High frequency of cultivable human subgroup F adenoviruses in stool samples from a paediatric population admitted to hospital with acute gastroenteritis

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The family Adenoviridae consists of five genera of which the genus Mastadenovirus includes human viruses classified into 57 serotypes clustered into seven subgroups (A–G). Serotypes 40 and 41 (subgroup F) are specifically associated with childhood gastroenteritis and are the most common cause of acute gastroenteritis in young children after rotaviruses and noroviruses. Standard methods for laboratory diagnosis of adenovirus infection include electron microscopy (EM) and conventional cell culture (CCC), although it is widely considered that adenoviruses 40 and 41 are difficult to cultivate, such that their circulation is most likely underestimated. One hundred and ten faecal specimens from paediatric patients with gastroenteritis were confirmed positive for adenovirus by EM and/or CCC at the Virology Unit of the University Hospital of Parma, Italy, during the period January 2010–December 2012. They were analysed to determine the actual prevalence of adenovirus 40 and 41 in these patients using PCR and restriction endonuclease analysis, and to evaluate their ability to be cultivated in standard cell lines. The results showed a high prevalence of subgroup F (62.7 %), with serotype 41 (89.8 %) predominating over serotype 40 (10.2 %). Surprisingly, among the 75 adenoviruses isolated by CCC, 37 (49 %) belonged to subgroup F, suggesting a higher capacity of adenovirus 40 and 41 to replicate in cell culture than previously thought. PCR and restriction enzyme techniques provide an efficient means of diagnosing enteric adenoviruses correctly, including subgroup F adenovirus strains in young children with gastroenteritis.

INTRODUCTION

Gastroenteritis in paediatric patients is a global health problem, causing approximately 2–3 million deaths every year, mainly in developing, but also in developed countries (Elliott, 2007; Chow et al., 2010; Dennehy, 2011). Viruses are the most frequent cause of gastroenteritis, in particular rotaviruses, followed by noroviruses, adenoviruses (AdVs) and astroviruses (Tran et al., 2010; Medici et al., 2012, 2014).

Besides gastroenteritis, human AdVs can cause several different clinical pictures, including respiratory diseases, conjunctivitis, haemorrhagic cystitis and exanthema. They are classified into 57 different serotypes, which are further divided into seven subgroups, A to G (De Jong et al., 1999; Wold & Horwitz, 2007; Jones et al., 2007; Harrach et al., 2011), based on their immunological, biological (e.g. tissue tropism) and genetic characteristics (Jacobs et al., 2004; Zhang & Bergelson, 2005; Jones et al., 2007; Lenman et al., 2011). Among the subgroups with members affecting the gastrointestinal tract (A, G and F), subgroup F, composed of serotypes 40 and 41, is the most diffuse and is responsible for 1–20 % of diarrhoeal episodes worldwide (Li et al., 2004; Shimizu et al., 2007; Verma et al., 2009).

Standard methods for the laboratory diagnosis of AdV infection from stool samples consist of electron microscopy (EM) and virus isolation by conventional cell culture (CCC). However, while the cell lines most frequently used for virus cultivation readily permit the isolation of respiratory tract AdVs, they are not ideal for the cultivation of subgroup F (Dey et al., 2011) and this is of great significance considering the high prevalence and severity of infections caused by these viruses. Furthermore, neither EM nor CCC is able to identify the infecting AdV serotype.
Considering that CCC does not permit the reliable isolation of subgroup F AdVs and that respiratory tract AdVs, which are comparatively very easy to cultivate, are frequently ingested and eliminated with stools, it is highly probable that the actual prevalence of circulating enteric AdVs is underestimated or, at least, misrepresented. On the basis of these facts, this study aimed at implementing a more reliable molecular diagnostic method for AdV 40 and 41, and to investigate the epidemiology of these agents in a representative cohort of paediatric patients with gastroenteritis admitted to the University Hospital of Parma, in the north of Italy. The ability to cultivate these viruses in standard cell culture was also analysed.

METHODS

Clinical specimens. Stool samples (n = 2,325) collected from hospitalized children with acute gastroenteritis between January 2010 and December 2012 were analysed at the Virology Unit of the University Hospital of Parma, Italy, for the presence of viral agents. Faecal samples were processed as previously described (Arcangeletti et al., 2005) and analysed using routine diagnostic methods, including EM, CCC (Intestine 407, Hep-2 cell lines: ATCC CCL-6 and CCL-23, respectively), neutralization tests and real-time PCR (PCR only applied to norovirus RNA detection). Faecal suspensions were stored at −80 °C.

Of these samples, 741 (31.8%) were positive for viral agents, 110 (14.8%) of which were positive for AdVs and were included in this retrospective study. Thirty samples (ten from each year considered), which were negative for AdV by traditional methods and negative for other infectious agents, were also included in the study.

DNA extraction, amplification and restriction enzyme analysis. An aliquot (250 μl) from each 5 ml faecal suspension was used for total DNA extraction using the commercial QIAamp Blood Mini Kit (Qiagen), according to the manufacturer’s instructions. The extracted DNA was subjected to PCR amplification using degenerate primers specific for the hexon gene of AdV: forward: 5′-GCSCARTGKGC-WTACATGCACATC-3′; reverse: 5′-CGGCACCCCGCIGATGTCAAA-3′ (Allard et al., 2001). The 301 bp amplification product was common to the seven AdV subgroups and was used for both RFLP analysis and sequencing. The amplification was carried out in a volume of 25 μl containing 2.5 μl 10× buffer (proprietary formulation containing both KCl and (NH₄)₂SO₄ to provide high specificity of primer annealing, and 20 mM MgCl₂), 0.1 mM deoxynucleotide triphosphates, 2.5 mM MgCl₂, 0.5 mM of each primer, 1.25 U Taq polymerase and H₂O (all reagents were from Thermo Scientific). The reaction tubes were placed in a thermal cycler and were held at 94 °C for 3 min, followed by 40 cycles at 94 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min, and by an extension time of 5 min at 72 °C.

The amplicons were digested using specific restriction enzymes: TaqI from Thermus aquaticus and Hinfl from Haemophilus influenzae. These enzymes allowed us to identify and discriminate between ‘non-F’ (not digested by TaqI) and F (digested by TaqI) AdV subgroups, and between AdV 40 (not digested by Hinfl) and 41 (digested by Hinfl). All digestion reactions were executed in 30 μl reaction mixtures with 10 U of enzyme, 3 μl 10× reaction buffer and 10 μl PCR amplification product (all reagents were from Thermo Scientific). Incubations were carried out for 16 h at 37 °C for Hinfl and at 65 °C for TaqI. The digestion products were loaded onto 2% agarose gels containing ethidium bromide (0.01 mg ml⁻¹) along with a 1 kbp molecular mass ladder (Life Technologies Euro). TaqI cleavages generate two bands of 191 bp and 110 bp, while Hinfl cleavages generate two bands of 120 bp and 80 bp.

In order to confirm the results, two other primer pairs specific for AdV 40 (forward: 5′-ACCAGATGAACCAGACA-3′; reverse: 5′-ACTTTGTAAGAGGTAAGGCGTTCC-3′) and 41 (forward: 5′-TGCCACCCTCGATGA-3′; reverse: 5′-TTTAGAGCGGCCAGGAGTTATA-3′) were used as already described (Samarbaf-Zadeh et al., 2010). The reaction products were analysed on 1% agarose gels containing ethidium bromide (0.01 mg ml⁻¹) along with a 1 kbp molecular mass ladder (Life Technologies Euro). All products were visualized using a Gel Doc XR instrument (Bio-Rad Laboratories).

DNA purification and sequencing. The 301 bp amplification products derived from AdV hexon genes were loaded onto a 1% agarose gel and purified using a QIAquick Gel Extraction kit (Qiagen) following the manufacturer’s protocol. DNA sequencing was performed using the dyeoxy chain-termination method on an automated sequencer (CEQ 2000XL DNA Analysis System; Beckman Coulter). Sequence data were aligned using Seqman, Version II (DNASTAR).

RESULTS

Restriction endonuclease analysis

The 110 stool specimens (from children with acute gastroenteritis) used in this study had all been previously identified as positive for AdVs using traditional diagnostic methods (EM and CCC); specifically, 31.8% were found to be positive by EM, 31.8% by CCC and 36.4% by both methods. The faecal suspensions were stored at −80 °C and used for total DNA extraction. To determine the specificity of the degenerate primers designed to amplify a fragment of the hexon encoding gene, preliminary testing of the DNA extracted from other viruses including human cytomegalo-virus (Towne strain, ATCC VR-977) and human herpes simplex virus type 1 (C35 strain, ATCC VR-260) was performed with the PCR assay devised. No amplicons generating a specific 301 bp band were detected, confirming the primers’ specificity (data not shown). The same primers were also used to run PCR on 30 samples (ten from each year considered) derived from as many paediatric patients with gastroenteritis; all samples were negative for AdV by both EM and CCC (and were also negative for other infectious agents). Once again, no AdV-specific amplicons were detected (data not shown).

Fig. 1. PCR amplification of DNA from 14 samples positive for AdV by traditional methods (EM and/or CCC). DNA was extracted and amplified using primers specific for a region of the hexon gene common to all the seven subgroups (A–G). The PCR products were revealed on a 1% agarose gel, which showed the characteristic 301 bp band.
These primers were then used with the AdV-positive samples, generating a specific 301 bp amplicon for all specimens (Fig. 1). As outlined in Methods, the use of specific restriction endonucleases (Allard et al., 2001; Filho et al., 2007) allowed us to identify the enteric subgroup F (TaqI), as well as the AdV 40 and 41 serotypes (Hinfl) belonging to this subgroup. The digestion steps are depicted in the flow chart in Fig. 2. The band pattern resulting from the endonuclease digestion of the 301 bp amplicon derived from 14 representative samples which were positive for AdV are shown in Fig. 3.

Fig. 2. Flow chart of the restriction endonuclease analysis used for PCR product typing. Only the 301 bp amplicons derived from subgroup F AdVs could be cleaved by TaqI (‘Digested AdV F’); if these amplicons confirmed as subgroup F AdVs belonged to serotype 41, they were also cleaved by Hinfl (‘Digested AdV 41’).

The results arising from this retrospective cohort of 110 specimens revealed that 41 (37.3%) AdVs belonged to a ‘non-F’ subgroup, while the remaining 69 (62.7%) belonged to the F subgroup (Table 1).

Among the ‘non-F’ subgroup specimens, 15/41 (36.5%) were most likely respiratory AdVs ingested and eliminated through the faeces (pharyngeal swab samples from the same patients had also been identified as positive for AdV). No pharyngeal swab data were available for the remaining 26 specimens. The ‘non-F’ specimens were not further investigated in the present study which was focussed on the 40 and 41 subtypes – the most common enteric AdVs causing acute gastroenteritis in young children.

Among the 69 specimens identified as belonging to the F subgroup, there was a significantly higher prevalence of AdV 41 (62/69, corresponding to 89.8%) compared with that of AdV 40, which was only found in seven samples (10.2%).

These results were also confirmed using two sets of specific primer pairs for AdV 40 and AdV 41, as described in the Methods section (data not shown).

Among the 75 samples which were positive for AdV by CCC, 37 (49.3%) belonged to the F subgroup (Table 2); this result was surprising considering that F subgroup AdVs have been repeatedly reported as being difficult to cultivate (Kim et al., 2010; Dey et al., 2011).

All the 110 specimens positive for AdV had also been tested for the presence of other viral agents at the time of sampling: six (5.4%) were positive for rotavirus and another six (5.4%) were positive for norovirus. Furthermore, 97 of the

Fig. 3. Restriction endonuclease digestion profiles of PCR products from 14 representative samples positive for AdV. (a) 301 bp amplicons digested with TaqI allowed us to discriminate between AdV F (lanes 1, 2, 4, 6, 7, 8, 11, 14, digested amplicons) and AdV ‘non-F’ (lanes 3, 5, 9, 10, 12, 13, amplicons not digested). (b) 301 bp amplicons (the same as those shown in (a)) digested with Hinfl allowed us to discriminate between AdV 41 (lanes 1, 2, 6, 7, 8, 11, 14, digested amplicons) and AdV 40 (lane 4, amplicons not digested). The PCR products that were not digested by either TaqI or Hinfl [(a) and (b): lanes 3, 5, 9, 10, 12, 13] belong to ‘non-F’ AdV subgroups. The digestion products were revealed on a 2% agarose gel. Digestion product lengths are shown to the right of the panels.
of this procedure and the variability of AdV concentration in stool samples. Moreover, as discussed above, the fact that respiratory tract AdVs are very easy to cultivate and are frequently ingested and eliminated with stools may have led to incorrect diagnoses and conclusions on the prevalence of circulating enteric AdVs. Indeed, of the 2325 stool samples collected and analysed by routine diagnostic methods during a 3-year period (January 2010–December 2012) at the Virology Unit of the University Hospital of Parma, Italy, 110 tested positive for AdV, but no information was obtained about the infecting serotype such that the true prevalence of serotypes 40 and 41 might be established.

The principal aim of the present study was to develop a more reliable molecular diagnostic method for AdV 40 and 41 identification, and to investigate the epidemiology of these agents and their actual circulation in a representative cohort of paediatric patients with gastroenteritis admitted to the University Hospital of Parma, Italy.

The data obtained using endonuclease and sequence analyses revealed that 69 out of 110 AdV-positive samples belonged to the F subgroup, and that among these, AdV 41 strains (89.8%) were more prevalent than AdV 40 (10.2%). It is interesting to note that the opposite trend was shown during the 1980s, with a higher frequency of AdV 40 with respect to AdV 41, but this tendency gradually changed throughout the 1990s, with a gradual increase in the incidence of AdV 41 versus AdV 40 being described (de Jong et al., 1993; Grimwood et al., 1995). Thus, the distribution of AdV 40 and 41 found in this study was in accordance with this trend. It is also supported by similar results obtained in other recent studies (Fukuda et al., 2006; Samarbaf-Zadeh et al., 2010; Sibanda & Okoh, 2012), confirming this significant change in enteric AdV circulation.

Another surprising result was the high rate of ability to be cultivated for the F subgroup AdVs, which have previously been reported as barely cultivable using CCC techniques (Ko et al., 2003; Kim et al., 2010; Dey et al., 2011). In fact, of the 69 F subgroup AdVs detected, 37 (53.6%) could be cultivated using standard cell lines (such as Intestine 407); four of these were identified as AdV 40, and 33 as AdV 41 using PCR and restriction endonuclease analyses. Other studies report that human embryonic kidney cells (HEK293) transfected with fragments of AdV 5 DNA are permissive to replication of viruses belonging to the AdV F subgroup (Graham et al., 1977; Louis et al., 1997). The transforming region contains the early 1 sequence (E1) of human AdV 5 DNA, consisting of two transcription units, E1a and E1b, whose expression may render these cells permissive to enteric AdV replication, possibly defective in E1 function. Indeed, although the exact cause of this difficulty to grow in CCC is still unknown (Zou et al., 2011), it has been postulated that the E1 gene of AdV 40 and 41 is deficient in its ability to transactivate its own genes (Tiemessen & Kidd, 1994; Kim et al., 2010), and this could explain why transformed HEK293 cells are permissive to F subgroup infection. Other studies report that only E1b

### Table 1. Distribution of F and ‘non-F’ AdV in the studied population

<table>
<thead>
<tr>
<th></th>
<th>F AdV</th>
<th>‘Non-F’ AdV</th>
</tr>
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<tbody>
<tr>
<td>40</td>
<td>41</td>
<td>41</td>
</tr>
<tr>
<td>7</td>
<td>62</td>
<td>41</td>
</tr>
<tr>
<td>Total</td>
<td>69 (62.7%)</td>
<td>41 (37.3%)</td>
</tr>
</tbody>
</table>

110 samples had also been subjected to bacteriological analysis, and six (5.4%) had given positive results (one *Staphylococcus aureus*, three *Campylobacter*, one *Salmonella typhimurium* and one *Clostridium difficile*). Ten of the 110 samples had been subjected to parasitological analysis and three to mycological analysis, all of which had given negative results. These results are summarized in Table 3. These enteric pathogens were more frequently associated with ‘non-F’ AdVs (12/18) than with AdV40 (1/18) or AdV41 (5/18).

### Sequence analysis of amplicons

The 301 bp amplicons were also subjected to DNA sequence analysis (Fig. 4) as further proof of the presence of the AdV 40 and 41 serotypes.

The results confirmed those obtained by restriction endonuclease analysis. In particular, the sequence derived from ‘non-F’ AdVs did not present the *TaqI* restriction site (highlighted in red), which was present, on the other hand, in AdVs belonging to the F subgroup. For the latter, the sequence analysis revealed the presence of the *HinFI* restriction site (highlighted in green) in AdV 41, which was absent in AdV 40.

### DISCUSSION

AdV is considered to be one of the main viral agents causing gastroenteritis in young children, together with rotavirus, norovirus and astrovirus (Tran et al., 2010; Medici et al., 2012, 2014). Of the enteric AdVs, serotypes 40 and 41 (belonging to the F subgroup) are the most common (Li et al., 2012, 2014). Of the enteric AdVs, serotypes 40 and 41 found in this study was in accordance with this trend.

### Table 2. Isolation rate of F and ‘non-F’ AdV by CCC

<table>
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<tr>
<th></th>
<th>F AdV</th>
<th>‘Non-F’ AdV</th>
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<tbody>
<tr>
<td>40</td>
<td>41</td>
<td>33</td>
</tr>
<tr>
<td>Total</td>
<td>37 (49.3%)</td>
<td>38 (50.7%)</td>
</tr>
</tbody>
</table>

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seems to be involved in growth defectiveness in AdV 40 (Mautner et al., 1999; Mautner, 2007), and that some defects may exist in its sensitivity to type 1 interferon (Sherwood et al., 2007). With regard to AdV 41, it has been postulated that defects in the virion assembly may also occur (Pieniazek et al., 1990). Furthermore, the poor infectivity of AdV 40 and 41 in standard cell cultures in vitro might reflect the need for viral capsids to undergo modification by gastric and intestinal fluids in order to generate infectious particles (Mangroo & Brown, 2013).

Studies addressing the difficulty to cultivate other viral agents, such as calicivirus and birnavirus, have hypothesized that the onset of novel genetic mutations in particular regions (such as those occurring in genes encoding binding proteins) could enhance the ability of these agents to be cultivated, or change their tissue tropism (Yamaguchi et al., 1996; White et al., 1996; Guo et al., 1999; Lim et al., 1999).

In conclusion, the combination of PCR and RFLP represents a more reliable and rapid diagnostic method for AdV typing.

### Table 3. Distribution of infective agents detected in the studied population

<table>
<thead>
<tr>
<th>Infective agents</th>
<th>Viruses</th>
<th>Bacteria</th>
<th>Parasites</th>
<th>Mycetes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of agents identified</td>
<td>RV</td>
<td>NoV</td>
<td>AdV</td>
<td>S. aureus</td>
</tr>
<tr>
<td>Number of tested samples</td>
<td>6 (5.4 %)</td>
<td>6 (5.4 %)</td>
<td>110 (100 %)</td>
<td>1 (0.9 %)</td>
</tr>
</tbody>
</table>

RV, rotavirus; NoV, norovirus; AdV, adenovirus; S. aureus, Staphylococcus aureus; S. typhimurium, Salmonella typhimurium; C. difficile, Clostridium difficile.

![Fig. 4. Sequence electropherograms of the relevant portion of the 301 bp amplicons. The parts of the hexon sequence containing the TaqI (in red) and HinfI (in green) restriction sites are highlighted. TaqI restriction site 5’...TCGA...3’, shown in red, is only present in AdVs belonging to subgroup F (b, c), while it is absent in ‘non-F’ AdV subtypes (a). The HinfI restriction site 5’...GANTC...3’ (where ‘N’ can be any nucleotide), shown in green, is present in AdV 41 (c), but is absent in ‘non-F’ AdV subtypes and AdV 40 (a and b, respectively).](Image)
considering that traditional techniques (EM and CCC) do not allow for AdV identification at the serotype level and that this is traditionally carried out by means of long and cumbersome methods (i.e. neutralization or haemagglutination inhibition tests). Furthermore, we show here, we believe for the first time, a more accurate picture of the distribution of AdV 40 and 41 serotypes occurring in Italian children admitted with acute gastroenteritis to the Maternal–Infantile Department of the University Hospital of Parma, one of the main hospitals in northern Italy, not yet considered for this aspect in the recent literature.

Moreover, the presence of a high rate of ability to be cultivated, in particular for the AdV 41 serotype, which has recently replaced serotype 40 as the most widespread AdV in paediatric gastroenteritis, could reflect an increased ability of this viral serotype to infect enteric cells in vivo, most likely due to specific genetic mutations or to post-transcriptional or post-translational modifications counteracting the possible defects originally present in these viruses, such as those mentioned above.

Work is in progress in our laboratory to shed light on the possible mechanisms underlying this unexpected easy cultivation of more than half of the F subgroup AdVs analysed in CCCs, with the aim of identifying the specific viral determinants that could account for this observation, which may also be related to an increased virulence in vivo.

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REFERENCES


