Comparative Medicine
Copyright 2004
by the American Association for Laboratory Animal Science

Vol 54, No 4
August 2004
Pages 410-417

Reverse Transcription-Polymerase Chain Reaction Detection and Nucleic Acid Sequence Confirmation of Reovirus Infection in Laboratory Mice with Discordant Serologic Indirect Immunofluorescence Assay and Enzyme-Linked Immunosorbent Assay Results

Mina Hicks Wright,[1] Lee M. Cera, DVM, PhD,[1] Nicolene A. Sarich,[2] and John A. Lednicky, PhD[2,*]

Reovirus infections are typically subclinical in weaned mice, and are best detected using serologic tests. After exposure to the soiled bedding of some mice obtained from various sources, numerous sentinel mice tested reovirus seropositive by use of indirect immunofluorescence assays (IIFA) in our institution. A major commercial rodent pathogen testing laboratory verified our IIFA results, but since the same samples were reovirus seronegative using their "more specific" enzyme-linked immunosorbent assay (ELISA), the IIFA results were reported as "false positives." As past in-house observations suggested transmission of the virus to sentinel and other animals, we sought to determine whether the IIFA results were always "false positives." An opportunity to test this notion arose after receipt of reovirus IIFA-positive transgenic mice from an academic source. Using reverse transcriptase-polymerase chain reaction (RT-PCR) assays, the presence of reovirus RNA was detected in fecal specimens taken from some sentinel animals that subsequently seroconverted from IIFA-negative to IIFA-positive for reovirus. The virus could not be isolated by use of tissue culturing methods. Nucleotide sequence analysis established the presence of unique reovirus sequences. These results indicate that contemporary reovirus infections may not be detected by use of some serologic tests, and that RT-PCR analysis may be useful for confirmation of active reovirus infection in certain situations.

Mammalian reoviruses (genus *Orthoreovirus*) are non-enveloped 78- to 82-nm-diameter viruses that contain a genome of 10 double-stranded RNA (dsRNA) segments (10). There are three reovirus serotypes, designated Reo-1, Reo-2, and Reo-3, which can be differentiated by use of serologic procedures (13, 16). Reoviruses have wide geographic distribution, and have been detected in nearly all mammals, including humans and house mice (*Mus musculus*), that have been tested (20). Natural infections in mice are almost always subclinical (2, 18, 20). Reovirus infections occur throughout the year and are frequently described as short-lived in mammals, with clearance within 1-2 weeks under experimental virus challenge conditions (15). These viruses are commonly found in environmental water sources (1, 3, 12, 17), and human fecal contamination may be a major source of reovirus in water supplies (9). From October 1999 to January 2002, we observed that 202 of 984 sentinel mice tested positive for reovirus exposure/infection by use of an indirect immunofluorescence assay (IIFA) after they were exposed to soiled bedding obtained from research mice that had been acquired from various commercial and academic sources. The sentinel mice (obtained from two commercial sources) were IIFA-seronegative for reovirus prior to study. Many serum samples obtained from the sentinel mice were sent to a large commercial rodent pathogen-testing laboratory for independent evaluation, where they were analyzed by use of a "more sensitive" industry-standard enzyme-linked immunosorbent assay (ELISA) as well as an IIFA. Whereas the commercial testing laboratory repeatedly verified our IIFA results, all ELISA results were always negative, and the positive IIFA results were reported as "false positives." Identical negative ELISA results were obtained when some matched serum samples were sent to a major midwestern university testing facility.

During the same period (October 1999 to January 2002), results of IIFAs conducted in house also indicated occasional reovirus transmission to research mice in cages without Micro-Isolator™ (Lab Products Inc., Seaford, Del.) tops after reovirus-seropositive mice were placed in the same rooms. Inter-cage reovirus transmission was effectively prevented by mandating use of Micro-Isolator™ (Lab Products Inc.) tops for all cages (data not shown).

Since there was evidence of reovirus transmission to sentinel (and other) animals, we sought to determine whether the IIFAs were detecting true reovirus infections. An opportunity arose to resolve this question on recent receipt from an academic source (year 2002) of transgenic mice that were apparently shedding reovirus, as sentinel mice exposed to soiled bedding obtained from the cages of transgenic mice had seroconverted to reovirus on the basis of results of IIFAs.

Virus isolation remains the gold standard for verifying that an

Received: 12/16/03. Revision requested: 3/08/04. Accepted: 3/30/04.
[1]Department of Comparative Medicine, and [2]Department of Pathology, Loyola University Medical Center, 2160 South First Street, Maywood, Illinois 60153.
[*]Corresponding author.

animal has active infection with a particular virus. During the acute phase of infection and shortly thereafter, primary reovirus isolates may be obtained from mouse fecal specimens. This is usually accomplished by use of tissue culturing methods. A variety of commercially available cell types have been used for this purpose, though monkey kidney cells are generally considered the most permissive (14, 18). Nevertheless, techniques for virus cultivation are often insensitive, and often result in inability to detect reovirus. Precisely for this reason, various diagnostic strategies based on detection of reovirus RNA have been developed, and many rely on sensitive and specific reverse transcription-polymerase chain reaction (RT-PCR) assays (7, 11, 17, 19, 21).

In the study reported here, we used recently described RT-PCR methods (7, 17) and attempted reovirus isolation to resolve whether IIFA-positive, ELISA-negative sentinel animals had been exposed to reovirus. We report that the animals indeed had been infected, that viral isolation procedures by use of various cell lines were ineffective, and that the RT-PCR method combined with DNA sequencing was essential for sensitive detection and confirmation of reovirus shedding. The nucleotide (nt) sequences of the reovirus that was detected by use of RT-PCR analysis appeared unique and more closely related to recently described reovirus strains from the 1990s onward than were the commonly studied reovirus type-1 Lang, type-2 Jones, and type-3 Dearing strains, which were isolated in the 1950s.

Materials and Methods

Animals and husbandry. Mice were used in accordance with the humane care and use guidelines of the Committee on the Care and Use of Laboratory Animal Resources, National Research Council. All procedures were pre-approved by the Loyola University Medical Center Institutional Animal Care and the Rodent User's Committees. Mice were housed in cages and bedding that were autoclaved prior to use, and were fed irradiated Harlan Tekland LM-485 rodent diet (Harlan, Indianapolis, Ind.). Twelve newly acquired transgenic mice (from a non-commercial source) were placed in a quarantine room of our institution. Six virus antibody-free, 8-week-old CD-1 mice (Charles River Laboratories, Inc., Wilmington, Mass.) were used as sentinel animals. Results of confirmatory in-house IIFAs indicated that, prior to use, the CD-1 mice were antibody negative to mouse hepatitis virus and reovirus, and their feces were RT-PCR negative for reovirus. Two sentinel mice in separate cages with Micro-Isolator™ (Lab Products Inc.) tops were shelved, with each cage containing 4 transgenic mice. Soiled bedding from the transgenic mice was added weekly to the cages containing sentinel mice.

Serum samples and fecal specimens from sentinels. One and three months after exposure to soiled bedding, retro-orbital blood sample collection was performed to obtain serum for serologic testing. Serum was separated from clotted blood, followed by centrifugation in a microtainer tube with serum separator and without anticoagulant (Beckton Dickinson, Franklin Lakes, N.J.), then storage at –70°C. Fecal specimens were collected monthly for 3 months for virus isolation attempts. Fecal pellets were separated from bedding and debris and weighed, and aliquots were stored in sterile containers at –70°C.

Cell lines. African green monkey kidney cells (Vero and CV-1), tertiary rhesus monkey kidney cells (LLC-MK2), and murine connective tissue cells (L929) were grown in monolayer culture in Dulbecco's modified Eagle's medium (DMEM; Cellgro, Mediatech Inc., Herndon, Va.) supplemented to contain 10% heat-inactivated fetal bovine serum (HI-FBS; Cellgro), 2 m*M* L-glutamine, 100 U of penicillin/ml, and 100 µg of streptomycin/ml. Hamster BHK-21 cells were similarly grown in (50:50 [vol:vol]) EMEM/Hank's modified Eagle's medium (EMEM/HMEM; Invitrogen Corp., Carlsbad, Calif.) with 10% HI-FBS.

Control reovirus stock. Reovirus-3 Dearing was a laboratory stock that is available from the American Type Culture Collection (Manassas, Va.) and was prepared in BHK-21 cells. The morphology of the virus was confirmed by use of electron microscopy, and the genetic identity of the stock virus was verified by use of RT-PCR analysis with the primers mentioned herein, followed by nucleic acid analysis. It had a 100% match with the corresponding sequences deposited at GenBank. The stock virus was quantitated by use of plaque assay in L929 cells (4, 5); plaques were read at postinfection day 6, and indicated viral titer of 2×10^7 plaque-forming units/ml.

Virus isolation attempts. Efforts were made to isolate virus in cells grown in medium containing 10% serum, as well as in parallel cultures of cells in serum-free medium containing trypsin, as the addition of exogenous protease facilitates reovirus infection in many restrictive cells (6). For isolation of virus in medium containing serum without trypsin activation of the virus, a 15% (wt/vol) feces suspension in phosphate-buffered saline (PBS) was prepared, and centrifuged at 10,000 ×*g* for 10 min at room temperature. The supernatant was passed through a 0.45-µm filter, and the filtrate was then diluted 1:20 in complete growth medium. Growth medium was removed from cells growing in 25-cm^2 flasks, and the cells were inoculated with 0.7 ml of the filtered feces suspension. After incubation for 1 h at 37°C, 5 ml of complete growth medium was added and the cells were incubated for 24 to 48 h (reovirus control) for up to 21 days (test specimens), with feedings at 4-day intervals. Under these conditions, all CV-1, LLC-MK2, and Vero cells and most BHK-21 cells were killed within 48 h after infection by addition of 10^4 reovirus-3 particles.

In parallel with these attempts to isolate virus in cells grown in serum-containing medium, "activated feces" (fecal specimens pre-digested with trypsin) were also prepared essentially as described (8). Briefly, a 15% (wt/vol) feces suspension in serum-free growth medium containing 15 mg of trypsin/ml was prepared, and was centrifuged at 10,000 ×*g* for 10 min at room temperature. The supernatant was passed through a 0.45-µm filter, then the filtrate was diluted 1:20 in serum-free medium containing 2 µg of trypsin per milliliter and was incubated at 37°C for 30 min to form "activated feces." Cells (in 25-cm^2 flasks) used for virus recovery were washed once with sterile PBS for 15 min before inoculation, then were bathed in 0.7 ml of serum-free medium containing 2 µg of trypsin per milliliter. The growth medium was subsequently removed, and the cells were inoculated with a 0.7-ml aliquot of activated feces and were incubated at 37°C for 1 h. Subsequently, 5 ml of serum-free growth medium containing 2 µg of trypsin per milliliter was added. Under these conditions, the reovirus-3 control preparation killed all cells within 48 h. Cell deterioration caused by the addition of trypsin prevented incubation times past 72 h in BHK-21, CV-1, and Vero cells, whereas LLC-MK2 cells survived for up to 14 days when refed at 3-day intervals with serum-free medium containing 2 µg of trypsin per milliliter.

Oligonucleotide primers. A segment of the reovirus *lambda*

3 (*L3*) gene that encodes part of the major core L1 protein was amplified, using REOL3F (forward; 5'-CAGTCGACACATTTG TGGTC-3') and REOL3R (reverse; 5'-GCGTACTGACGTGGAT CATA-3'), which yield a 320-bp product (17). A separate reovirus subgenomic sequence from the *L1* gene that partially encodes the minor core protein lambda 3 was also amplified. The primers for the primary *L1* gene RT-PCR assay L1.rv5 (forward; 5'-GCATCCATTGTAAATGACGAGTCTG-3') and L1.rv6 (reverse; 5'-CTTGAGATTAGCTCTAGCATCTTCTG-3'), which form a 416-base pair (bp) RT-PCR product (7). The primers for the secondary *L1* gene PCR assay were L1.rv7 (forward; 5'-GCTA GGCCGATATCGGGAATGCAG-3') and L1.rv8 (reverse; 5'-GTCTCACTATTCACCTTACCAGCAG-3'), which form a 344-bp RT-PCR product (7).

Nucleic acid extractions. Attempts were made to isolate viral RNA from three sources: cell culture supernatant, infected cells, and fecal specimens. Viral RNA was purified from cell culture supernatant by use of a viral RNA kit (Qiagen Inc.) following the manufacturer's recommendations. Total RNA was extracted from infected cells using a Qiagen RNeasy Kit and following the manufacturer's recommendations. Fecal specimens were emulsified in PBS to form a 10% (wt/vol) solution and were centrifuged at low speed, then the RNA was extracted from 140 µl of the clarified supernatant using a viral RNA kit. Since animals experimentally infected with reovirus-3 were not available, reovirus particles were added to feces obtained from a known reovirus-negative sentinel animal (pretested by use of serologic testing and RT-PCR assay of the feces) for positive-control determinations; individual fecal specimens for each RNA extraction contained 10^3 infective reovirus particles.

Reverse transcriptase-polymerase chain reaction analysis. Reverse transcription reactions were performed in a PTC-200 DNA Engine Peltier Thermal Cycler (M. J. Research, Inc., Waltham, Mass.), using Qiagen Omniscript reverse transciptase. Two methods were tested: reverse transcription primed by specific forward and reverse primers and reverse transcription in the presence of 250 p*M* random hexamers (Ambion, Austin, Tex.). The former procedure was superior in our hands and is the method discussed. A 15-µl aliquot of RNA and primers at a concentration of 1 µ*M* were heated to 95°C for 2 min, then were placed on ice. Following the manufacturer's recommendations, chilled Omniscript buffer, dNTP mix, RNase-free water, 10 U of SUPERase-In RNase inhibitor (Ambion), and 4 U of Omniscript RT were added to a 10-µl aliquot of the annealed RNA-primer mixture (final total volume of 20 µl). The RT reaction was then incubated at 37°C for 1 h. An RT control that contained water in place of purified nucleic acids was run in parallel and served as a negative control. The PCR reactions (50 µl) were performed using Eppendorf MasterTaq (Brinkmann, Westbury, N.Y.) and contained 2.5 U of *Taq* DNA polymerase, 50 pmoles of each primer, 0.2 m*M* each dNTP, 1X MasterTaq buffer (1.5 m*M* Mg^{+2}), and 5 µl of cDNA. The PCR reactions were initially denatured at 94°C for 2 min, then were cycled 44 times under the following conditions: 94°C for 15 sec, 50°C for 20 sec, and 72°C for 20 sec, and ended with one terminal annealing step of 94°C for 3.5 min.

Sequencing of DNA. The PCR amplicons were purified from a 2% agarose gel using a QIAquick Gel Extraction Kit (Qiagen), and were sequenced by use of the same PCR primers used for reovirus detection and with LI-COR (Lincoln, Nebr.) automated sequencing and dye-terminator chemical analysis at the Loyola University Medical Center Molecular Core Research Facility.

Indirect immunofluorescence assay procedure and antibodies. The BHK-21 cells infected with Reovirus-3 Dearing were used for positive controls, and non-infected BHK-21 cells were used for negative controls. The BHK-21 cells were seeded onto glass slides, air-dried, fixed with ice-cold acetone for 20 min, and stored at –70°C. Mouse anti-reovirus antibody 93712-20 (Section of Comparative Medicine, Yale University School of Medicine, New Haven, Conn.) and matched pre-immune serum (Yale University School of Medicine) were used as primary antibodies. Fluorescein isothiocyanate-conjugated goat anti-mouse IgG (Antibodies Inc., Davis, Calif.) was used as a secondary antibody. Cells were reacted with a 1:10 dilution of primary antibody at 37°C for 15 min, then were rinsed and soaked in PBS for 5 min. The cells were then reacted with secondary antibody (1:50 dilution) at 37°C for 15 min, after which they were rinsed and soaked in PBS for 5 min. Finally, the cells were mounted with 50% glycerol in PBS, cover-slipped, and viewed using an Olympus BX60 fluorescent microscope (Olympus America, Inc., Melville, N.Y.). Aliquots of the sera were also sent to a major rodent testing laboratory for confirmation testing.

Enzyme-linked immunosorbent assay (ELISA). Sera from laboratory and sentinel mice of this study were sent to a major commercial rodent testing laboratory for evaluation by use of an industry-standard ELISA.

Results

After exposure to soiled bedding, in-house IIFA results indicated that 6 of 6 sentinel animals of this study had seroconverted to reovirus positive. An IIFA result for sentinel mouse 02-11, using serum collected 3 months after exposure to soiled bedding, is shown in Fig. 1, and is representative of our findings for the 5 other sentinels of this study. The serum samples were IIFA-negative 1 month after exposure to soiled bedding. The fluorescence intensity was somewhat reduced compared with that of the positive control, though the staining pattern was identical. For verification and quality-control testing, matched serum samples were sent to a major commercial rodent testing facility for IIFA and ELISA evaluation. Whereas the commercial laboratory obtained reovirus-positive IIFA results identical to ours, all ELISA results were negative. The IIFA results were reported as "false positives" by the commercial laboratory.

Virus isolation attempts were unsuccessful using feces collected monthly up to 3 months after exposure to soiled bedding. Whereas the control Reovirus-3 Dearing stock replicated with high efficiency in simian and rodent cells, regardless of the presence of trypsin, reovirus-type cytopathic effects were not detected in any of the cells tested, even with prolonged incubation (21 days for cells in serum-containing medium; LLC-MK2 cells in serum-free medium plus trypsin for 14 days). Use of serial RT-PCR assays performed at 24-h intervals for up to 2 weeks also failed to detect reovirus except in cells inoculated with the Reovirus-3 Dearing control preparation.

Unlike our failure to isolate reovirus by use of cell culturing methods, reovirus RNA was detected by use of RT-PCR analysis in fecal specimens collected from the six sentinels 2 months after exposure to soiled bedding. Reovirus RNA was not detected by use of RT-PCR analysis in feces collected 1 and 3 months after exposure to soiled bedding. Results representative for the six sentinels (Fig. 2) indicate that reovirus RNA was detected in a fe-

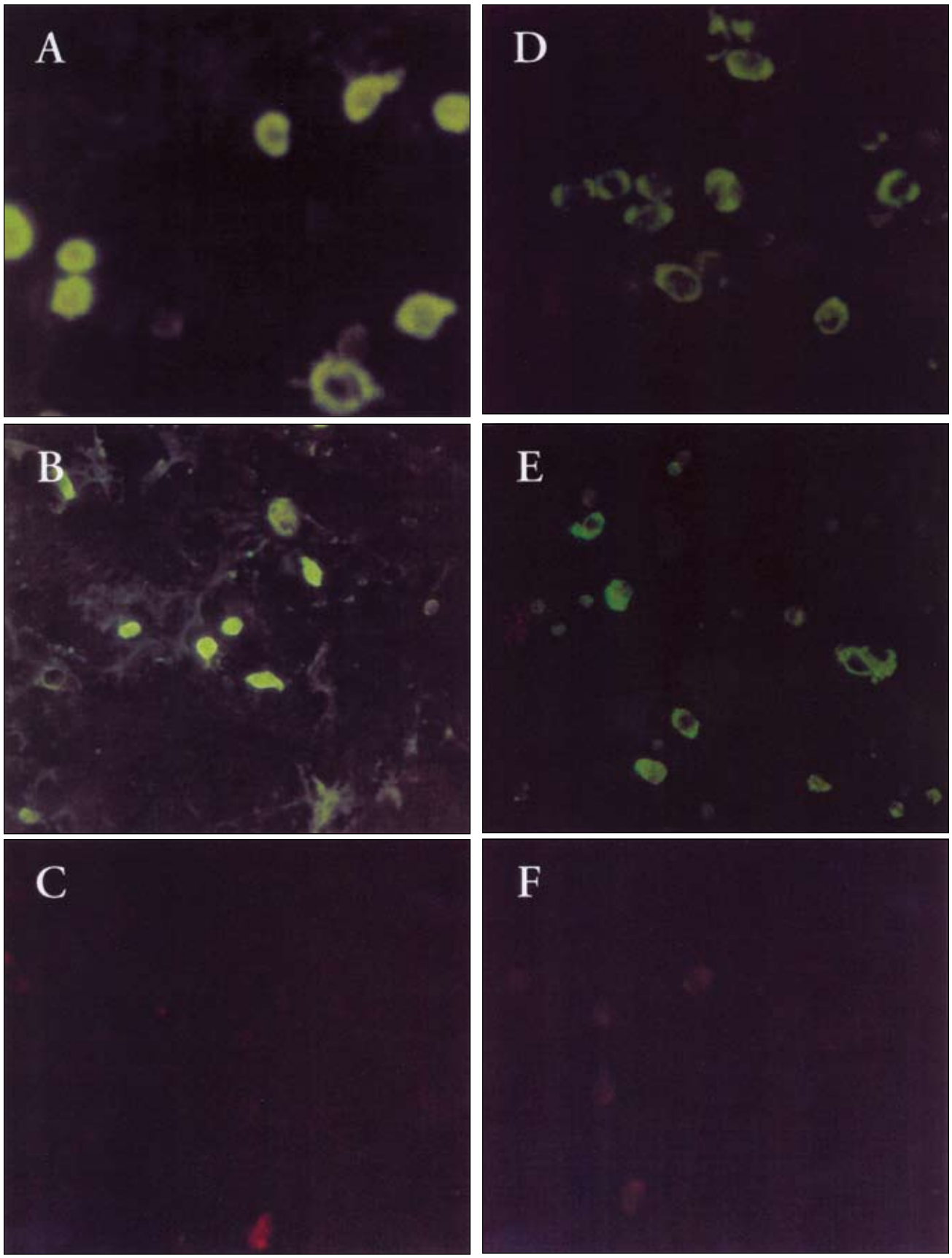

Figure 1. Indirect immunofluorescence assay (IIFA) for reovirus antibody. Panels: (A) and (B) positive-control serum; (C) negative-control serum; (D) and (E) serum from animal 02-11; (F) serum from pre-immune sentinel animal. Original magnifications: (A) and (D) 200×; (B) (C) (E) and (F)

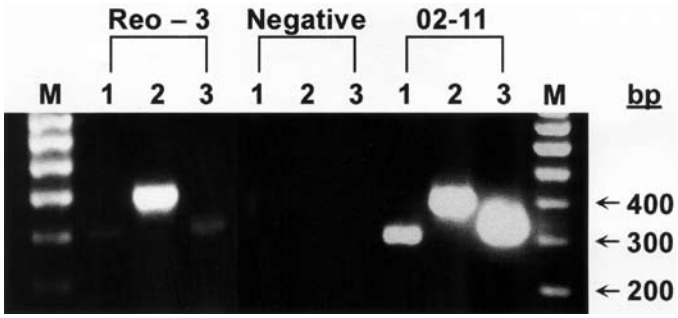

Figure 2. Reverse transcriptase-polymerase chain reaction (RT-PCR) detection of reovirus in a fecal specimen from animal 02-11 and its analysis by use of ethidium bromide 2% agarose gel electrophoresis. Molecular weight (MW) markers are loaded on the extreme right and left of the gel in lanes topped with the letter M. The size (base pairs [bp]) of relevant MW markers is indicated on the right side of the gel. Shown are the results obtained using 5 µl in each lane of the positive control (Reo-3) and negative control (Negative) RT-PCR-amplified products, and 10 µl in each lane of the RT-PCR-amplified products obtained for a fecal specimen from animal 02-11. Primer pairs REOL3F and REOL3R, L1.rv5 and L1.rv6, and L1.rv7 and L1.rv8 formed RT-PCR products sized 320, 416, and 344 bp, and these are loaded in lanes marked 1, 2, and 3, respectively.

cal specimen to which Reovirus-3 Dearing was added and in the feces of animal 02-11, but not in the negative-control specimen (feces from a freshly acquired sentinel animal that was RT-PCR negative in a prior pre-test and was IIFA-negative for reovirus). Nucleotide sequence analyses were performed on the L1 and L3 PCR products from animals 02-11 and 02-17, two of the sentinels of this study that were in separate cages. Both products yielded identical sequence results, which confirmed presence of the same reovirus strain in the feces of the two animals.

GenBank accession numbers for the reovirus sequences of this study are: *L1* gene segment, AY496276, and *L3* gene segment, AY494858. Additional L1 sequence analysis of the other four sentinels yielded sequences identical to those obtained for 02-11 and 02-17. Finally, the same sequences were detected after a separate RNA extraction from fecal specimen 02-11 and other fecal specimens, and concomitantly repeated RT-PCR and sequence analyses (data not shown).

Incomplete GenBank sequence entries make it impossible to fully compare the entire PCR amplicon sequences generated in this study with a large pool of sequences. For large-scale database analyses, we were limited to an internal 249-bp sequence within the L3 amplicon and a 365-bp internal sequence of the L1 amplicon. On analysis using the programs Align 2 sequences (bl2seq) and Nucleotide-nucleotide BLAST (blastn) (both programs from the National Center for Biotechnology Information and accessible at: http://www.ncbi.nlm.nih.gov/BLAST), the 02-11 *L3* sequence had higher homology to corresponding sequences from Reovirus species RVH (91% homology) (17) and Reovirus species RVG (90% homology) (17) than to reoviruses type 1 (85%), type 2 (76%), or type 3 (85%), as indicated in Table 1. Similarly, the *L1* sequence had higher homology to reoviruses 780-99, 491-99, and 469-99 (7) than to reovirus types 1, 2, and 3 (Table 2). The experimentally determined reovirus sequences of this study were thus clearly different from those obtained from our Reovirus-3 Dearing stock virus. The deduced amino acid (aa) sequences of the reovirus species 02-11 PCR amplicons are shown in Fig. 3B and 4B.

Discussion

Reovirus infections are generally subclinical in immunocompetent rodents, and are easiest to detect by use of serologic test

Table 1. Percentage (%) of L3 amplicon homology as detected by use of reverse transcriptase-polymerase chain reaction (RT-PCR) analysis

Virus[a]	% Homology[b]							
	Type 1 Lang	Type 2 Jones	Type 3 Dearing	RVH	RVG	RVE	RVA	02-11
Type 1 Lang	100							
Type 2 Jones	78	100						
Type 3 Dearing	97	78	100					
RVH	81	78	81	100				
RVG	86	80	85	82	100			
RVE	97	79	97	81	86	100		
RVA	95	78	95	81	86	95	100	
02-11	85	76	85	91	90	86	86	100

[a]GenBank accession numbers: type 1 Lang, AF129820; type 2 Jones, AF129821; type 3 Dearing, AF129822; RVH, AF325768; RVG, AF325767; RVE, AF325766; RVA, AF325764; 02-11, AY494858.
[b]Sequence analyzed: internal 249 base pairs (bp) within REOL3F- and REOL3R-primed amplicon, corresponding to nucleotides (nt) 3215–3463 of the *L3* gene of Reovirus type 3 Dearing.
RVH = Reovirus species (sp.) RVH; RVG = Reovirus sp. RVG; RVE = Reovirus sp. RVE; RVA = Reovirus sp. RVA (8).

Table 2. Percentage of L1 amplicon homology as detected by use of RT-PCR analysis

Virus[a]	% Homology[b]							
	Type 1 Lang	Type 2 Jones	Type 3 Dearing	780-99	491-99	469-99	T3C8-60	02-11
Type 1 Lang	100							
Type 2 Jones	78	100						
Type 3 Dearing	99	78	100					
780-99	87	78	87	100				
491-99	87	78	87	100	100			
469-99	87	78	87	100	100	100		
T3C8-60	88	77	88	97	97	97	100	
02-11	88	78	88	91	91	91	90	100

[a]GenBank accession numbers: type 1 Lang, M24734; type 2 Jones, M31057; type 3 Dearing, M31058; 780-99, AY007392; 491-99, AY007391; 469-99, AY007390; T3C8-60, AY007393; and 02-11, AY496276.
[b]Sequence analyzed: internal 365 bp within r5, 6-primed amplicon, corresponding to nt 1919–2277 of the *L1* gene of Reovirus type 3 Dearing.

A.

```
Reovirus 02-11  ── Primer REOL3F ──►tccattggcgccaccaccggacctagtgtttgatcgtgacacgcctggtg
Reo-3 Dearing   cagtcgacacatttgtggtca..gc....t..t.....t.....g.............t..c.......
Reo-2 Jones     ..........................................t.............a..t.....a..c.
Reo-1 Lang      ...................a..gc....t..t.....t.....g.................t.......

Reovirus 02-11  tgcatatcttcgggcgagactgccgtatttcgtttggaatgaacggagccgccccaatgattagagacga
Reo-3 Dearing   .t..c........a.....t........a..............t..c.....g.............t..
Reo-2 Jones     .t..cg.g.....ca.............c..a..c..c.....t..c..t..t..g.....cc.t..t..
Reo-1 Lang      .t..c........a.....t.....c..a..............t..c.....g.............t..

Reovirus 02-11  gactggcatgatggtgcctttcgaaggaaattggatttttcctttggcgctttggcagatgaatacgcga
Reo-3 Dearing   ......ac.............t.................c..ac.............a........a...
Reo-2 Jones     ...c..g........a..g..t.....t..............c.c..tt.g...........c...a.g
Reo-1 Lang      ......a.............t..............c..c..ac.............a........a...

Reovirus 02-11  tatttcaaccaacagttcgatgcgtggattaagacgggagaacttaggattcgtattgagatgggtgcct
Reo-3 Dearing   .....t..t...........c..............a.....gt.gc.a..c..c...........c..g.
Reo-2 Jones     ........t..g..a........t.....c..a.....t..gt.ac.t...a.a..a........g..g.
Reo-1 Lang      .....t..t.....a.....c..............a.....gt.gc.a..c..............c..g.

Reovirus 02-11  atccgtacatgctacactac◄── Primer REOL3R ──  GenBank # AY494858
Reo-3 Dearing   ....a..t...t.g..t...tatgatccacgtcagtacgc  GenBank # AF129822
Reo-2 Jones     ....a........g..t..t...................  GenBank # AF129821
Reo-1 Lang      ....a..t...t.g..t......................  GenBank # AF129820
```

B.

```
Reovirus 02-11  PLAPPPDLVFDRDTPGVHIFGRDCRISFGMNGAAPMIRDETGMMVPFEGNWIFPLALWQMNTRYFNQQF
Reo-3 Dearing   ..........................................L.........................
Reo-2 Jones     ..................V.......................................................
Reo-1 Lang      ...........................................................................

Reovirus 02-11  DAWIKTGELRIRIEMGAYPYMLHY
Reo-3 Dearing   ........................
Reo-2 Jones     ........................
Reo-1 Lang      ........................
```

Figure 3. Nucleotide (nt) and deduced amino acid (aa) sequence of the REOL3F and REOL3R-primed RT-PCR product obtained from reovirus in feces of animal 02-11. (A) Nucleotide sequence between the primer binding sites. Directional arrows and letters in bold font identify the primer binding sites. Dots (·) indicate nt identity. (B) Deduced aa sequence of the reovirus sp. 02-11 nt sequence of panel A. Dots (·) indicate aa identity.

methods. In this study, we determined that results of current serologic tests must be interpreted cautiously, as IIFA-positive, ELISA-negative animals were truly infected with reovirus. As observed in most reovirus infections of immunocompetent animals, the infections that developed in the sentinel animals were subclinical, and were not detected by physical examination. The immunologic status of the transgenic mice of this report was uncertain, but they also did not exhibit visible signs of any illness during the course of this work. Whereas we cannot formally state that all current IIFA-positive, ELISA-negative reovirus results represent true infections, our study establishes that such situations are possible.

The source of the virus that infected the transgenic mice of this study is unknown. Reoviruses infect virtually all mammals, and it is possible that the reovirus that infected the transgenic mice had been transmitted from a human source to the mice through sub-optimal husbandry practices. Partial resolution of this question will necessitate isolation of the virus and determination of its genetic sequences for molecular tracking and comparison with known reovirus strains circulating in humans and animals. Concerning virus isolation, it is clear that our reovirus isolation methods must be re-evaluated. The isolation of reovirus by use of tissue culturing is known to often be insensitive (7). Additional established cell lines and primary monkey cells must be tested in future attempts to isolate reoviruses of the type described in this study. However, the RT-PCR assays were effective, possibly because they were performed on feces collected during (or close to) the acute phase of infection. It is possible that test-positive feces were detected only for specimens collected at the end of 2 months due to the temporal sequence: a certain duration was required for the sentinel animals to become infected with reovirus and to develop an active infection with fecal shedding of the virus, and the infections were likely cleared by the time fecal specimens were collected at 3 months. These findings are consistent with the known biology of reoviruses. The nt sequences of the reovirus detected in the sentinel mice was clearly different

A.

```
                gcattcattgtaaatgacgagtctg→(L1.rv5)        gcTagGccGatatcGggaatgcag→(L1.rv7)
Reovirus 02-11  ——— Primer L1.rv5 ——→tcgtcggggtgagagctgccagaccaatatcaggaatgcagaatatgattcagcatctctcaaagt
Reo-3 Dearing   ........c..................t..a..........t..g..g....g..........c.............a..g..ac
Reo-2 Jones     ..g.....c.....c........a..g.....t........a..tc.g..t.....t.............g.c......t.a..c....
Reo-1 Lang      ................t..........t..a..........t..g..g....g..........c.............a..g..ac

Reovirus 02-11  tatatcagcgtggattttcatatagagtgaacgattctttttctcctggtaatgattttacccatatgactacaactttcccatcaggttc
Reo-3 Dearing   .....a.a.....................a................a.....c.......t..........c........g........
Reo-2 Jones     .....a....g..t......c.cc.t..c.....c..g.....a..a..a........c..g..c.....c..g........t..c..g..
Reo-1 Lang      .....a.a.....................a................a.....c.......t..........c........g........

Reovirus 02-11  aacagctacctctactgagcataccgctaataatagtacaatgatggaaacttttctaacagtatgggggccggagcatactgacgagcct
Reo-3 Dearing   ......c................t.............g.............c..g..........a..c..a..........c...
Reo-2 Jones     c..g.....t..c........c..t..a.....c.....c........g..c...t.g.ac........a..a.....c...a.t..c...
Reo-1 Lang      ......c................t.............g.............c..g..........a..c..a..........c...
                                                                                          (L1.rv8)←ctg
Reovirus 02-11  gatgttttgcgactaatgagatctttaactatccaaagaaattatgtatgtcaaggtgacgacggattaatgattatcgacgggaatactg
Reo-3 Dearing   ..c..c..a..tt......ag..........t.....g.....c..............t..t................t....c.....
Reo-2 Jones     .....a......t.....c.c..a...........gc.c..c..c..c..c........t..t...........c..a..t....ca..g.
Reo-1 Lang      ..c..c..a..tt......ag..........t.....g.....c..............t..t................t....c.....
                ctggtaaggtgaatagtga
Reovirus 02-11  ctggtaaggtgaatagtgagactatt←——— Primer L1 RV6 ———   GenBank # AY498276
Reo-3 Dearing   ............c.....a......cagaagatgctagagctaatctcaag  GenBank # M31058
Reo-2 Jones     .g.....................................................  GenBank # M31057
Reo-1 Lang      ............c.....a.....................................  GenBank # M24734
```

B.

```
Reovirus 02-11  VGVRAARPISGMQNMIQHLSKLYQRGFSYRVNDSFSPGNDFTHMTTTFPSGSTATSTEHTANNSTMMETFLTVWGPEHTDEPDVLRLMRSL
Reo-3 Dearing   ......................K.....................................................D.......K..
Reo-2 Jones     ..............V.......K.....................................................N.......ND.........
Reo-1 Lang      ......................K.....................................................D.......K..

Reovirus 02-11  TIQRNYVCQGDDGLMIIDGNTAGKVNSETI
Reo-3 Dearing   ..................T..........
Reo-2 Jones     ..................T.....S....
Reo-1 Lang      ..................T..........
```

Figure 4. Nucleotide and deduced aa sequence of the L1.rv5 and L1.rv6-primed RT-PCR product obtained from reovirus in feces of animal 02-11. Labeled similarly as for Fig. 3. Nucleotides in the sequence of primer L1.rv7 that do not match the sequence of reovirus sp. 02-11 are capitalized.

from those of Reovirus-3 Dearing, the only other reovirus present in this laboratory (Fig. 3A and 4A). Thus, the possibility of laboratory contamination of the RT-PCR assays was ruled out. Instead, the sequences may be representative of contemporary reovirus strains currently in circulation that may not be detected by commonly used ELISAs. This study raises the question of how prevalent these types of IIFA-positive, ELISA-negative reovirus infections are in mice in the United States at the present time, and the consequences of these infections.

References

1. **Adams, D. J., D. N. Ridinger, R. S. Spendlove, and B. B. Barnett.** 1982. Protamine precipitation of two reovirus particle types from polluted waters. Appl. Environ. Microbiol. **44:**589-596.
2. **Committee on Infectious Diseases of Mice and Rats.** 1991. Digestive system, p. 121. *In* Infectious diseases of mice and rats. National Academy Press, Washington, D.C.
3. **Dahling, D. R., R. S. Safferman, and B. A. Wright.** 1989. Isolation of enterovirus and reovirus from sewage and treated effluents in selected Puerto Rican communities. Appl. Environ. Microbiol. **55:**503-506.
4. **Dermody, T. S., J. D. Chappell, J. G. Hofler, W. Kramp, and K. L. Tyler.** 1995. Eradication of persistent reovirus infection from a B-cell hybridoma. Virology **212:**272-276.
5. **Fields, B. N. and W. K. Joklik.** 1969. Isolation and preliminary genetic and biochemical characterization of temperature-sensitive mutants of reovirus. Virology **37:**335-342.
6. **Golden, J. W., J. Linke, S. Schmechel, K. Thoemke, and L. A. Schiff.** 2002. Addition of exogenous protease facilitates reovirus infection in many restrictive cells. J. Virol. **76:**7430-7443.
7. **Leary, T. P., J. C. Erker, M. L. Chalmers, A. T. Cruz, J. D. Wetzel, S. M. Desai, I. K. Mushahwar, and T. S. Dermody.** 2002. Detection of mammalian reovirus RNA by using reverse transcription-PCR: sequence diversity within the lambda3-encoding L1 gene. J. Clin. Microbiol. **40:**1368-1375.
8. **Lipson, S. M., L. Svenssen, L. Goodwin, D. Porti, S. Danzi, and R. Pergolizzi.** 2001. Evaluation of two current generation enzyme immunoassays and an improved isolation-based assay for the rapid detection and isolation of rotavirus from stool. J. Clin. Virol. **21:**17-27.
9. **Matsuura, K., M. Ishikura, T. Nakayama, S. Hasegawa, O. Morita, K. Katori, and H. Uetake.** 1993. Ecological studies on reovirus pollution of rivers in Toyama Prefecture. II. Molecular epidemiological study of reoviruses isolated from river water. Microbiol. Immunol. **37:**305-310.
10. **Nibert, M. L., L. A. Schiff, and B. N. Fields.** 1996. Reoviruses and their replication, p. 1557-1596. *In* B. N. Fields, D. M. Knipe, and P. M. Howley (ed.), Fields virology, 3rd ed. Lippincott-Raven, Philadelphia.
11. **Parashar, K., M. J. Tarlow, and M. A. McCrae.** 1992. Experimental reovirus type 3-induced murine biliary tract disease. J. Pediatr. Surg. **27:**843-847.
12. **Ridinger, D. N., R. S. Spendlove, B. B. Barnett, D. B. George, and J. C. Roth.** 1982. Evaluation of cell lines and immunofluorescence and plaque assay procedures for quantifying reoviruses in sewage. Appl. Environ. Microbiol. **43:**740-746.

13. **Rosen, L.** 1960. Serologic grouping of reovirus by hemagglutination-inhibition. Am. J. Hyg. **71:**242-249.
14. **Rosen, L.** 1979. Reoviruses, p. 577-584. *In* E. H. Lennette and N. J. Schmidt (ed.), Diagnostic procedures for viral and rickettsial infections. American Public Health Association, New York.
15. **Rosen, L., F. R. Abinanti, and J. F. Hovis.** 1963. Further observations on the natural infection of cattle with reoviruses. Am. J. Hyg. **77:**38-48.
16. **Sabin, A. B.** 1959. Reoviruses. Science **130:**1387-1389.
17. **Spinner, M. L. and G. D. Di Giovanni.** 2001. Detection and identification of mammalian reoviruses in surface water by combined cell culture and reverse transcription-PCR. Appl. Environ. Microbiol. **67:**3016-3020.
18. **Stanley, N. F.** 1974. Diagnosis of reovirus infections: comparative aspects, p. 385-421. *In* E. Kurstak and K. Kurstak (ed.), Comparative diagnosis of viral diseases. Academy Press, New York.
19. **Steele, M. I., C. M. Marshall, R. E. Lloyd, and V. E. Randolph.** 1995. Reovirus 3 not detected by reverse transcriptase-mediated polymerase chain reaction analysis of preserved tissue from infants with cholestatic liver disease. Hepatology **21:**697-702.
20. **Tyler, K. L. and B. N. Fields.** 1996. Reoviruses, p. 1597-1623. *In* B. N. Fields, D. M. Knipe, and P. M. Howley (ed.), Fields virology, 3rd ed. Lippincott-Raven, Philadelphia.
21. **Tyler, K. L., R. J. Sokol, S. M. Oberhaus, M. Le, F. M. Karrer, M. R. Narkewicz, R. W. Tyson, J. R. Murphy, R. Low, and W. R. Brown.** 1998. Detection of reovirus RNA in hepatobiliary tissues from patients with extrahepatic biliary atresia and choledochal cysts. Hepatology **27:**1475-1482.