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Development and Evaluation of a New Commercial Test Allowing the Simultaneous Detection of Noroviruses and Sapoviruses by Reverse Transcription-PCR and Microplate Hybridization

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This work describes the design and initial evaluation of a commercial test allowing the detection of noroviruses and sapoviruses by reverse transcription-PCR (RT-PCR) in a single tube followed by microplate hybridization, as well as the detection of PCR inhibitors. The test was shown to be broadly reactive (except for Melksham-like strains), sensitive, and specific and thus should be useful for calicivirus detection in clinical practice.

Human caliciviruses (HuCVs) are important pathogens causing sporadic cases, winter epidemics, and outbreaks of acute gastroenteritis in children and adults worldwide (4, 5, 11). Two HuCV genera have been described, *Norovirus* and *Sapovirus* (previously called Norwalk-like and Sapporo-like viruses, respectively) (12), which are further divided into at least two distinct genogroups. In addition, for noroviruses, the genogroups are further subdivided into genetic types, and although there is no consensus for such a subclassification, seven genogroup I (GGI) and eight genogroup II (GGII) types have been proposed (7). Until now, detection of HuCVs remained difficult in clinical laboratories because of the lack of a broadly reactive test. The recent development of a commercial enzyme-linked immunosorbent assay (15) for detecting norovirus antigen in feces should improve the surveillance of outbreaks caused by these viruses, but enzyme-linked immunosorbent assay-negative organism outbreaks should be confirmed by reverse transcription-PCR (RT-PCR), which remains the most sensitive method (2). The RNA polymerase gene is the main target, and many primers have been designed, especially for noroviruses (2, 8). However, the genetic variability of HuCVs has made it difficult to detect a great variety of strains from both genera in a single test, and to our knowledge, few methods allowing this have been reported (9, 14) and no commercial test has been developed. This work describes the design and initial evaluation of a commercial test (Calicivirus Consensus; Argene-Biosoft, Varilhes, France) allowing the broad detection of both noroviruses and sapoviruses by RT-PCR in a single tube followed by the confirmation of PCR products by microplate hybridization as well as the detection of PCR inhibitors.

Alignment of a region (positions 4201 to 4620) within the Lordsdale virus sequence (X86557), located in the RNA polymerase gene, from 28 HuCVs (25 noroviruses [GGI and GGII] and three sapoviruses) allowed us to select a primer set targeting the YGDD motif (3′ end) and giving a 320-bp fragment and probes for hybridization. Sequence information related to the primers and probes is considered proprietary.

A panel of 35 HuCV strains previously amplified with one or more of the primer pairs ("classical method") NVp110 (10)-NVp36 (17), NVp110–SR48-50-52 (1), NVp110-NI (6), and JV12-JV13 (16) for noroviruses or NVp110-SR80 (13) for sapoviruses was tested. These strains had been characterized by sequencing of the amplified region in the polymerase gene and, for 12 of them, of a region in the capsid. The panel included four sapoviruses and 31 noroviruses including some nonclassified strains. In addition, 93 stool samples from patients in 11 outbreaks (December 2001 to November 2002) were analyzed by both the Argene and the classical methods.

To verify the specificity, 54 stool samples which were negative for HuCVs by use of the primer pairs described above were assayed; 19 of them contained another enteric virus (group A rotavirus, 12; adenovirus 40/41, 2; astrovirus, 2; enterovirus, 2; and hepatitis A virus, 1).

All reagents except the HotstarTaq DNA polymerase were included in the Calicivirus Consensus kit (Argene-Biosoft). Viral RNA was extracted from 140 μl of 20% stool suspensions in phosphate-buffered saline with the QIA Amp viral RNA kit (Qiagen, Hilden, Germany) included in the Argene kit according to the manufacturer's instructions. Samples were analyzed through a one-step RT-PCR. Ten microliters of RNA was denatured in 37 μl of RT-PCR premix containing 1.5 U of HotstarTaq DNA polymerase for 10 min at 60°C. Omniscript reverse transcriptase (10 U) was added, and the RT-PCR program was performed for 45 min at 50°C, directly followed by the following amplification program in a Perkin-Elmer 2400 thermocycler: 15 min at 94°C for *Taq* activation followed by 5 cycles of 30 s at 94°C, 1 min at 59°C, and 1 min at 72°C; 15 cycles of 30 s at 94°C, 1 min at 47°C, and 1 min at 72°C; 20 cycles of 30 s at 94°C, 1 min at 54°C, and 1 min at 72°C; and a final 2-min extension at 72°C. The increase from annealing to elongation temperature was slowed to 3 s/°C. Amplified products were stored at −20°C until detection.

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Each sample was also coamplified in a second tube in a PCR mixture containing an inhibition control to display inhibitory agents present in the sample, if any. This standard is a composite plasmid containing sequences which are recognized by the primers flanking a plasmidic sequence different from the viral sequences but with similar thermodynamics. The PCR mixture and amplification program were the same for amplification and inhibition control. Thus, this standard serves as both an inhibition and a positive control.

Five microliters of amplified products was analyzed by microplate hybridization with the Hybridowell kit (Argene Biosoft) (3; C. Barranger, S. Ott, M. Joannes, and G. Somme, abstr. from the 10th Papillomavirus Workshop, 1991). The amplified product was chemically denatured and incubated in a microwell plate for 1 h at 37°C. Hybridization with a mixture of biotinylated probes specific for either noroviruses or sapoviruses (generic probe) or a control probe for inhibition controls was performed for 30 min at 37°C. After washing, streptavidin peroxidase conjugate was added for 15 min at room temperature and the microplate was again washed five times. Hydrogen peroxide associated with tetramethyl benzidine as a chromogen was used as a substrate for 30 min at room temperature in the dark. The optical density (OD) reading at 450 versus 620 nm gave the final result. Cutoff (CO) calculation was performed using the equation CO = OD (blank) + 0.075. CO calculation was performed using the equation CO = OD (blank) + 0.075. This value (0.075) corresponds to three times the mean of negative values, as determined by the manufacturer on negative stool samples. Samples giving an OD of the CO value ± 10% must be tested again, with another extraction. Samples are then determined as positive on the second run when the OD is >CO.

Detection of previously characterized norovirus and sapovirus strains. A panel of 35 strains representative of most genetic HuCV groups (four sapovirus and 31 norovirus strains) was then tested (Table 1). All the sapovirus strains tested were amplified and hybridized, as well as all the norovirus strains tested, which were related to the Norwalk, Southampton, Chiba, and Hesse strains for GGI and to the Hawaii, Toronto, Bristol, Leeds, and Amsterdam strains for GGII in the polymerase gene. Strains clustering in a new genetic variant designated GGIIb (H. Vennema, E. Kohli, D. Brown, E. Schreier, C. H. von Borsdorff, L. Svensson, F. Hanon, and M. Koopmans, Abstr. XIIth Int. Congr. Virol., abstr. 93-V624, p. 212, 2002) were detected, as well as one Alphatron-like strain (which may define a third genogroup) and nonassigned strains. Four strains could not be detected by the Argene test: two Melksham-like strains (L164 and SH8) were amplified but not hybridized and two strains could not be detected at all, one Desert Shield strain (E80) and one Melksham strain (S53). Thus, the Argene test is broadly reactive and sensitive since it allowed detection of 91.5% of the strains tested representing all the sapoviruses and GGI and GGII noroviruses related to 10 out of 11 different genotype reference strains. In addition, all the nonclassified and nonassigned strains tested were also detected, suggesting that new circulating strains may be detected using this test. Concerning the two Melksham-like strains (GGII) which were amplified but not hybridized, the addition to the probe mixture of an additional specific probe should improve the detection of such strains.

TABLE 1. Detection of 35 previously characterized sapoviruses and noroviruses by the Argene assay

Virus (*n*)	Genotype reference strain[a]	Sample	Argene assay result
Sapovirus (4)	Sapporo	S52	+
		S94	+
	London	S14	+
		L43	+
Norovirus GGI, (9)	Norwalk/1968/US	L198	+
	Southampton/1991/UK	E59	+
		S112	+
		S127	+
	Desert Shield/395/1990/SA	L18	+
		L152	+
		E80	−
	Chiba 407/1987/JP	E150	+
	Hesse	E394	+
Norovirus GGII (14)	Hawaii/1971/US	E38	+
	Melksham/1994/UK	SH8	−[b]
		L164	−[b]
		S53	−
	Toronto 24/1991/CA	E10	+
		GEA82	+
		E25	+
		L28	+
	Bristol/1993/UK	GEA152	+
		L207	+
		L161	+
		L206	+
	Leeds	S73	+
	Amsterdam/1998/NL	S26	+
Other nonclassified norovirus strains (8)	New genetic variant GGIIb	E78	+
		L110	+
		E132	+
	Alphatron/1998/NL	L248	+
	Nonassigned strains	S55	+
		S63	+
		L23	+
		E3	+

[a] Genotype reference strain showing ≥90% identity in the analyzed region (polymerase gene) except for Desert Shield-like strains, which showed 78 to 80% identity but 90% in the capsid gene.

[b] Strains SH8 and L164 were amplified but not hybridized.

Detection of HuCVs in patients involved in acute gastroenteritis outbreaks. Ninety-three stool specimens from patients in 11 outbreaks that occurred from December 2001 to November 2002 were systematically tested with the classical method used in the laboratory and the Argene assay (Table 2). Fifty samples (53.8%) were found positive by both methods whereas 21 (22.5%) gave discordant results, 2 samples being positive only with the classical method and 19 samples being positive only with the Argene assay. None of the stool samples tested gave an indeterminate result. Among the 19 samples which were positive only with the Argene test, 5 were found positive after RNA reextraction because of the detection of PCR inhibitors by use of the inhibition control. Indeed, among the 93 samples, 10 were found to be inhibited. After RNA reextraction, two samples remained inhibited, three were still negative, and five were positive. This result illustrates the relevance of the inhibition control. In fact, stool samples contain a lot of inhibitors such as polysaccharides and salts which may inhibit PCRs. Their detection allows us to repeat amplification after RNA reextraction and/or dilution and finally to confirm

TABLE 2. Viral investigation of 11 gastroenteritis outbreaks, comparing the method used in our laboratory with the Argene assay

Outbreak	Setting(s)	No. of samples	No. (%) positive		Genetic identity
			Classical method[a]	Argene assay	
La-12-01	Nursing home	7	5 (71)	7 (100)	Bristol
Lo-12-01	Geriatric hospital	4	3 (75)	4 (100)	Bristol
Au-12-01	Nursing home and geriatric hospital	14	9 (64)	10 (71)	Bristol
Se-04-02	Infant care group	22	8 (36)	13 (59)	Bristol and new variant GGIIb
Re-04-02	Nursing home	7	5 (71)	7 (100)	New variant GGIIb
Fo-04-02	Nursing home and geriatric hospital	9	6 (67)	5 (56)	Bristol
Ar-07-02	Recreation center	7	6 (86)	6 (86)	Desert Shield
Es-09-02	Primary school	4	3 (75)	3 (75)	Hesse
Yv-10-02	Geriatric hospital	6	1 (17)	3 (50)	Bristol
Va-11-02	Nursing home	4	3 (75)	4 (100)	Bristol
Is-11-02	Waterborne	9	3 (33)	7 (78)	Desert Shield and Bristol
Total		93	52 (56)	69 (74)	

[a] Classical method: primers NI-NVp110, SR48-50-52–NVp110, JV12-JV13, and SR80-NVp110 were used, except for outbreaks Fo-04-02 and Es-09-02, samples from which were analyzed using only JV12-JV13 and SR80-NVp110.

whether the RT-PCR can be validated. Finally, if accidental carryover contamination with the control were introduced, it could not lead to a false-positive result, since the only sequences shared with caliciviruses are the primer sequences. In addition, because the control contains a limited number of copies and is amplified with a large excess of primers, the risk of a competition leading to a false-negative result is limited.

Specificity of the Argene assay. Fifty-four specimens from sporadic case patients included in previous studies, negative for HuCVs with the primers described above, were found negative with the Argene assay; 19 of them contained another enteric virus, indicating that the test showed a high specificity. One must be careful about false-positive reactions by RT-PCR, notably because of the YGDD motif, which is included in most primers because of its high degree of conservation among caliciviruses. However, this motif, especially the GDD motif, is also conserved among other RNA viruses. Thus, the confirmation of amplification by hybridization is necessary. The test described here offers the advantage of minimizing nonspecific amplifications because of the combination of optimized PCR conditions and the use of hybridization.

In conclusion, this work reports the first evaluation of a commercial test allowing the detection of both sapoviruses and noroviruses in stool samples in a single reaction tube as well as the detection of PCR inhibitors. Viral detection is based on amplification by RT-PCR of a region located in the polymerase gene followed by microplate hybridization. This standardized test was found to be broadly reactive, showed a good sensitivity associated with high specificity, and should be useful for calicivirus detection in clinical practice. Even if it already detects 31 strains out of the 35 tested, continuous study and improvement of RT-PCR primers and probes to extend the detection spectra of noroviruses and sapoviruses will be performed. Further characterization of infections such as discrimination of noroviruses and sapoviruses may also be an enhancement of this technique.

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