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Detection of Newly Recognized Rodent Parvoviruses by PCR

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Several autonomous parvovirus isolates distinct from the prototypic rodent parvoviruses have recently been identified. These include variants of a mouse orphan parvovirus (MOPV) and a hamster isolate designated hamster orphan parvovirus (HOPV). In this study, a PCR primer set specific for these newly identified rodent parvoviruses was designed on the basis of DNA sequence comparisons of these isolates with other autonomous parvoviruses. The specificity of the primer set was determined by testing viral preparations of seven different parvoviruses and eight other viruses known to infect rodents. The PCR assay amplified the expected 260-bp product only in the presence of DNA from MOPV, HOPV, or LuIII, a parvovirus of unknown species origin. The assay was able to detect as little as 10 pg of MOPV viral DNA or 1 pg of HOPV viral DNA, and it was able to detect MOPV in tissues from naturally infected mice and HOPV in tissues from experimentally infected hamsters. In contrast, the 260-bp product was not amplified from tissues of MOPV-negative mice or mock-infected hamsters. Our findings indicate that this PCR assay provides a rapid, specific, and sensitive method for the detection of MOPV in mice, HOPV in hamsters, and MOPV and HOPV in cell culture systems and that it may also be useful for the detection of LuIII contamination of cell culture systems.

Several rodent parvoviruses that are distinct from the prototypic rodent parvoviruses minute virus of mice (MVM), H-1 parvovirus, and Kilham rat virus (KRV) have recently been identified. These include variants of a mouse orphan parvovirus (MOPV) and a hamster isolate designated hamster orphan parvovirus (HOPV). MOPV1, also referred to as mouse parvovirus 1 and Fitch orphan parvovirus, was first isolated from murine cytotoxic T cells as a cell culture contaminant, and the DNA sequence of this isolate has since been determined (1, 5, 19, 26). A field strain of MOPV, hereafter referred to as MOPV2, was sequenced directly from a naturally infected Swiss mouse, but this isolate has not yet been propagated in vitro (5). Both MOPV1 and MOPV2 cause subclinical infections in mice with no associated gross or histologic lesions (3, 26). HOPV was originally detected in hamsters experiencing runting, tooth loss, and neonatal mortality (14). This isolate has been successfully propagated in vitro, and experimental infections with HOPV in neonatal hamsters have reproduced the pathology observed during the initial outbreak of disease (4). DNA sequence and antigenic analyses have shown MOPV1, MOPV2, and HOPV to be closely related to each other and distinct from MVM, H-1, and KRV (1, 5, 19). Sequence comparisons with other autonomous parvoviruses indicate that the capsid protein-encoding regions of the genomes of MOPV1, MOPV2, and HOPV are most similar to LuIII, a parvovirus of unknown species origin isolated as a contaminant of a human lung culture (16, 25). In this paper we will refer to MOPV, HOPV, and LuIII collectively as orphan parvoviruses.

The potential effects of orphan parvoviruses on research are largely unknown. MOPV1 demonstrates cytolytic activity against cloned cytotoxic T cells in vitro (19), suggesting that mice infected with MOPV may have altered immune responses. Precedence for this exists in that other rodent parvoviruses have been shown to alter in vitro and in vivo lymphocyte activity (6–8, 11). In addition, parvoviruses are known to produce persistent, subclinical infections, and they frequently contaminate cell cultures and transplantable tumors (2, 9, 12, 13, 16, 21, 22). This raises concerns about orphan parvovirus contamination of these biologic systems and the effects that this contamination might have on studies that utilize these systems. Because of the potentially deleterious effects that these viruses may have on research, identification of infected laboratory animals and contaminated biological materials is critical.

A limited number of methods to diagnose orphan parvovirus infections in rodents and biological materials are currently employed. Serological evaluation for the presence of antiparvovirus antibodies has typically been used to diagnose MOPV in mice. However, serologic assays are unable to detect acute infections with MOPV in which seroconversion has not occurred, and they are not routinely used for monitoring of HOPV infections in hamsters. Histopathology can be used to diagnose acute HOPV infections in hamsters, but it cannot be used to diagnose MOPV infections in mice, since no gross or histologic lesions are associated with such infections. Virus isolation has long been considered the "gold standard" for detection of viral infections, but the method is expensive and labor-intensive and has a slow turnaround time. In addition, the sensitivity of virus isolation for the detection of MOPV, HOPV, and LuIII has not been determined. This latter concern is emphasized by the inability to propagate MOPV2 in vitro. Detection of MOPV, HOPV, or LuIII contamination of biological materials currently relies upon virus isolation or the mouse antibody production test. Mouse antibody production testing is based on the induction of antibody against a contaminating virus when the biological material is inoculated into a mouse. Serum from the mouse is collected at 3 to 4 weeks postinoculation and is screened by serologic assays currently used to diagnose mouse viral infections. In addition to the slow turnaround time, the ability of the mouse antibody production test to detect contamination by HOPV or LuIII is unknown. Given the limitations of the available diagnostic methods, a rapid, direct method for the detection of orphan parvoviruses in infected animals and biological materials is needed.

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TABLE 1. Parvovirus isolates used for determination of the specificity of the PCR assay

Isolate	Host of Origin	Reference
MOPV1	Mouse	19
HOPV	Hamster	14
LuIII	Unknown	16
MVM(i)	Mouse	6
MVM(p)	Mouse	10
H-1	Rat	28
KRV	Rat	18

In this study, gene amplification as a method for specific diagnosis of MOPV infections in mice and HOPV infections in hamsters was investigated. Primer sequences were selected from regions of the capsid genes conserved among MOPV, HOPV, and LuIII but distinct from MVM, H-1, and KRV. These primers were synthesized, and a DNA amplification procedure to identify viral DNA in infected cell cultures, tissues from mice naturally infected with MOPV, and tissues from hamsters experimentally infected with HOPV was developed. The results obtained indicate that this PCR assay provides a rapid, specific, and sensitive method for identification of MOPV in infected mice, HOPV in infected hamsters, and MOPV and HOPV in infected cell culture systems and that it may also prove to be useful for the detection of LuIII contamination of cell culture systems.

MATERIALS AND METHODS

Viral isolates and propagation. The rodent parvovirus isolates used in this study are listed in Table 1. MOPV1 was grown in murine cytotoxic T cells (CTLL-2 [ATCC TIB 214]), HOPV was grown in baby hamster kidney cells (BHK-21 [ATCC CCL 10]), LuIII and H-1 parvoviruses were grown in 324K simian virus 40-transformed human newborn kidney cells (24), the immunosuppressive strain of MVM [MVM(i)] was grown in murine T-lymphoma cells (S49.1TB.2 [ATCC TIB 30]), the prototype strain of MVM [MVM(p)] was grown in murine $A9_{2L}$ fibroblasts (27), and KRV was grown in rat glial tumor cells (C_6 Glial [ATCC CCL 107]). All cells were grown in Dulbecco's modified Eagle's medium (Hazleton, Lenexa, Kans.) containing 10% Serum-plus (JRH Biosciences, Lenexa, Kans.) at 37°C in a 10% CO_2 atmosphere, except for CTLL-2 cells, which were grown in RPMI medium (Sigma Chemical Co., St. Louis, Mo.) supplemented with 10% Serum-plus, 15 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid), 2 mM sodium pyruvate, and 10 U of human recombinant interleukin-2 (Gibco BRL, Grand Island, N.Y.) per ml of medium.

The median (50%) tissue culture infective dose for each viral stock was determined in 96-well microtiter plates by using seeding densities of 2.5×10^2 cells per well for CTLL-2 cells and 5×10^3 cells per well for all other cell lines. Concentrated viral preparations were then obtained by infecting mammalian cells at a multiplicity of infection of 0.1 and incubating cultures at 37°C. Cell pellets were collected by centrifugation (10 min at $500 \times g$) when approximately 90% of the cells exhibited cytopathic effect. Each cell pellet was resuspended in a 1/10 volume of Tris-EDTA (50 mM Tris, 10 mM EDTA [pH 8.5]) and subjected to four freeze-thaw cycles. Cellular debris was then removed by centrifugation (10 min at $1,000 \times g$). The DNA content of each viral preparation was determined by dot blot quantitation (23), and the preparations were diluted to 20 μg of DNA per ml to equilibrate the DNA template concentration for the PCR assays.

Oligonucleotide primers. Two oligonucleotide primers, 3759f (5′-GCAG CAATGATGTAACTGAAGCT-3′) and 4018r (5′-CCATCTGCCTGAATCA TAGCTAA-3′), were synthesized at the DNA Core Facility, University of Missouri, Columbia. Primer sequences were selected on the basis of sequence alignments generated by the EuGene software package (Baylor College of Medicine, Houston, Tex.). All sequence data were obtained from GenBank with the exception of the sequences for MOPV1, MOPV2, and HOPV, which were determined in our laboratories (5), and the sequence for KRV (29) (kindly provided by Lisa Ball-Goodrich, Yale University, New Haven, Conn.). Primers were designed from segments which were highly conserved among MOPV1, MOPV2, HOPV, and LuIII but exhibited maximum heterology with all other rodent parvoviruses.

PCR amplification. All reactions were performed in a 50-μl volume in an automated Perkin-Elmer model 9600 thermocycler. Each reaction mixture contained various amounts of template DNA (see below), 1 μM each oligonucleotide primer, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCl, 0.2 mM each deoxynucleoside triphosphate (dATP, dCTP, dGTP, and dTTP), and 2.0 U of *Taq* polymerase (Boehringer Mannheim, Indianapolis, Ind.). PCR consisted of 30 s of denaturation at 94°C followed by 35 cycles of 2 s of denaturation at 94°C, 2 s of annealing at 55°C, and 30 s of elongation at 72°C. PCR products (10 μl) were electrophoretically separated in a 3% NuSieve agarose gel (FMC BioProducts, Rockland, Maine), stained with ethidium bromide, and visualized by UV light. DNA markers of known sizes were run on each gel to facilitate determination of the sizes of the reaction products.

To test the specificity of the assay, PCR was performed with 100 ng of template DNA from concentrated viral preparations of MOPV1, HOPV, LuIII, MVM(i), MVM(p), H-1, and KRV. The specificity of each assay was also evaluated with viral stocks of K virus, mouse adenovirus 2, mouse cytomegalovirus, mouse hepatitis virus, polyomavirus, pneumonia virus of mice, reovirus 3, and Sendai virus, all of which were obtained from the University of Missouri Research Animal Diagnostic and Investigative Laboratory (Columbia, Mo.). All viral preparations were of tissue culture origin except for K virus, which was of mouse liver tissue homogenate origin (Charles River Laboratories, Boston, Mass.), and Sendai virus, which was cultivated on embryonated chicken eggs.

To test the sensitivity of the PCR assay, 10-fold serial dilutions of MOPV1 or HOPV DNA, ranging from 100 ng to 1 fg, were used as template in the PCR assay. To simulate diagnostic conditions, the PCRs for the MOPV sensitivity determination were performed in the presence of 1.25 μg of DNA extracted from the kidney of a heterozygote nude mouse from a colony serologically negative for MOPV. Likewise, the PCRs for the HOPV sensitivity determination were performed in the presence of 1.25 μg of DNA extracted from the kidney of a mock-infected Syrian hamster.

Animals. Ten 6-week-old, heterozygote nude (*nu*/+) female mice were obtained from a colony determined to be free of parvovirus infection by repeated serologic testing, and the same number of age-, strain-, and sex-matched mice were obtained from a colony that was enzootically infected with MOPV as evidenced by repeated serologic testing. Mice were euthanized and bled by cardiocentesis, and liver, kidney, uterus, intestine, and brain tissues were collected from each mouse and frozen at −80°C.

Breeding pairs of Syrian hamsters were obtained from a colony determined to be free of parvovirus infection by serologic testing and were housed in microisolator cages in accordance with the National Institutes of Health's *Guide for the Care and Use of Laboratory Animals* (20). Two groups of six 3-day-old hamsters were inoculated oronasally with 10-μl volumes containing either 10^4 50% tissue culture infective doses of HOPV or Tris-EDTA. At 7 days postinoculation, hamsters were euthanized and liver, kidney, testicle or uterus, intestine, and brain tissues were collected from each hamster and frozen at −80°C.

DNA isolation. DNA was extracted from mouse and hamster tissues with a QiAmp Tissue Kit (Qiagen Inc., Chatsworth, Calif.) by following the manufacturer's instructions. The DNA content and purity of the tissue DNA extracts were determined by measuring the A_{260}/A_{280} optical density ratio with a Perkin-Elmer Lambda 3B UV-visible spectrum spectrophotometer. The amount of DNA used as the template in PCR testing of tissues from mice and hamsters was 1.25 μg.

DNA sequencing. PCR products amplified from mouse and hamster tissues were purified on 3.5% polyacrylamide gels, and the sequences were determined by the *Taq* dideoxy-chain termination method with a commercially available kit (*Taq* dye deoxy terminatory cycle sequencing kit; Applied Biosystems, Inc., Foster City, Calif.). Sequence data were analyzed with the EuGene software package.

Serology. Serum samples collected from the mice were diluted 1:5 in normal saline, heat inactivated at 55°C for 30 min, and tested by hemagglutination inhibition (HAI). Duplicate wells containing twofold serial dilutions of each serum sample, ranging from 1:10 to 1:80, were prepared in 96-well V-bottom microtiter plates with 0.1% bovine serum albumin in phosphate-buffered saline as a diluent. An equal volume (25 μl per well) containing 8 hemagglutination units of MOPV1 was added to one set of dilutions per serum sample, and an equal volume of diluent was added to the other set. After a 1-h incubation at 35°C, 25 μl of a 0.5% mouse erythrocyte suspension was added to each well, and 40 min later the wells were examined for hemagglutination. An HAI titer of 1:20 or greater was interpreted as a positive result.

RESULTS

Evaluation of PCR primers. Comparisons of the nucleotide sequences of MOPV1, MOPV2, HOPV, and LuIII with those of the other rodent parvoviruses revealed several regions conserved among the orphan parvoviruses but heterologous with regard to the prototypic rodent parvoviruses. On the basis of these comparisons, a primer set specific for MOPV, HOPV, and LuIII was designed. The primer set consisted of a forward primer, 3759f (5′-GCAGCAATGATGTAACTGAAGCT-3′), and a reverse primer, 4018r (5′-CCATCTGCCTGAATCAT AGCTAA-3′). Primers were designed to provide maximum

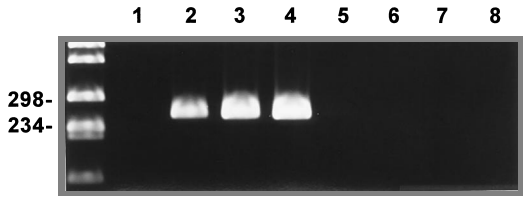

FIG. 1. Ethidium bromide-stained agarose gel demonstrating the specificity of the PCR assay when used with rodent parvoviruses. PCR products resulting from amplification using 100 ng of viral DNA as a template are shown. Lanes: 1, no-template control; 2, MOPV1; 3, HOPV; 4, LuIII; 5, MVM(i); 6, MVM(p); 7, H-1; 8, KRV. The migration of molecular size markers is shown in the leftmost lane of the gel. Sizes are in base pairs.

heterology with regard to the aligned regions from the other rodent parvoviruses and were named according to the MOPV1 genomic sequence position (1) of the nucleotide at the 5′ end of each primer.

The specificity of the primer set was determined by using as the template preparations of parvoviruses, including MOPV1, HOPV, LuIII, MVM(i), MVM(p), H-1, and KRV, and preparations of other viruses that infect rodents, including K virus, mouse adenovirus 2, mouse cytomegalovirus, mouse hepatitis virus, polyomavirus, pneumonia virus of mice, reovirus 3, and Sendai virus. The primer set amplified only MOPV1, HOPV, and LuIII viral DNA and produced the expected 260-bp product (Fig. 1). The sensitivity of the PCR assay was determined by amplifying 10-fold serial dilutions of viral DNA in the presence of 1.25 μg of DNA isolated from the kidney of either a mouse from an MOPV-seronegative colony or a mock-infected hamster. The PCR assay detected a 260-bp product when 10 pg of MOPV1 viral DNA was used as a template (Fig. 2A) or when as little as 1 pg of HOPV viral DNA was used as a template (Fig. 2B).

PCR of DNA from tissue samples. DNA amplifications with the primer set were performed on DNA extracted from the tissues of mice obtained from either an MOPV-seropositive colony or an MOPV-seronegative colony. The PCR assay amplified the expected 260-bp product in tissues from 10 of 10 mice from the MOPV-seropositive colony. Of the 10 PCR-positive mice, 5 were positive with both kidney and intestinal DNA as the template, 4 were positive with only intestinal DNA as the template, and 1 was positive with only kidney DNA as the template. No amplified product was detected for any of the mice from the MOPV-seronegative colony when kidney or intestinal DNA was used as a template (Fig. 3A). DNA extracts of liver, uterine, and brain tissues from two of the PCR-positive mice were also tested by the PCR assay, and the 260-bp product was amplified from each of these tissues from both mice, while no product was amplified from the same tissues from two of the PCR-negative mice (data not shown). HAI testing was performed on serum obtained from the mice used in our study to provide corroborative evidence of their MOPV statuses. The HAI test specifically detects antibody directed against the MOPV viral hemagglutinin and confirms exposure to MOPV. Serum from 7 of the 10 mice from the MOPV-seropositive colony inhibited hemagglutination by MOPV, while serum from 0 of 10 mice from the MOPV-seronegative colony inhibited hemagglutination by MOPV.

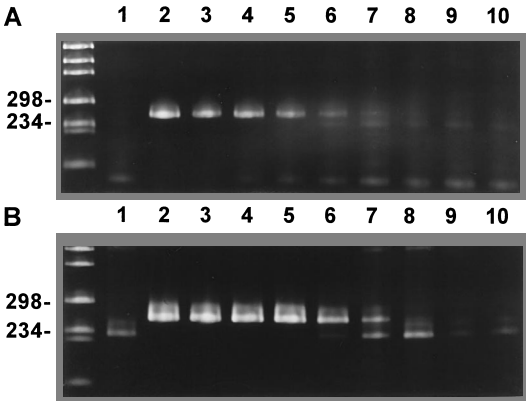

FIG. 2. Ethidium bromide-stained agarose gel demonstrating the sensitivity of the PCR assays. Shown are PCR products resulting from amplification of serial dilutions of MOPV1 viral DNA in the presence of 1.25 μg of kidney DNA from an uninfected mouse (A) and HOPV viral DNA in the presence of 1.25 μg of kidney DNA from an uninfected hamster (B). Lanes: 1, kidney DNA only; 2, 100 ng of viral DNA; 3, 10 ng of viral DNA; 4, 1 ng of viral DNA; 5, 100 pg of viral DNA; 6, 10 pg of viral DNA; 7, 1 pg of viral DNA; 8, 100 fg of viral DNA; 9, 10 fg of viral DNA; 10, 1 fg of viral DNA. The migration of molecular size markers is shown in the leftmost lane of each gel. Sizes are in base pairs.

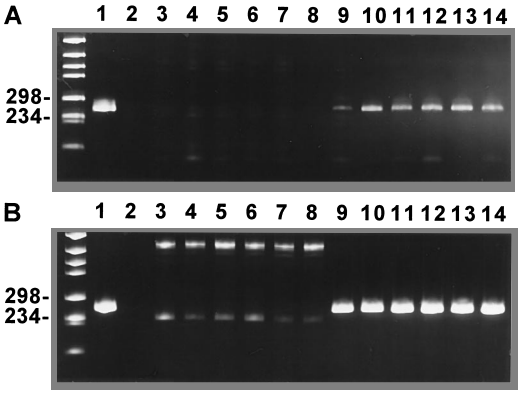

FIG. 3. PCR amplification of kidney DNA from mice from the MOPV-seronegative colony, mice from the MOPV-seropositive colony, mock-infected hamsters, and hamsters experimentally infected with HOPV. (A) PCR products resulting from amplification of 1.25 μg of kidney DNA from six of the mice from the MOPV-seronegative colony (lanes 3 to 8) and six of the mice from the MOPV-seropositive colony (lanes 9 to 14). (B) PCR products resulting from amplification of 1.25 μg of kidney DNA from mock-infected hamsters (lanes 3 to 8) and HOPV-inoculated hamsters (lanes 9 to 14). Lane 1 is the positive control and lane 2 is the no-template control for each assay. The migration of molecular size markers is shown in the leftmost lane of each gel. Sizes are in base pairs.

DNA amplifications with the primer set were performed on DNA extracted from the tissues of hamsters that were either experimentally infected with HOPV or mock infected with Tris-EDTA. The PCR assay generated the expected 260-bp product from six of six HOPV-infected mice when DNA extracted from kidney or intestinal tissues was used as the template, while no amplified product was detected for any of the mock-infected hamsters when kidney or intestinal DNA extracts were used as the template (Fig. 3B). DNA extracts of liver, uterine, and brain tissues from two of the PCR-positive hamsters were also tested by the PCR assay, and the 260-bp product was amplified from each of these tissues for both hamsters, while it was not amplified from the same tissues of two mock-infected hamsters (data not shown).

The PCR products amplified from the MOPV-seropositive mice and the HOPV-infected hamsters were sequenced to confirm the identities of the products as MOPV and HOPV sequences, respectively. The sequence for each product aligned exactly with the previously determined capsid sequence for each virus (data not shown).

DISCUSSION

In this study a PCR assay to detect MOPV, HOPV, and LuIII viral DNA was developed. An oligonucleotide primer set specific for this group of viruses was designed and synthesized on the basis of nucleotide sequence comparisons of the orphan parvoviruses and the prototypic rodent parvoviruses. The PCR assay was evaluated for specificity by testing the primer set against a panel of parvoviruses and a panel of other viruses that infect rodents. The expected 260-bp product was produced only when MOPV1, HOPV, or LuIII viral DNA was present, indicating that the assay was specific for these viruses.

The PCR assay was able to detect the expected PCR product when either 10 pg of DNA of the MOPV1 viral preparation or 1 pg of DNA of the HOPV viral preparation was used as the template. A 10-fold difference in sensitivity was also observed when the same two templates were used in a PCR assay that detects all autonomous rodent parvoviruses (data not shown). The latter PCR assay utilizes primers designed on the basis of segments of the viral genome that are highly conserved among MOPV, HOPV, LuIII, MVM, H-1, and KRV; therefore, the same level of sensitivity would be expected with each virus when equivalent amounts of viral DNA are used as the template (3). These data suggest that the difference in sensitivity observed is due to viral DNA concentrations in the samples tested and not to an inherent difference in the abilities of this PCR assay to detect MOPV and HOPV.

The PCR assay was able to detect viral DNA in mice naturally infected with MOPV and hamsters experimentally infected with HOPV. The assay was able to detect MOPV viral DNA in tissues from 10 of 10 6-week-old mice from an MOPV-seropositive colony, and it did not detect viral DNA for any of the 10 age-, strain-, and sex-matched mice from an MOPV-seronegative colony. Viral DNA was detected for 9 of the 10 PCR-positive mice with intestinal DNA as the template, compared with 6 mice that were PCR positive with kidney DNA as the template. These data may reflect an increased ability of intestinal tissues to support MOPV replication in 6-week-old mice, since parvoviruses require rapidly dividing cells for replication (25). The fact that not all PCR-positive mice could be detected by the use of either kidney or intestinal DNA alone as the template suggests that it may be appropriate to test both intestinal and kidney DNA to identify a larger percentage of MOPV-positive mice. Seven of the 10 PCR-positive mice were confirmed positive by the MOPV HAI assay, while all PCR-negative mice were negative by the MOPV HAI assay. The fact that only 7 of the 10 PCR-positive mice were found to be seropositive may be due to the decreased sensitivity of the HAI assay or to a lack of seroconversion in mice recently exposed to MOPV. It is also of interest that the PCR assay detected MOPV DNA in the uterus and brain tissues of two of the mice from the MOPV-seropositive colony, whereas previous studies have been unable to detect MOPV DNA in these tissues by in situ hybridization (17, 26). This finding probably reflects the increased sensitivity of PCR compared with in situ hybridization in their relative abilities to detect viral DNA (15). As expected, the PCR assay was able to detect HOPV DNA in tissues from six of six hamsters experimentally infected with HOPV but it did not detect viral DNA in tissues from any of the mock-infected hamsters.

The PCR assay described in this report may also be utilized to detect contamination of biological materials. Detection of MOPV, HOPV, or LuIII contamination of biological materials currently relies upon virus isolation or mouse antibody production testing. As an alternative to these time-consuming and expensive techniques, PCR represents a rapid, specific, and sensitive assay that is independent from the immune response. The specificity and sensitivity of the PCR assay reported here suggest that this assay should be useful for detecting contamination of biological materials by MOPV and HOPV and that it may be useful for detection of LuIII contamination of these materials.

In summary, a PCR assay specific for MOPV, HOPV, and LuIII was able to distinguish these viruses from the prototypic rodent parvoviruses, as well as from other rodent viruses. The results of our study indicate that this PCR assay should be useful as a diagnostic tool for rapid detection of MOPV infection in mice, HOPV infection in hamsters, and orphan parvovirus contamination of cell culture systems.

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