, and photographed using a Bio-Rad GelDoc System (Bio-Rad Laboratories Ltd., Hemel Hempstead, United Kingdom).

PCR amplicon cloning and sequencing. The majority of samples were sequenced directly using HAsV primers, with the exception of the PCR amplicon from strain NewcastleGH-F3/2004/UK (Table 2), which was weakly reactive and was cloned using a TA cloning system (TOPO; Invitrogen, United Kingdom), as previously described (17).

Purified DNA was sequenced in both directions using the Beckman Coulter CEQ2000 dye terminator cycle sequencing quick-start kit (according to the manufacturer's instructions) (Beckman Coulter, High Wycombe, United King-
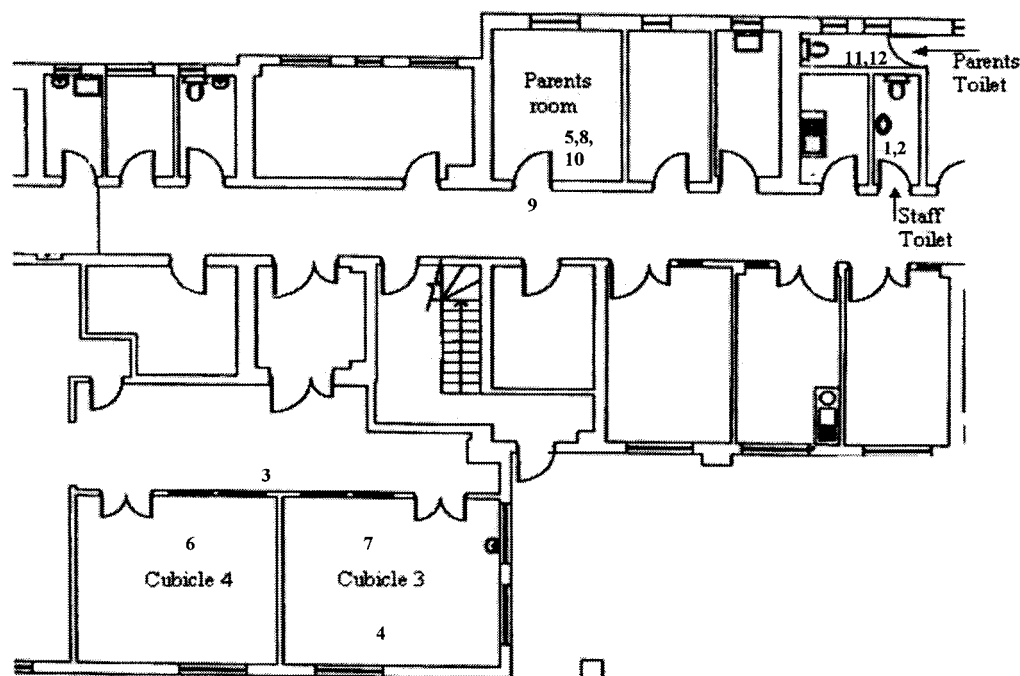
FIG. 1. Plan of the primary immunodeficiency unit. Numbers 1 to 12 are swab sample sites; see Table 1.

dom) and a Beckman Coulter CEQ2000 capillary sequencer (Beckman Coulter). Generation of contiguous sequences and pairwise alignments of the 429-bp interprimer region (Mon269 and Mon270) and 402-bp interprimer region (Mon269N and Mon270) of astrovirus ORF2 sequences was performed using Genebuilder and Clustal in Bionumerics version 3.5 (Applied Maths, Kortrij, Belgium).

RESULTS

Single-round and heminested RT-PCR assay. Astrovirus RNA was detected by single-round and heminested PCR in two fecal samples each from patients 1 and 3. Specimens from two other patients in the unit were negative. Astrovirus RNA was detected by heminested PCR in four swab samples, including cubicle 3 outside flow-syringe pumps, cubicle 4 outside flow-syringe pumps, television and games console, and parents' room toilet taps (Table 1 and Fig. 2). Swab samples taken postcleaning (18 April) were all negative.

Astrovirus strain characterization. The majority of astroviruses characterized were identical, were typed as HAsV-3 (GenBank accession no. AF117209), and had 93% identity to the prototype strain of this genotype, with the exception of the

swab sample from the parents' room toilet taps, which had 97% identity to BCN1.8 (GenBank accession no. AF348760) and 89% identity to HAsV-1 (GenBank accession no. Z25771) (Fig. 2).

DISCUSSION

A heminested RT-PCR assay was developed to ascertain if environmental contamination of medical and hospital equipment and surfaces was a possible reservoir of astrovirus during an outbreak in the Primary Immunodeficiency Unit (ward 23) at Newcastle General Hospital. Results demonstrate that while single-round PCR is likely sufficiently sensitive for human diagnosis, heminested (or nested) PCR is required to reliably detect astrovirus in environmental specimens.

Patient 3 was admitted to the unit with astrovirus gastroenteritis among other infections. Fecal samples from two patients from cubicles 3 (patient 3) and 4 (patient 1) were shown to contain a HAsV-3 strain (Fig. 2). Six other patients were in the unit during the outbreak, and two of these tested negative for astrovirus by RT-PCR. It was demonstrated using a hemin-

TABLE 2. Patient fecal specimens examined and astrovirus PCR and genotyping data

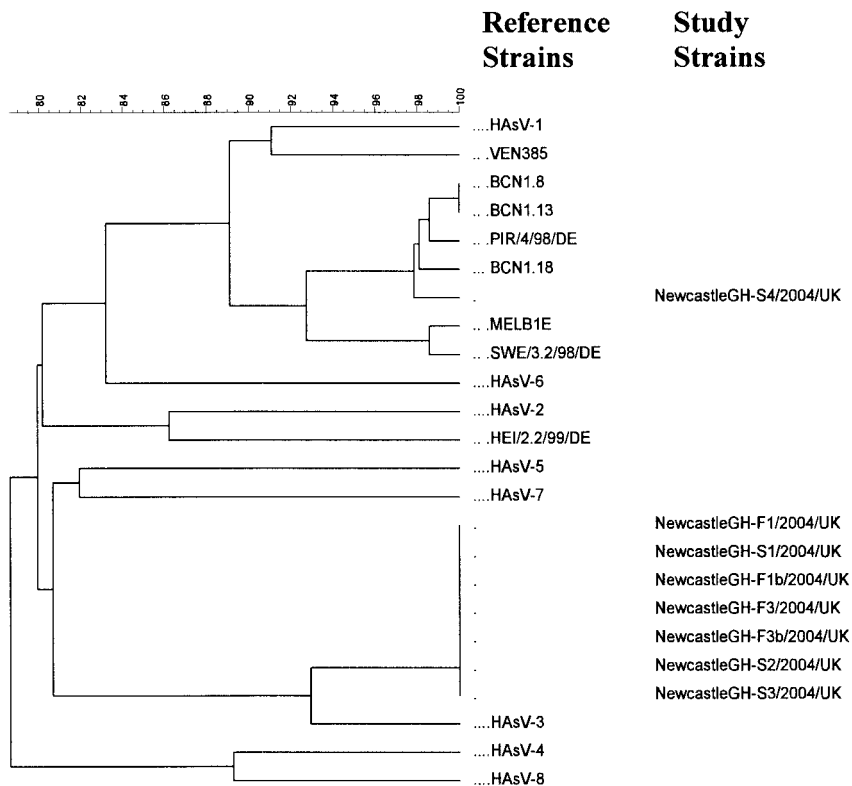| Patient sample | PCR result | | | |
	Single-round PCR	Hemnested PCR	Strain designation	Astrovirus genotype
Patient 1 (03/24/04)	+	+	NewcastleGH-F1/2004/UK	HAsV-3
Patient 2 (03/26/04)	−	−		
Patient 3 (03/25/04)	+	+	NewcastleGH-F3/2004/UK	HAsV-3
Patient 4 (03/25/04)	−	−		
Patient 1b (06/15/04)	+	+	NewcastleGH-F1b/2004/UK	HAsV-3
Patient 3b (06/15/04)	+	+	NewcastleGH-F3b/2004/UK	HAsV-3

FIG. 2. Dendrogram of astrovirus strains in stool specimens and environmental swabs from the outbreak. GenBank strains are HAsV-1 (accession no. Z25771), VEN385 (AF211596), BCN1.8 (AF348760), BCN1.13 (AF348765), PIR/4/98/DE (AY007585), BCN1.18 (AF348770), MELB1E (AF175253), SWE/3.2/98/DE (AY007583), HAsV-6 (Z46658), HAsV-2 (L13745), HEI/2.2/99/DE (AY007584), HAsV-5 (U15136), HAsV-7 (Y08632), HAsV-3 (AF117209), HAsV-4 (Z33883), and HAsV-8 (AF260508). Study strains are as follows: S1 = cubicle 3 outside flow, swab; S2 = cubicle 4 outside flow, swab; S3 = television and games console, swab; S4 = parents toilet taps, swab; F1 and F1b = patient 1, feces; F3 and F3b = patient 3, feces. Sequences from strains detected in this study are available from the corresponding author.

ested PCR assay that three environmental swabs were also positive for this strain. The route of transmission from patient 3 to patient 1 may have been via the transfer of virus from the syringe pumps attached to the wall outside the laminar-flow air curtain of cubicles 3 and 4, since the surfaces of both pumps were found to be contaminated. A breakdown in the infection control procedure prior to the outbreak appears to have allowed the HAsV-3 strain excreted by patient 3 to be transmitted to patient 1 and elsewhere in the unit. It is possible that contact with patient 3, by either a parent or a member of the staff, was responsible for the initial contamination of the syringe pump in patient 3's cubicle (cubicle 3).

Only staff and parents were allowed through the laminar-flow curtain (not siblings); however, parents, other relatives, and siblings were allowed access to the area outside the laminar-flow curtain within the cubicle. Parents of one patient do not enter cubicles in which other patients are treated; however, there is a shared corridor in front of cubicles 3 and 4 (Fig. 1).

Interestingly, HAsV-3 was also found to be contaminating the TV/games console in the parents' room. It was also noted that the parents' room (where the TV/games console was housed) is not frequented by members of the staff (Fig. 1). Therefore, the contamination of the TV/games console was probably through contact by a parent or sibling, suggesting that

the transmission route may have been from patient 3 to the parents' room and back to patient 1.

The unit has strict guidelines and training for hand washing and other procedures, applicable to both staff and parents to limit cross-infection between patients and to prevent nosocomial infections.

It was only feasible to swab a selected number of sites; however, it was thought that the sites chosen were representative of the unit and the focal point of infection, cubicles 3 and 4.

An unrelated HAsV-1 strain was detected on a parents' toilet tap, indicating that another astrovirus strain was also present as an environmental contaminant. HAsV-1 was not detected in samples collected from any of the patients in the unit, and its detection in the environment highlights the problem of continuous introductions into hospital settings by visiting parents and siblings. HAsV-1 strains are more commonly seen in the United Kingdom (9) and elsewhere in Europe (14), and therefore, it was not surprising for this strain to be detected in the environment as well as the outbreak strain.

From this study, it is recommended that in situations in which virus infections causing acute gastroenteritis are life threatening, such as in a bone marrow transplant or immuno-deficiency disorder units, environmental sampling should be

undertaken to determine if enteric viruses are present on surfaces and equipment during a suspected or known viral gastroenteritis outbreak. It is more problematic when asymptomatic excretion of noroviruses (8), astroviruses (16, 24), or other gastroenteric viruses occurs in staff, patients, or relatives. In this situation, prospective environmental monitoring for gastroenteric viruses in high-dependency units may reduce the risk of patients acquiring these infections.

Although virus was detected in environmental swabs, this does not exclude transmission by another route, nor does it determine the viability or infectivity of the virus within the environment. The coincidence of environmental contamination on both syringe pumps and the detection of the outbreak HAsV strain within the environment strongly suggest an association between environmental contamination and transmission.

This highlights the need for regular, thorough cleaning schedules together with strict infection control measures, especially if patients are known to be clinically infected and are likely to be shedding high viral loads. This study has also identified those surfaces that may be associated with transmission, such as door handles, taps, equipment, and other items that are frequently in contact with hands, many of which may be overlooked or difficult to clean, e.g., a TV and games console.

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