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Reverse Transcription Multiplex PCR for Differentiation between Polio- and Enteroviruses from Clinical and Environmental Samples

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For the rapid detection of polioviruses and their differentiation from nonpoliovirus enteroviruses, we developed a protocol in which clinical or environmental specimens are first inoculated onto cell cultures in tubes. After overnight incubation, the cultures are subjected to reverse transcription multiplex PCR with a primer pair which detects all enteroviruses (T. Hyypiä, P. Auvinen, and M. Maaronen, J. Gen. Virol. 70:3261–3268, 1989) and two newly designed primer pairs specific for all 36 poliovirus strains tested. The PCR products can unequivocally be identified by their lengths in agarose gels, whereas the genetic heterogeneity of the poliovirus strains precludes identification by back-hybridization with internal probes. The proposed protocol is highly insensitive to the inhibitory effects of substances in the sample (stool, sewage). It allows for the detection of polioviruses and for polioviruses to be distinguished from nonpoliovirus enteroviruses within 24 h, and it allows for the concomitant isolation of a viable strain suitable for further typing.

In the picornavirus family, the genera *Enterovirus*, *Rhinovirus*, and *Hepatitis A virus* are pathogenic for humans. The traditional ultimate goal of diagnostic virology is the identification of the virus serotype that is causing a given infection. With more than 70 serotypes in the enterovirus (EV) group and more than 100 different rhinoviruses, this goal cannot and does not always have to be met for reasons of cost-effectiveness, available personnel, and clinical relevance. In the EV group, a rapid and reliable means of differentiating between poliovirus (PV) and non-PV EV (NPEV) is important, particularly when rapid therapeutic and/or public health measures must be taken. The final identification of the virus serotype may often be less urgent or, especially for NPEV, may be of minor clinical or practical importance. This holds true particularly in a PV surveillance program (28) in which patients with flaccid paralysis or environmental samples must be monitored primarily for the presence of PV. If environmental surveillance programs rely on the analysis of sewage, an additional complication is the abundance of NPEV among which the relatively few PVs must be detected.

There are two different approaches for the detection of EVs in clinical or environmental samples. First, viral RNA can be identified, directly or after PCR amplification, by hybridization techniques. This approach has the drawback that a series of different hybridization probes must be used to detect all EV serotypes and genetic variants (quasispecies) of a given serotype (25, 26, 30; this report). In addition, substances interfering with reverse transcription or with PCR amplification have been described (21, 27), especially when stool or sewage is the sample to be tested. The second approach used for all except certain coxsackievirus type A strains is the isolation and identification of EVs in cell culture. Again, toxic substances and/or a high bacterial load in the sample may interfere with the assay, leading to low levels of virus multiplication and premature culture degeneration. Cells normally used for EV isolation are similarly sensitive for PVs and NPEVs, which may lead to the overgrowth of abundant NPEVs. The use of transgenic L cells carrying the PV receptor (10, 17) was proposed to solve this problem.

In order to circumvent the drawbacks mentioned above, we propose in the present report that the cultivation of EVs in cell culture be combined with a reverse transcription (RT) multiplex PCR with a primer pair designed to detect all EVs (11; for a recent compilation of EV-specific primers, see reference 13) and two primer pairs to simultaneously identify them as PV or NPEV. Since no primer pair has been reported to hybridize to all PV strains (1), we designed, in the P2 genomic region, two new primer pairs which, when used together, compensate for this shortcoming.

The protocol proposed here has a sensitivity of 3 PFU or less. It is rapid in that an overnight incubation of the inoculated cell culture is sufficient to multiply such low input titers of EV to levels detectable by RT-PCR and is relatively insensitive to the adverse effects caused by substances in the sample because the cell cultures need to tolerate suboptimal conditions only for a few hours. Thus, it is possible to detect EVs and to identify them as NPEV or PV within 24 h and, at the same time, to isolate a viable strain suitable for further detailed identification and typing.

MATERIALS AND METHODS

Cells and virus strains. For isolation of EVs from stool, samples were suspended in Eagle's minimal essential medium (MEM-E; approximately 20% by volume); penicillin, streptomycin, and gentamicin were added; and the suspension was clarified by centrifugation at 3,000 × *g* for 20 min. EVs were isolated from cerebrospinal fluid without pretreatment.

Isolation was carried out in RD-6, Vero, and MRC-5 cells. To grow virus stocks, RD-6, HEp-2, BSC-1, and MRC-5 cells were used. The cells were cultivated in tubes as monolayers at a density of 10^5 cells per tube in minimal essential medium (MEM), Joklik modified medium, or MEM-E, all of which were supplemented with newborn or fetal calf serum (Seromed, Berlin, Germany).

To test the specificity and sensitivity of the RT-PCR, a panel of prototype and archival isolates of EV was used, including an EV collection originally obtained

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TABLE 1. EV- and PV-specific primers[a]

Primer region and map position[b]	Sequence[c]	Primer designation	Product length (bp)	Specificity
5′ NTR				
163–178	5′-AAGCACTTCTGTTTCC-3′	E1	297	EV
443–460	5′-CATTCAGGGGCCGGAGGA-3′	E2		EV
P2 region				
4460–4478	5′-CAGTTCAAGAGCAA (A/G)CACC-3′	Po1	193	PV
4634–4653	5′-TC(A/G)TCCAT(A/G)AT(A/C)AC(T/C)AC(T/A)CC-3′	Po2		PV
P2-P3 region:				
4922–4941	5′-GAAATGTGTAAGAACTGTCA-3′	Po3	565	PV
5467–5487	5′-GTAACAATGTTTCTTTTAGCC-3′	Po4		PV

[a] Primers E1 and E2 were described previously (11); primers Po1-Po4 were described in this report.
[b] Map position and sequence of PV type 1 (Sabin) are given. 5′ NTR, 5′-nontranslated region.
[c] For the degenerate primers Po1 and Po2, the sequence of the Sabin strain is underlined.

from M. F. Paccaud, formerly at Institut d'Hygiène, Geneva, Switzerland. All virus strains used in the present study are listed in the Results section.

PV isolates were identified either by the microtechnique (28) with commercial neutralizing antisera obtained from the Institut Pasteur (Paris, France) or by immunofluorescence on paraformaldehyde-fixed, Triton X-100-permeabilized cells with PV type-specific antibodies (Chemicon International, Temecula, Calif.) (unpublished data).

Isolation of EV RNA for PCR. For isolation of RNA from cell culture, an infected culture in a tube was scraped into the medium with a bacteriological plastic loop (Difco, Detroit, Mich.) and was sedimented. If it was not used immediately, the sediment was frozen at −20°C. For RNA isolation, the cell sediment was digested with 500 μl of proteinase K solution (200 μg/ml in 100 mM Tris [pH 8.0] and 0.25% sodium dodecyl sulfate [SDS]) for 30 min at 37°C, diluted to 800 μl, and extracted twice with an equal volume of phenol-chloroform-isoamyl alcohol (PCI) (25:24:1); this was followed by a chloroform-isoamyl alcohol (CI) (24:1) extraction. After extraction, the water phase was equally distributed into three tubes, yeast tRNA was added as a carrier, and RNA was precipitated with ethanol at −70°C. Alternatively, the cell sediment was extracted with 800 μl of TRIzol (Gibco-BRL, Gaithersburg, Md.), and the RNA was precipitated, in three aliquots, according to the protocol of the manufacturer.

For isolation of RNA from stool, samples were made approximately 20% in MEM-E, and either 600 μl was extracted once with phenol, twice with PCI, and once with CI or 150 μl was extracted with TRIzol and then RNA was precipitated as described above.

RT, PCR, and multiplex PCR. One aliquot of the precipitated RNA was washed once with 70% ethanol by centrifugation at 4°C, dissolved in 3 μl of RNase-free distilled water, and added to 17 μl of the RT mixture (GeneAmp RNA PCR kit; Perkin-Elmer, Norwalk, Conn.) containing random hexamer primers. The mixture was incubated for 10 min at room temperature and for 30 min at 42°C. After heating for 5 min at 95°C, the RT mixture was added to 80 μl of PCR mixture (Perkin-Elmer) containing $MgCl_2$ (2 mM), *Taq* polymerase (2.5 U), and appropriate primers (0.15 μM). The primer pairs used in the study are listed in Table 1. After 35 cycles (1 min at 94°C, 2 min at 48°C, and 1 min at 72°C), 10 μl of the PCR mixture was subjected to electrophoresis in 3% agarose (NuSieve; FMC, Rockland, Maine) in TBE buffer (44.5 mM Tris, 44.5 mM boric acid, 1 mM EDTA). The gel contained 0.5 μg of ethidium bromide per ml and was run for 45 min at 80 V. Bands were photographed under UV light.

Sequencing and Southern hybridization of PCR products. Direct sequencing of the PCR products was performed by previously published methods (7).

For Southern blot analysis of the PV-specific amplicons Po1-Po2 and Po3-Po4 (Table 1), 10 μl of the respective PCR-amplified samples was electrophoresed on 1.5% agarose gels; this was followed by transfer to Zeta-Probe blotting membranes (Bio-Rad, Hercules, Calif.) as described previously (24).

The hybridization probe (140 bp) for the Po1-Po2 amplicon was obtained by PCR with the primer pair 5′-GAACCAGTATGTTTGCTAGT-3′ (Sabin type 1 map positions 4484 to 4503) and 5′-TATCCGTCGAAGTGTGATGG-3′ (positions 4604 to 4623) and the plasmid pVR106 (19) as a target. The hybridization probe (487 bp) for the Po3-Po4 amplicon was obtained by using the primer pair 5′-TAGTGTGTGGTAAGGC-3′ (positions 4974 to 4989) and 5′-ACTGCGTA ATCGAACCCTGG-3′ (positions 5441 to 5460) on the same DNA target. The amplified fragments were electrophoresed on a 1.5% agarose gel, and the individual bands were isolated by centrifugation of the gel slices through siliconized glass wool as described previously (8). Both hybridization probes were labeled with [α^{32}P]dATP by random priming (DNA labeling kit; Boehringer Mannheim, Mannheim, Germany). Prehybridization and hybridization were in 7% SDS–1% bovine serum albumin (fraction V; Boehringer Mannheim)–0.5 M Na_2HPO_4 at pH 7.2 and 65°C. After hybridization, the membranes were washed twice for 5 min each time with 1% SDS in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at room temperature and twice for 15 min each time with 0.1% SDS in 0.1% SSC at 65°C.

Autoradiographs were obtained by exposing the filters overnight to X-OMAT AR films (Kodak, Rochester, N.Y.).

RESULTS

Specificities of the primers. A series of 81 EV strains (Table 2) grown in cell culture were positive when they were tested in the RT-PCR with the E1-E2 primer pair (Table 1), yielding an amplicon of approximately 300 bp. This is in agreement with information on these primers presented in the original publication (11). All NPEVs tested except the closely PV-related coxsackievirus type A21 (18) were negative with the PV-specific primers Po1-Po4 (Table 2). Among the PV strains tested with the PV-specific primer pair Po1-Po2, only 1 type 1 isolate of 36 PV strains was negative. With the PV-specific primer pair Po3-Po4, six isolates of all three types and the prototype strain Leon A (type 3) were negative.

To confirm the specificities of the 200- and the 600-bp PCR products, Southern hybridizations with type 1 Mahoney-derived internal probes (see Materials and Methods) were performed. For the Po1-Po2 (200-bp) product, 11 of 21 PV strains tested gave positive hybridizations, and for the Po3-Po4 (600-bp) product, 10 of 12 PV strains tested gave positive hybridizations. Positive and negative results were found for strains of all three serotypes.

The hybridization-negative, 200-bp PCR products of the prototype strains were sequenced. When they were compared with their respective consensus sequences, identity was found to vary between 87.5 and 80%. Identity to the corresponding sequence of type 1 Mahoney, however, was only 66%, which may account for the observed negative hybridization with the Mahoney probe under stringent conditions. These results indicate that testing the specificity of our PV PCR products by back-hybridization is not usually feasible, since strain-specific rather than generic probes would have to be used.

Multiplex PCR with EV- and PV-specific primers. In an effort to facilitate routine diagnostic work, we combined the EV-specific primers E1 and E2 and the PV-specific primers Po1-Po4 in a multiplex PCR. The patterns of the amplicons of different sizes obtained with our PV and NPEV strains are given in Table 3. Twenty-five (70%) of the PV strains showed an additional PV-specific band of approximately 1,000 bp resulting from a read through between primers Po1 and Po4.

TABLE 2. Specificities of primers for PV and EV amplification

Virus type	Strain(s)	PCR result with primers:		
		E1-E2	Po1-Po2	Po3-Po4
PV type 1	Mahoney	+	+	+
	Brunhilde	+	+	+
	Chat Koprowski	+	+	+
	Lsc2ab, Sabin	+	+	+
	Three isolates from Switzerland, 1978–1992	+	+	+
	Isolate from Oman, 1988	+	+	+
	Isolate from India, 1983	+	+	+
	Isolate from England	+	−	+
	Isolate from Israel, 1988	+	+	−
	Two isolates from Switzerland, 1975–1978	+	+	−
PV type 2	Lansing	+	+	+
	MEF	+	+	+
	P7 12ch2ab, Sabin	+	+	+
	Isolate from Sweden, 1977	+	+	+
	Ten isolates from Switzerland, 1960–1994	+	+	+
	Two isolates from Switzerland, 1959, 1961	+	+	−
PV type 3	Saukett	+	+	+
	Leon A	+	+	−
	Leon 12ab Sabin	+	+	+
	England	+	+	+
	Two isolates from Switzerland, 1961, 1979	+	+	+
	Isolate from Finland, 1984	+	+	−
Coxsackievirus type A	2, 4, 5, 7, 9, 10, 11, 13, 16	+	−	−
	21 (see text)	+	+	−
Coxsackievirus type B	1, 2, 3, 4, 5, 6	+	−	−
Echovirus	1, 2, 3, 4, 5, 6, 7, 8, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 24, 25, 26, 27, 29, 30, 31, 32, 33	+	−	−
EV	70	+	−	−

Twenty (55%) PV strains showed all three PV-specific bands of 200, 600, and 1,000 bp, 10 (27%) PV strains showed two of the three PV-specific bands, and six (16%) PV strains showed only the 200-bp PV-specific band. All NPEV and PV strains were positive with the EV-specific primers, and as in PCRs with the primers used separately (Table 2), only 1 (coxsackievirus A21) of 45 NPEV strains showed a band with a PV-specific primer pair. The band patterns observed by multiplex PCR of the Sabin strain types 1 to 3 are shown in Fig. 1. With a Sabin type 3 strain, the 600-bp band of the PV-specific Po3-Po4 primers was missing, which was also observed with some other type 3 strains (Table 3, pattern 4). This is due to mismatches of

TABLE 3. Patterns of amplicons obtained by multiplex PCR

Pattern	Result with amplicon size (primer pair) of:				Virus type, strains
	300 bp (E1-E2)	200 bp (Po1-Po2)	600 bp (Po3-Po4)	1,000 bp (Po1-Po4)	
1	+	+	+	+	PV type 1, Mahoney, Brunhilde, Chat, Lsc2ab Sabin, three isolates from Switzerland (1978 to 1992); PV type 2, Lansing, P7 12ch2ab Sabin, nine isolates (one from Sweden, 1977; eight from Switzerland, 1960 to 1994); PV type 3, two isolates from Switzerland (1961 and 1979)
2	+	+	+	−	PV type 1, two isolates (one from India, 1983; one from Oman, 1988); PV type 2, MEF, two isolates from Switzerland (1994)
3	+	−	+	+	PV type 1, one isolate from the United Kingdom
4	+	+	−	+	PV type 3, Leon A, Saukett, Leon 12ab Sabin, one isolate from the United Kingdom
5	+	+	−	−	PV type 1, three isolates (one from Israel, 1988; two isolates from Switzerland, 1975 and 1978); PV type 2, two isolates from Switzerland (1959 and 1961); PV type 3, one isolate from Finland (1984); coxsackievirus type A21
NPEV	+	−	−	−	44 coxsackievirus and echovirus strains[a]

[a] See Table 2.

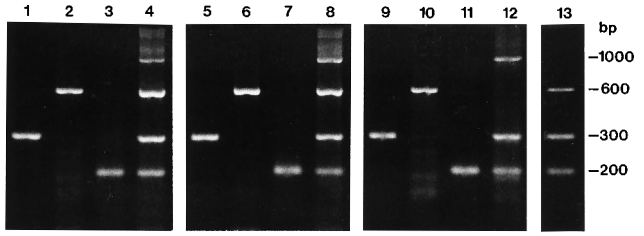

FIG. 1. Band patterns observed with PCR of the Sabin strains type 1 (lanes 1 to 4), type 2 (lanes 5 to 8), and type 3 (lanes 9 to 12) with the primer pairs E1-E2 (lanes 1, 5, and 9), Po3-Po4 (lanes 2, 6, and 10), and Po1-Po2 (lanes 3, 7, and 11). The primers were tested singly and in combination (multiplex, lanes 4, 8, and 12). In single reactions, all three strains can be amplified with all primer pairs. In the multiplex PCR, the Sabin type 3 strain does not show the Po3-Po4 band (600 bp) because read through between Po1 and Po4 is favored over that between Po3 and Po4 (see text). Lane 13, PCR products (marker).

primer Po3 with type 3 sequences (data not shown), which favors read through between the perfectly matching Po1 and Po4 primers.

To test the specificity of the 1,000-bp product, a small amount of the band, obtained by amplification of a Sabin type 1 strain, was harvested from the gel by stabbing the band with a needle (6). The DNA thus obtained was amplified with the Po1-Po2 and the Po3-Po4 primer pairs. The expected bands of 200 and 600 bp, respectively, were obtained.

Our RT multiplex PCR detected 10^2 PFU of partially purified PV (data not shown). The overall sensitivity of our protocol, combining overnight cell culture with the RT multiplex PCR, was tested by adding known amounts of PV-infected cells to a culture of 10^5 uninfected cells of the same type immediately before processing the cells for PCR. The RT multiplex PCR detected three infected cells in such a reconstructed culture.

Two examples of PV detection by RT multiplex PCR in clinical samples are given in Fig. 2. A stool specimen from a patient with PV vaccination-associated transient paralysis and one from a symptomless baby receiving primary PV vaccination were analyzed in parallel directly by PCR and by PCR of an infected cell culture as described in this report. By the latter protocol, PV was readily demonstrated, whereas the PCR performed with a stool suspension was negative. Among 10 other stool samples (data not shown), all of which were positive for PV by our protocol, only one sample was positive by direct RT-PCR.

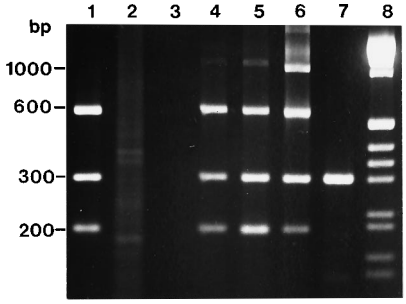

FIG. 2. Two examples of PV detection by RT multiplex PCR in clinical samples. Aliquots of stool suspensions were either processed directly for PCR or inoculated onto RD-6 cells, incubated overnight, and then subjected to PCR. The two short-term cultures yielded a positive PCR (patient A, lane 4; patient B, lane 5), whereas the PCRs performed directly with the stool suspensions were negative (patient A, lane 3; patient B, data not shown). Controls were uninfected cell culture (lane 2), PV type 1 Mahoney (lane 6), and coxsackievirus type A11 (lane 7). Lane 1, PCR products (marker); lane 8, 1-kb DNA ladder (Gibco).

In conclusion, we propose a protocol for the routine detection of EV and their identification as NPEVs or PVs as outlined in Fig. 3. The specimen is inoculated in at least two suitable cell culture tubes, and after overnight incubation, regardless of whether a cytopathic effect is visible or not (9), the cell sediment of one of the tubes is used to perform the RT multiplex PCR described here. If EVs or PVs are found to be present, the second tube can be used for virus isolation and conventional typing (12) by neutralization or, for PV, by immunofluorescence (unpublished data).

DISCUSSION

The RT multiplex PCR described here was developed for the rapid and sensitive detection of PV and its discrimination from NPEVs. This distinction is important in PV surveillance programs, e.g., in the World Health Organization polio eradication campaign. Such surveillance programs rely on the identification of PV in stool and sewage samples, which cannot be subjected to RT-PCR because they contain inhibitors (21, 27). We circumvented this problem by combining short-term cell culture with RT-PCR. Short-term cultures seem to be much less affected by contaminating substances from stool or sewage than RT-PCR, as shown by the results obtained with our PV-positive stool samples.

The primers, which are specific for either EV or PV, were chosen so that they can easily be combined in a multiplex PCR and give rise to amplicons of different sizes. Comparable to nested PCR (13, 23), a set of amplicons (in our case, two to four) of the correct size can be considered diagnostically significant, without the need for back-hybridization of the PCR products. This renders our routine PV detection protocol straightforward and rapid and, in addition, obviates the use of a series of different hybridization probes which would be necessary to compensate for the genetic diversity of PVs (see Results).

The sensitivity of our RT multiplex PCR, as measured with RNA extracted from a virus suspension, was found to be 100 PFU. With clinical samples spiked with EV, RT-PCR has a demonstrated sensitivity in the wide range of between 5 PFU (15) and 10^3 50% tissue culture infective doses (20). By combining short-term culture and PCR we were able to detect three infected cells in a cell culture (which corresponds, by definition, to 3 PFU in the inoculum). Thus, the overall sensitivity of our protocol is at least as sensitive as but is likely up to 2 orders of magnitude more sensitive than RT-PCR performed directly with clinical specimens. Such a small amount of virus can be detected in conventional cell culture by observation of a cytopathic effect only after prolonged incubation, if at all. Our protocol allows results to be obtained within 24 h.

It should be noted, however, that this diagnostic approach relies on viable virus. This is not a problem in the investigation of clinical cases of infection, whereas in environmental samples inactivated virus might be present (14), and these would be missed by our protocol. However, looking for infectious, and thus freshly shed, virus reflects more accurately the epidemiological situation in a given human population.

Genetic heterogeneity is a general phenomenon with RNA viruses, including those of the PV group (25, 26). In investigating the role of the proteins of the P2 genomic region in the PV life cycle (4, 5) we found, using anti-2C monoclonal antibodies (3, 16), that this genomic region might contain conserved species-specific regions (unpublished data). In designing the PV-specific primers, however, it was found that it was

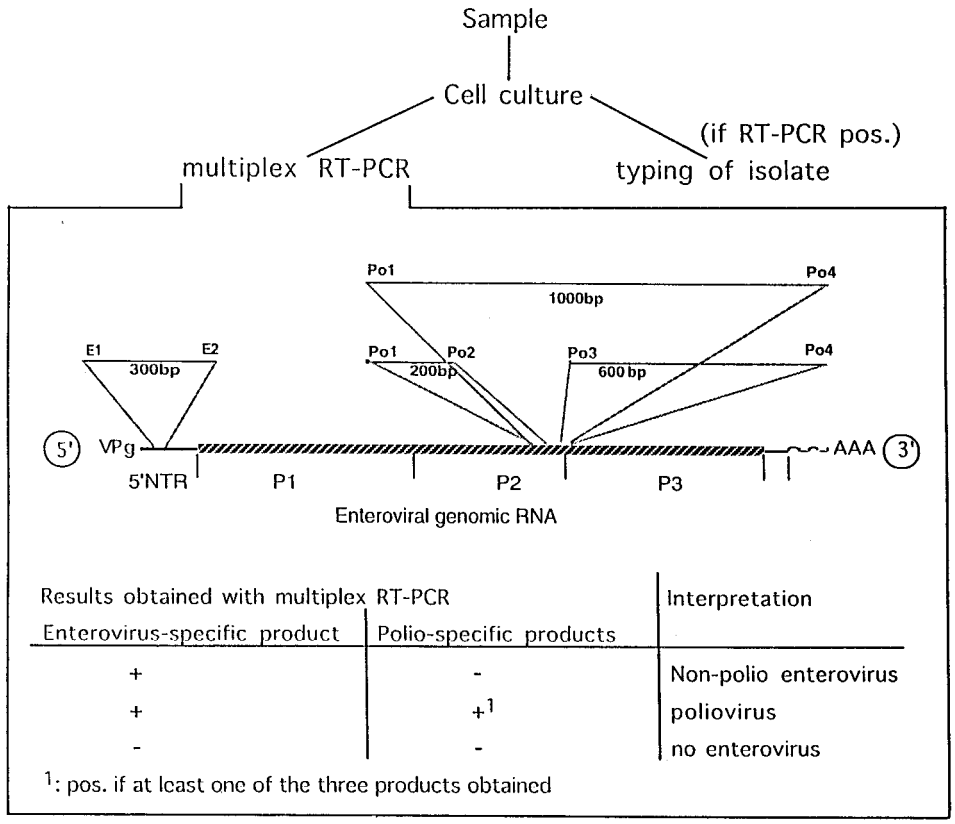

FIG. 3. Proposed protocol for the routine detection of EVs and their identification as PV or NPEV. The specimen is inoculated into cell culture tubes. After overnight incubation, the cell sediment of one of the tubes is used to perform the RT multiplex PCR described in the text. If the PCR is positive, a second inoculated cell culture tube may be used for virus isolation and typing. 5′ NTR, 5′-nontranslated region.

necessary to use degenerate primers (Po1 and Po2) in order to overcome the genetic heterogeneity within the PV species. Still, our two primer pairs, used alone, do not recognize all PV strains. No strains were missed only when they were used in combination, notably, strains which have been isolated in very different geographic regions. Abraham et al. (1) described primers which recognize all but certain PV type 2 strains but which still cross-react with two echovirus strains. With our primers, we found cross-reactivity only with the NPEV strain coxsackievirus type A21. This virus has a special position in that it is more closely related to the PVs than to other NPEVs (18). In the P2 genomic region, in which the sequences of the PV-specific primers are located, coxsackievirus type A21 shows 90% similarity to PV types 1 to 3 (10a).

In addition to the sensitivity, specificity, and speed of the PV detection protocol presented here, there is the advantage that in parallel to the demonstration of PV and its distinction from NPEVs, the virus in question can be isolated and, subsequently, can be subjected to inter- and intrastrain-specific typing, e.g., by serological (12) or genetic methods, such as restriction fragment length polymorphism analysis (2, 22) or strain-specific PCR (29).

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