JOURNAL OF CLINICAL MICROBIOLOGY, July 1996, p. 1722–1725 Vol. 34, No. 7
0095-1137/96/$04.00+0


# Rapid Diagnosis of Poliovirus Infection by PCR Amplification

CLAUDIA CHEZZI*

*Department of Virology, University of the Witwatersrand, and National Institute for Virology, Johannesburg, South Africa*

Received 17 October 1995/Returned for modification 5 January 1996/Accepted 27 March 1996

**A single-tube, single-primer-set reverse transcription-PCR assay was developed for the rapid detection of polioviruses in infected tissue culture fluids and clinical materials. The poliovirus-specific PCR primers are located in the VP1-2A region of the poliovirus genome. They generate a 290-bp product and can be used in duplex reactions with general enterovirus primers. The primers span the region used for genotype determination, so that genotype analysis of wild-type polioviruses can be performed by direct sequencing of the PCR products. Of 125 virus isolates typed as polioviruses by neutralization assays, 125 (100%) were also positive by PCR, and of 38 isolates typed as non-polio enteroviruses by conventional techniques, 38 (100%) were also negative by PCR. The assay described here is rapid, highly sensitive, and specific and has clinical applicability in the diagnosis of poliovirus infections.**

The enteroviruses, including polioviruses, coxsackieviruses, echoviruses, and the more recently classified enteroviruses (10), are among the most common and important of the human viral pathogens. Acute flaccid paralysis surveillance and laboratory diagnosis of poliomyelitis is a critical part of the World Health Organization's initiative for poliomyelitis eradication by the year 2000 (19, 20), and it is thus necessary that rapid and reliable techniques be available for differentiating between polioviruses and non-polio enteroviruses.

Diagnosis of enterovirus infection still relies largely on cell culture techniques (8), with subsequent typing of the enteroviruses by neutralization (9), techniques which are both lengthy and expensive. Differentiation between polioviruses and non-polio enteroviruses by microneutralization or immunofluorescence with poliovirus-specific antisera is less expensive but is still time-consuming. Recently, mouse L cells transfected with the poliovirus receptor have been developed for the selective isolation of polioviruses and the exclusion of other enteroviruses (4, 12). However, confirmatory serotyping subsequent to isolation in these cells and difficulties in maintaining the cell lines (personal observation) preclude routine use of these cells.

PCR techniques for the detection of enteroviruses in clinical specimens and infected tissue culture supernatants have been well established (2, 5, 6, 11, 14, 16, 21). However, the identification of polioviruses by these techniques requires subsequent hybridization with poliovirus-specific probes (2) or restriction enzyme analysis (6, 15). PCR primers specific for polioviruses have been described, but they are not able to detect all poliovirus strains (1) or several combinations of primer pairs need to be used simultaneously in order to obtain positive results (3).

This report describes the rapid detection of polioviruses in a simple reverse transcription (RT)-PCR (RT-PCR) assay in which a single primer pair is used. The assay is sensitive (4 PFU) and highly specific for polioviruses. In addition, the primers span the VP1-2A region used for genotype determination (13). By this technique it is possible to identify polioviruses, to differentiate them from other enteroviruses within 24 h, and to determine the genotypes of wild-type polioviruses by direct sequencing of the PCR products.

* Mailing address: National Institute for Virology, Private Bag X4, Sandringham, 2131, South Africa. Phone: (01027-11) 882-9910. Fax: (01027-11) 882-0596. Electronic mail address: claudiac@niv.ac.za.

## MATERIALS AND METHODS

**Virus strains.** A panel of enterovirus isolates was used to test the sensitivity and specificity of the PCR. Seven reference laboratory poliovirus strains, representative of each of the three serotypes, were used: P1/Mahoney, P1/Brunenders, P1/LSc2ab Sabin 1, P2/MEF, P2/P712ch2ab Sabin 2, P3/Saukett, and P3/Leon 12ab Sabin 3. Coxsackievirus types B1 to B6 and enterovirus 70 were originally obtained from the American Type Culture Collection. All other poliovirus and enterovirus isolates were from clinical specimens sent to the National Institute for Virology for typing. The virus strains used in the present study are listed in Table 2, in the Results section.

Polioviruses, coxsackievirus types A9, A16, B1 to B6, enterovirus 70, and echoviruses were grown on VK, Vero, HEp-2, and RD cell lines and were typed by the microneutralization technique with antiserum pools supplied by the National Institute for Public Health and Environmental Protection, Bilthoven, The Netherlands. All other coxsackievirus type A strains were archival isolates originally propagated and typed in suckling mice.

**Virus RNA extraction.** RNA was extracted from 160 μl of infected tissue culture fluid. Forty microliters of 5× lysis buffer (250 mM Tris-HCl [pH 8.3], 350 mM KCl, 25 mM $MgCl_2$, 2.5% Nonidet P-40) was added to each tube, and the tubes were incubated on ice for 15 min. Nucleic acids were extracted once with phenol, once with phenol-chloroform (1:1), and once with chloroform-isoamyl alcohol (24:1). The aqueous supernatants were used as templates for PCRs and were subsequently stored at −70°C.

For isolation of RNA from stool samples, the samples were diluted to 20% in Eagle's minimum essential medium and were clarified by vortexing and centrifugation. RNA was extracted from 200 μl of clarified stool samples by using the Qiamp Blood kit (Qiagen GmbH, Hilden, Germany) and was eluted in a final volume of 50 μl.

For RNA extraction from mouse tissue, mice which had been stored at −20°C in sterile McCartney bottles were allowed to thaw at room temperature in the bottles. One milliliter of phosphate-buffered saline was added to each bottle, the bottles were vortexed vigorously for a few minutes in order to release the cells into the liquid, and the cell suspension was transferred to a clean microcentrifuge tube. RNA was extracted from 200 μl of the cell suspension by using the Qiamp Tissue kit (Qiagen GmbH) according to the instructions supplied with the kit. RNA was eluted in a final volume of 50 μl and was stored at −70°C.

**Primers.** For amplification of enteroviral genomes, the primers described by Rotbart (14) were used. These primers are located in the conserved 5′ noncoding region of enterovirus genomes.

For poliovirus-specific amplification, primer 2A, previously described by Rico-Hesse et al. (13), was used as the reverse primer. Forward primer PVPCR2 was chosen from a conserved region of the poliovirus VP1 region after analysis of published Sabin and wild-type poliovirus sequences (GenBank). The primer pair PVPCR2-2A was checked for internal and 3′ end complementarity by using Primer Detective 1 primer design and analysis software. The specificities of these primers were checked against the GenBank and EMBL data banks. No homology with other non-polio enteroviruses for which sequences were available was detected.

The sequences and locations of the primers within the poliovirus type 1 genome are given in Table 1.

TABLE 1. Primers for enterovirus and poliovirus PCR

| Primer | Position in genome of poliovirus type 1[a] | Sequence (5′–3′) | Target size (bp) |
|---|---|---|---|
| Ent1 | 5′ NC 484–603 | ATT GTC ACC ATA AGC AGC CA | 154 |
| Ent2 | 5′ NC 450–479 | CCT CCG GCC CCT GAA TGC GGC TAAT | |
| PVPCR2 | VP1 3235–3254 | GTC AAT GAT CAC AAC CCAC | 290 |
| 2A | 2A 3508–3527 | AAG AGG TCT CTA TTC CAC AT | |

[a] Numbering is according to Toyoda et al. (17). NC, noncoding.

**RT and PCR.** RT and PCR (RT-PCR) were performed in a single step in a 100-μl reaction volume. An RT-PCR master mixture containing 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCl, 20 pmol of each primer pair (Ent1-Ent2 for enterovirus PCR or PVPCR2-2A for poliovirus PCR), 200 μM (each) deoxynucleoside triphosphate, 10 U of RNase inhibitor (Boehringer Mannheim), 5 U of avian myeloblastosis virus reverse transcriptase (Boehringer Mannheim), and 2.5 U of *Taq* polymerase (Boehringer Mannheim) was prepared for each reaction and was divided into 95-μl aliquots, and each aliquot was placed into a thin-walled 0.5-ml reaction tube. The reaction mixtures were overlaid with 3 drops of light mineral oil (Sigma) and were kept on ice. RNA templates were heat denatured (80°C, 2 min) and snap cooled on ice for 5 min. Five microliters of denatured template was added to each reaction tube.

RT-PCR amplification was performed by using the following program on a Biometra Trioblock thermal cycler: one cycle of reverse transcription at 42°C for 45 min, one cycle of denaturation at 95°C for 3 min, 30 cycles of denaturation at 95°C for 30 s, annealing at 50°C (enterovirus PCR) or 56°C (poliovirus PCR) for 45 s, and elongation at 72°C for 1 min followed by 1 cycle of elongation at 72°C for 7 min. Reaction mixtures were then held at 4°C.

For simultaneous amplification of both general enterovirus and poliovirus templates, 20 pmol of each primer, primer Ent1, Ent2, PVPCR2, and 2A, was added to the master mixture for each reaction. RT and amplification conditions were as described above, with annealing being performed at 50°C.

**Analysis of PCR products.** PCR products were analyzed by electrophoresis through 2.5% agarose gels. Ten microliters of amplified products was electrophoresed at 10 V/cm through 2.5% gels in 0.5× Tris-borate-EDTA buffer. Gels contained 0.4 μg of ethidium bromide per ml and were visualized under short-wave UV light.

**Sequencing of amplified products.** Amplified products were purified from agarose gels by using the Mermaid kit (Bio 101). Briefly, 40 μl of amplified product was electrophoresed on a 1.5% Biogel (Bio 101) in Mermaid buffer, and the target band was excised from the gel. DNA was purified from the agarose slice according to the manufacturers' instructions and was eluted in a final volume of 20 μl of distilled water. Five microliters of purified DNA was used for sequencing.

Dideoxy sequencing of the purified PCR products was performed with the Sequenase PCR product sequencing kit (United States Biochemicals) with [$^{35}$S]dATP. The amplified products were sequenced with primer 2A, and the sequences were confirmed by repeat sequencing with primer PVPCR2.

The sequenced products were resolved on 8% acrylamide gels containing 7 M urea and were vizualized by autoradiography (BMax film; Amersham). Sequence data were read manually and were entered into DNASIS (Hitachi) analysis software. The sequences of the PCR products were compared with those of reference poliovirus strains to confirm their identities and genotypes.

## RESULTS

One hundred twenty-five enterovirus isolates identified as poliovirus by conventional typing techniques and 39 non-polio enteroviruses were subjected to RT-PCR with primers PVPCR2 and 2A. All 125 isolates (100%) typed as poliovirus by tissue culture neutralization were also positive by PCR (Table 2). With primer set PVPCR2-2A, an amplification product of the expected size (290 bp) was obtained with all of the poliovirus-positive isolates but not with any of the non-polio enteroviruses (Fig. 1); minor variations in the size of the 290-bp amplicon were obtained with different templates. RNA extracted from noninfected cells used to grow the viruses was used as a negative control. All of the PCR products were sequenced to confirm their identities. Sequence data (data not shown) confirmed that the amplicons were indeed 290 bp in size and were from vaccine-like or wild-type poliovirus genomes. The slight variation in size appears to be the result of minor differences in the electrophoretic mobilities of the prod-

TABLE 2. Specificities of primers for poliovirus amplification

| Virus | Strain(s) | No. of PCR-positive isolates | |
|---|---|---|---|
| | | Ent1-Ent2 | PVPCR2-2A |
| Polioviruses | | | |
| Poliovirus type 1 | Mahoney | | 1 |
| | Brunenders | | 1 |
| | LSc2ab Sabin | | 1 |
| | 50 isolates from South Africa (1980–1989) | | 50 |
| | 40 isolates from Namibia (1993–1995) | | 40 |
| | 10 isolates from Central African Republic 1992–1993 | | 10 |
| | 2 isolates from Angola 1994 | | 2 |
| | 3 isolates from Tanzania 1995 | | 3 |
| | 2 isolates from Zimbabwe 1995 | | 2 |
| | 1 isolate from Curacao | | 1 |
| | 1 isolate from Brazil | | 1 |
| | 1 isolate from Greece | | 1 |
| Poliovirus type 2 | MEF | | 1 |
| | P712ch2ab Sabin | | 1 |
| | 2 isolates from South Africa (1980–1995) | | 2 |
| | 1 isolate from Cameroon | | 1 |
| | 1 isolate from Vietnam | | 1 |
| | 1 isolate from Brazil | | 1 |
| Poliovirus type 3 | Saukett | | 1 |
| | Leon 12ab Sabin | | 1 |
| | 1 isolate from Spain | | 1 |
| | 1 isolate from Vietnam | | 1 |
| | 1 isolate from Israel | | 1 |
| Total | | | 125 |
| Non-polio enteroviruses | | | |
| Coxsackievirus type A | 1, 2, 3, 4, 6, 7, 8, 9, 10, 11, 12, 13, 14, 16, 17, 18, 19, 20, 21, 22, 23, 24 | 22 | 0 |
| Coxsackievirus type B | 1, 2, 3, 4, 5, 6 | 6 | 0 |
| Echovirus | 2, 6, 7, 11, 14, 16, 20, 25, 27 | 9 | 0 |
| Enterovirus | 70 | 1 | 0 |
| Total | | 38 | 0 |

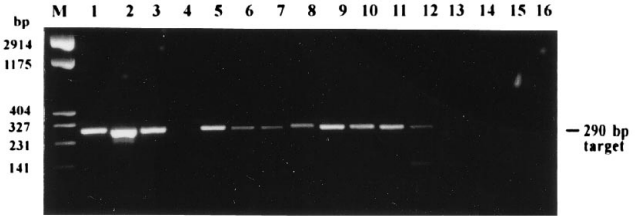


FIG. 1. Poliovirus-specific PCR amplification of reference, vaccine-like, and wild-type poliovirus strains with the primer pair PVPCR2-2A. Lane 1, P1/LSc2ab Sabin 1; lane 2, P2/P712ch2ab Sabin 2; lane 3, P3/Leon 12ab Sabin 3; lane 4, no RNA; lane 5, P1/Mahoney; lane 6, P2/MEF; lane 7, P3/Saukett; lane 8, P1/wild-type South Africa/1982; lane 9, P1/wild-type/South Africa/1989; lane 10, P1/wild-type/Greece; lane 11, P2/wild-type/Cameroon; lane 12, P3/wild-type/Vietnam; lane 13, coxsackievirus A9; lane 14, coxsackievirus A16; lane 15, coxsackievirus B3; lane 16, enterovirus 70; lane M, ϕX174/*Taq*1 molecular mass markers.

ucts because of nucleotide sequence differences between amplicons. In order to determine the endpoint sensitivity of the poliovirus PCR, 10-fold dilutions of polioviruses of known titer were made, and RNA was extracted from the diluted specimens and amplified with primers PVPCR2 and 2A. A detection limit of 4 PFU was observed with ethidium bromide staining (data not shown) when culture fluid was used as the source of viruses. A detection limit of approximately 4 PFU was also observed when the PCR assay was performed directly on stool specimens.

To ensure that the negative PCR results obtained with non-polio enteroviruses with primers PVPCR2-2A were due to the specificities of the primers and not due to the fact that the RNA was of unsuitable quality for amplification, all of the non-polio enterovirus isolates were amplified with the general enterovirus primers Ent1-Ent2. All isolates yielded a positive signal with the enterovirus-specific primers (Table 2).

In an effort to facilitate routine identification of polioviruses from other enteroviruses, primers PVPCR2, 2A, Ent1, and Ent2 were combined in a duplex PCR. Duplex PCR was performed for several of the isolates, and in all cases the 290-bp poliovirus-specific amplicon was detected only in reaction mixtures containing poliovirus RNA (Fig. 2). The 154-bp enterovirus amplicon was detected in all cases. An annealing temperature of 50°C was routinely used for the duplex amplification because higher annealing temperatures failed to amplify the enterovirus targets. The specificity of the 290-bp poliovirus amplicon was not affected by using a 50°C annealing temperature in the reaction.

The ability of the PCR assay to detect polioviruses directly in clinical specimens was measured by amplifying RNA extracted

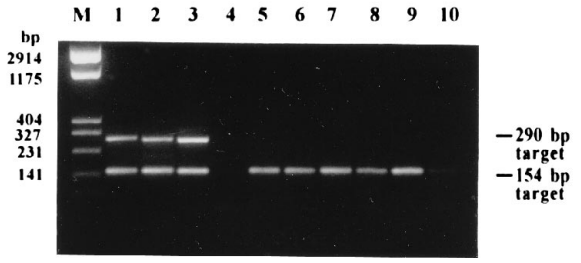


FIG. 2. Duplex PCR amplification of polioviruses and non-polio enteroviruses with primer pairs PVPCR2-2A and Ent1-Ent2. Lane 1, P1/LSc2ab Sabin 1; lane 2, P2/P712ch2ab Sabin 2; lane 3, P3/Leon 12ab Sabin 3; lane 4, no RNA; lane 5, coxsackievirus A9; lane 6, coxsackievirus A16; lane 7, coxsackievirus B3; lane 8, echovirus 11; lane 9, echovirus 25; lane 10, enterovirus 70; M, ϕX174/*Taq*1 molecular mass markers.

from stool specimens. RNA was extracted from 25 stool specimens from which polioviruses had been isolated; all 25 specimens were also positive for poliovirus by PCR (data not shown); amplicon bands, however, were less intense than those obtained when RNA extracted from infected tissue culture fluids was used as a template, but this reflects the much lower numbers of virions in stool specimens than in tissue culture supernatants. Negative controls included 13 stool specimens from which no virus isolate could be obtained, 3 stool specimens from which non-polio enteroviruses were cultured (coxsackievirus type A, coxsackievirus type B2, and echovirus 9), and 1 stool specimen from which a reovirus was isolated. All culture-negative and poliovirus-negative controls were also negative for poliovirus by PCR. In order to test the direct applicability of the PCR assay to stool specimens from patients with acute flaccid paralysis for which no tissue culture results were yet available, RNA was extracted from six stool specimens and was amplified with primers PVPCR2 and 2A. Poliovirus RNA was detected in all six stool specimens. Two of the six specimens yielded poliovirus upon isolation; no virus could be isolated from the remaining four specimens. It is possible that the culture-negative specimens contained no viable virus but contained enough poliovirus RNA for detection by PCR.

## DISCUSSION

The reliable diagnosis of poliovirus infection is of importance in public health programs, in particular the World Health Organization acute flaccid paralysis surveillance program, a critical part of the poliomyelitis eradication campaign (20). This paper describes a rapid, sensitive technique for the detection of poliovirus and its differentiation from other non-polio enteroviruses. A one-step RT-PCR assay was developed. The assay uses the same poliovirus-specific primers for both RT and amplification. The numbers of reagents and steps in the assay are kept to a minimum, thus keeping the assay rapid and cost-effective and reducing the possibility of cross contamination. The RT-forward PCR primer 2A, located in the conserved region of the poliovirus genome coding for protease 2A, has been shown to bind, albeit as a sequencing primer, to a large number of both vaccine-like and wild-type polioviruses (7, 13). Primer PVPCR2 was chosen from a conserved region of the poliovirus genome coding for the capsid protein VP1. Both primers were checked against sequences in GenBank and EMBL for their homologies with other nucleic acid sequences, particularly those of other non-polio enteroviruses; homology was observed only with poliovirus sequences. A single, 290-bp PCR product, easily detectable by standard gel electrophoresis, is generated with these primers.

Direct detection of polioviruses in clinical and environmental specimens will become of increasing importance as the poliovirus eradication campaign progresses, since relatively low levels of polioviruses will have to be detected from among the more abundant non-polio enteroviruses. The PCR assay described in this report is highly sensitive, detecting as few as 4 PFU of infectious virus, and can be performed directly on clinical material. Although amplicon bands obtained with RNA extracted from stool specimens were less intense than those obtained from tissue culture supernatants, the sensitivity of detection in clinical specimens is comparable to that in tissue culture supernatants; the titer of virus in stool specimens is considerably lower than that in tissue culture supernatants, with the numbers of viral particles in an aliquot of stool specimen seldom being higher than the minimum number required for the detection of a positive PCR result (4 PFU). Egger et al. (3) describe a method for circumventing both the problems of

a low input titer of virus, below the level detectable by RT-PCR, and of the presence of PCR inhibitors in stool samples; a combination of short-term (overnight) cell culture followed by RT-PCR. This method could be applied for the routine amplification of virus from clinical and environmental samples, in which low virus titers and PCR inhibitors could lead to false-negative results.

Abraham et al. (1) describe poliovirus primers in the 5′ noncoding region which recognize all polioviruses but type 2 Lansing. However, these primers exhibited cross-reactivity with echoviruses 11 and 32. Two primer pairs, located in the VP2 region, that can be used for the rapid detection of polioviruses have been described by Egger et al. (3); however, these two primer pairs, when used alone, did not recognize all poliovirus strains and showed cross-reactivity with coxsackievirus type A21. In the present study, a range of reference vaccine-like and wild-type polioviruses was tested. Wild-type viruses were from diverse geographical locations and represented several different genotypes, as determined by sequence analysis (18). With primers PVPCR2 and 2A, positive signals were obtained with all polioviruses tested, and no cross-reactivity with other non-polio enteroviruses was observed. Echovirus 32 was not available for testing with the present panel of enteroviruses. However, this enterovirus is very rarely isolated in this laboratory (9a), and in the event of cross-reactivity, PCR products can be sequenced to confirm their identities. A duplex PCR was developed in order to be able to differentiate polioviruses from other enteroviruses in a single reaction. No cross-reactivity between the poliovirus and enterovirus primers was observed; the 290-bp poliovirus-specific amplicon, which could be easily resolved from the 154-bp general enterovirus amplicon by electrophoresis, was generated only in reactions containing poliovirus RNA. In this duplex reaction, general enterovirus amplification can also serve as an internal positive control in the reaction; both general enterovirus- and poliovirus-specific amplicons will be expected if a specimen is positive for polioviruses. The sensitivity and specificity of the RT-PCR obviates the need for either restriction enzyme analysis or hybridization with poliovirus-specific probes, techniques which increase the time required for a positive diagnosis.

Primers PVPCR2 and 2A can be used in combination with Sabin-specific primers (21) for intratypic differentiation between vaccine-like and wild-type polioviruses. In reactions with both sets of primers, PVPCR2-2A acts as a positive control, and isolates that amplify as poliovirus positive, Sabin negative can then be typed as wild-type isolates. The primers were chosen deliberately to span the VP1-2A region commonly sequenced for genotype analysis (7, 13), so that genotyping of wild-type isolates could be performed by direct sequencing of the PVPCR2-2A product. All polioviruses used in the present study were sequenced and genotyped by this technique (unpublished data). This approach was also successfully used for the rapid detection and characterization of polioviruses isolated during the 1993-1994 poliomyelitis outbreak in Namibia (18) and is being used routinely for poliovirus characterization in this laboratory.

In conclusion, the results of the present study show that RT-PCR with primers PVPCR2 and 2A can be used to rapidly identify polioviruses and to differentiate them from non-polio enteroviruses and that further characterization of polioviruses can be performed by direct sequencing of the PCR product generated by these primers.

## ACKNOWLEDGMENTS

My thanks to Ezekiel Maselesele for preparing the viral isolates, to Barry D. Schoub for critical reading of the manuscript, and to Leona Rowland for preparation of the manuscript. Thanks also to Marion Taylor and Jim Phillips for help with the figures.

This work was supported in part by grants from the Poliomyelitis Research Foundation and the South African Medical Research Council.

## REFERENCES

1. **Abraham, R., T. Chonmaitree, J. McCombs, B. Prabhakar, P. T. Lo Verde, and P. L. Ogra.** 1993. Rapid detection of poliovirus by reverse transcription and polymerase chain reaction amplification: application for differentiation between poliovirus and non-poliovirus enteroviruses. J. Clin. Microbiol. **31:** 395–399.
2. **Chapman, N. M., S. Tracy, C. J. Gauntt, and U. Fortmueller.** 1990. Molecular detection and identification of enteroviruses using enzymatic amplification and nucleic acid hybridization. J. Clin. Microbiol. **28:**843–850.
3. **Egger, D., L. Pasamontes, M. Ostermayer, and K. Bienz.** 1995. Reverse transcription multiplex PCR for differentiation between polio- and enteroviruses from clinical and environmental samples. J. Clin. Microbiol. **33:**1442–1447.
4. **Hovi, T., and M. Stenvik.** 1994. Selective isolation of poliovirus in recombinant murine cell line expressing the human poliovirus receptor gene. J. Clin. Microbiol. **32:**1366–1368.
5. **Hypiä, T., P. Auvinen, and M. Maaronen.** 1989. Polymerase chain reaction for human picornaviruses. J. Gen. Virol. **70:**3261–3268.
6. **Kämmerer, U., B. Kunkel, and K. Korn.** 1994. Nested PCR for specific detection and rapid identification of human picornaviruses. J. Clin. Microbiol. **32:**285–291.
7. **Kew, O. M., B. K. Nottay, R. Rico-Hesse, and M. A. Pallansch.** 1990. Molecular epidemiology of wild poliovirus transmission, p. 199–221. *In* E. Kurstak, R. G. Marusyk, F. A. Murphy, and M. H. V. Van Regenmortel (ed.), Applied virology research, vol. 2. Plenum Publishing Corporation, New York.
8. **Lennette, E. H. (ed.).** 1992. Laboratory diagnosis of viral infections, 2nd ed. Marcel Dekker, Inc., New York.
9. **Lim, K. A., and M. Benyesh-Melnick.** 1969. Typing of viruses by combination of antiserum pools. Application to typing of enteroviruses (coxsackie and echo). J. Immunol. **84:**309–317.

9a. **Maselesele, E.** Personal communication.

10. **Melnick, J. L.** 1990. Enteroviruses: polioviruses, coxsackieviruses, echoviruses, and newer enteroviruses, p. 549–605. *In* B. N. Fields and D. W. Knipe (ed.), Virology, 2nd ed. Raven Press, New York.
11. **Muir, P., F. Nicholson, M. Jhetam, S. Neogi, and J. E. Banatvala.** 1993. Rapid diagnosis of enterovirus infection by magnetic bead extraction and polymerase chain reaction detection of enterovirus RNA in clinical specimens. J. Clin. Microbiol. **31:**31–38.
12. **Pipkin, P. A., D. J. Wood, V. R. Rancaniello, and P. D. Minor.** 1993. Characterisation of L cells expressing the human poliovirus receptor for the specific detection of polioviruses in vitro. J. Virol. Methods **41:**333–340.
13. **Rico-Hesse, R., M. A. Pallansch, B. K. Nottay, and O. M. Kew.** 1987. Geographic distribution of wild poliovirus type 1 genotypes. Virology **160:**311–322.
14. **Rotbart, H. A.** 1990. Enzymatic amplification of the enteroviruses. J. Clin. Microbiol. **28:**438–442.
15. **Schweiger, B., F. Schreir, B. Böthig, and J. M. Lópes-Pila.** 1994. Differentiation of vaccine and wild-type polioviruses using polymerase chain reaction and restriction enzyme analysis. Arch. Virol. **134:**39–50.
16. **Thorén, A., A. J. Robinson, T. Maguire, and R. Jenkins.** 1992. Two-step PCR in the retrospective diagnosis of enteroviral viraemia. Scand. J. Infect. Dis. **24:**137–141.
17. **Toyoda, H., M. Kohara, Y. Kataoka, T. Suganuma, T. Omata, N. Imura, and A. Nomoto.** 1984. Complete nucleotide sequences of all three poliovirus serotype genomes. J. Mol. Biol. **174:**561–585.
18. **Van Niekerk, A. B. W., J. B. Vries, J. Baard, B. D. Schoub, C. Chezzi, and N. K. Blackburn.** 1994. Outbreak of paralytic poliomyelitis in Namibia. Lancet **344:**661–664.
19. **World Health Organization.** 1988. Global eradication of poliomyelitis by the year 2000. Weekly Epidemiol. Rec. **63:**161–168.
20. **World Health Organization.** 1990. Global poliomyelitis eradication by the year 2000. Expanded programme on immunization and division of communicable diseases. World Health Organization, Geneva.
21. **Yang, C. F., L. De, B. P. Holloway, M. A. Pallansch, and O. M. Kew.** 1991. Detection and identification of vaccine-related polioviruses by the polymerase chain reaction. Virus Res. **20:**159–179.
22. **Zoll, G. J., W. J. G. Melchers, H. Kopecka, G. Jambroes, H. J. A. van Der Poel, and J. M. D. Galama.** 1992. General primer-mediated polymerase chain reaction for detection of enteroviruses: application for diagnostic routine and persistent infections. J. Clin. Microbiol. **30:**160–165.