Application of a Molecular Panel To Demonstrate Enterotropic Virus Shedding by Healthy and Human Immunodeficiency Virus-Infected Patients

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We used a molecular panel, targeting seven enteric viruses, to explore the advantage of using molecular methods to establish the etiology of enteric diseases and to evaluate the prevalence of enteric viruses in asymptomatic human immunodeficiency virus-infected patients. This approach favors rapidity and sensitivity of laboratory diagnosis of viral enteric syndromes.

Enteroviruses, adenoviruses, rotaviruses, astroviruses, and noroviruses are major etiological agents of both sporadic and epidemic gastroenteric syndromes, particularly in children and immunosuppressed patients (4, 5, 8, 10, 13, 18, 19). Many of these agents remained often unidentified in the past for their inability to grow in cell culture.

Gastrointestinal disorders are among the most common debilitating conditions affecting human immunodeficiency virus (HIV)-infected people, ranging from 50% to almost 100% of AIDS reports. In this respect, in these patients emerging viral enteric pathogens (i.e., picobirnaviruses, astroviruses, and caliciviruses) are more frequently associated with diarrhea than either “classic viruses” or bacterial and parasitic enteropathogens (9, 19). However, causal association between unusual viral pathogens and gastroenteric syndromes in HIV-infected patients is controversial (9, 14). Furthermore, prolonged viral excretion in the absence of gastroenteric symptoms has been reported in immunodeficient patients, including HIV infected (3, 6, 15).

The objective of the study was to explore the inclusion of molecular test panels in routine diagnostic virology, to improve the rapidity and sensitivity of laboratory diagnosis of viral enteric syndromes, since classical viral culture methods are scarcely sensitive, take a long time and are cumbersome to perform, and could not detect noncultivable viruses.

To this aim, a molecular biology test panel detecting seven enteric viruses (adenoviruses, astroviruses, enteroviruses, noroviruses, rotaviruses, hepatitis A virus [HAV], and hepatitis E virus [HEV]), was used in parallel with virus isolation. In addition, the same panel was used to support the concept that asymptomatic shedding of enteric viruses is common among HIV-infected patients.

Firstly, retrospective evaluation was performed on 103 fecal

specimens from patients with acute non-epidemic gastroenteritis sent to the laboratory in December 2000 to September 2002.

Secondly, 102 fecal samples were collected between May 2002 to May 2003 (excluding August and December 2003 and January 2004) from consecutive patients attending the National Institute of Infectious Diseases “L. Spallanzani” (Rome, Italy). Among these, 62 samples come from adult HIV-positive patients (12 with diarrhea, 50 without symptoms), 40 from HIV-negative individuals 25 with symptoms and 15 without, of whom 31 were infants, 20 with and 11 without diarrhea.

Fecal specimen suspensions (20% [wt/vol] in phosphate-buffered saline) were clarified, filtered, and extracted by the Boom method, with Nuclisens reagents (Biomerieux). cDNA was synthesized with random primers and Moloney murine leukemia virus reverse transcriptase (Invitrogen). Amplification was done with the GeneAmp DNA amplification reagent kit from Applied Biosystems.

Primers and conditions for PCR (astrovirus, calicivirus, and rotavirus) and nested PCR (enterovirus, HEV, and adenoviruses) were as previously described (1, 2, 7, 11, 12, 16, 17). The following external primers were used for HAV nested PCR: EPA1, 5'-TTTGTCTTTTAGTTGTTATTGTCTGT-3' (sense); and EPA2, 5'-AGTCACACCTCTCCAGGAAACTT-3' (antisense). The following internal primers were used: EPA3, 5'-TATT TGTCTGTACAGAACAATCAG-3' (sense); EPA4, 5'-CA TTATTTTCATGCTCCTCAG-3' (antisense) (M. Divizia, personal communication).

Positive and negative controls were included in each PCR and reverse transcription-PCR (RT-PCR) assay. Sensitivity of the PCRs was established using cloned target sequences in the Probit analysis. All reactions detected ≤ 50 copies of target nucleic acid. Specificity was verified by cross-testing each PCR against the others in the panel, and with herpes simplex virus type 1/2, human papillomavirus, and cytomegalovirus. No cross-amplification was observed. The presence of inhibitors from the sample matrix was ruled out by adding unrelated control DNA (β -globin DNA) and RNA (MS2 phage genome)

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TABLE 1. Virus detection by a seven-virus-based molecular panel and by virus isolation in feces from patients according to their HIV status, age class, and presence of symptoms

Age class and HIV status (n)	No./total (%) [no. of each type]			
	Symptomatic		Asymptomatic	
	PCR	Virus isolation	PCR	Virus isolation
Adults (71)				
HIV positive (62)	2/12 (16.7) [2 adenovirus]	0/12 (0)	4/50 (8.0) [2 adenovirus, 1 enterovirus, 1 HAV]	0/50 (0)
HIV negative (9)	1/5 (20.0) [1 norovirus]	0/5 (0)	0/4 (0)	0/4 (0)
Children (31)				
HIV negative (31)	8/20 (40.0) ^a [3 adenovirus, 1 rotavirus, 2 norovirus, 1 rotavirus + sapovirus, 1 adenovirus + enterovirus] ^c	2/20 (10.0) [1 adenovirus, 1 enterovirus]	2/11 (18.2) ^b [1 adenovirus, 1 enterovirus]	2/11 (18.2) [1 adenovirus, 1 enterovirus]
Total (102)	11/37 (29.7)	2/37 (5.4)	6/65 (9.2)	2/65 (2.9)

^a Children versus adults, symptomatic shedding, $P = 0.262$ in chi-square test.

^b Children versus adults, asymptomatic shedding, $P = 0.580$ in chi-square test.

^c In concomitance with *Shigella dysenteriae*.

to all samples before the extraction and amplifying the target control sequences. None of the samples showed inhibition. Spiking experiments were also performed on negative samples.

Molecular characterization was performed by direct sequencing of PCR amplicons, using the BigDye Terminator method and ABI Prism 3100 instrument (Applied Biosystems). Virus type was assigned with the aid of the GenBank sequence database.

For virus isolation, filtered specimen suspensions were inoculated onto a panel of cell lines (Hep2, Vero, Buffalo green monkey, A549, and diploid fibroblast). Cultures were incubated at 37°C for 15 days and screened daily for the presence of cytopathic effect. Isolates were identified molecularly and/or by seroneutralization (enteroviruses).

Results, based only on PCR, from the retrospective collection of 103 fecal specimens, indicated that 37 (35.9%) contained one or more viruses: 10 (9.7%) were positive to adenoviruses, 10 (9.7%) to enteroviruses, 12 (11.6%) to rotaviruses, 4 (3.9%) to astroviruses, and 5 (4.8%) to caliciviruses. Two were HAV positive (1.9%) and none was HEV positive. Multiple viral genomes were detected in five specimens (4.8%): two enterovirus plus rotavirus, one rotavirus plus adenovirus, one rotavirus plus astrovirus, one rotavirus plus adenovirus plus enterovirus.

For the 102 prospectively collected samples, molecular detection and characterization were performed in parallel with classical virus isolation. Seventeen (18.6%) were positive to 1 or more viruses by PCR or RT-PCR: 8 (7.8%) contained adenoviruses (1 type 1, 1 type 3, 2 type 41, 1 type 48, and 2 undetermined); 3 (2.9%) caliciviruses (all noroviruses), and 2 (1.9%) enteroviruses (1 ECHO 30 and 1 coxsackievirus, A22/19). Rotaviruses and HAV were each found in 1 case (0.9%); HEV or astroviruses were not found. Multiple viral genomes were detected in 2 cases (1.9%), involving 1 adenovirus (type 1) plus enterovirus (ECHO 1) and one rotavirus (A) plus calicivirus (sapovirus), in HIV-negative children with severe diarrhea. Caliciviruses and rotaviruses were exclusively associated with symptomatic cases, while adenoviruses and enteroviruses were found also in asymptomatic cases (Table 1). Virus

isolation was significantly less sensitive than molecular detection, showing only 4 positive samples (3.9%, $P = 0.006$ as compared to PCR). The isolated viruses were adenovirus type 3, adenovirus type 1, ECHO 30 and ECHO 1, consistent with PCR results.

The use of a molecular panel, based on previously published RT-PCR and PCR methods, is here described for the first time. This panel appears useful for detecting a wide group of viruses in the gastrointestinal tract, including also noncultivable viruses. Similar to the other viral syndromes (20), our initial results showed that molecular probes were more sensitive than culture techniques in detecting the presence of viruses usually associated with enteric diseases. In addition, our results on asymptomatic shedding, although restricted to a small group of HIV-infected patients, confirm that enteric virus excretion without specific symptoms is not rare. Further studies, including a larger number of subjects (both HIV infected and HIV negative), would be necessary to determine the clinical usefulness of this diagnostic approach in clinical practice.

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