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Sensitive and Broadly Reactive Reverse Transcription-PCR Assays To Detect Novel Paramyxoviruses[▽]

Suxiang Tong,* Shur-Wern Wang Chern, Yan Li, Mark A. Pallansch, and Larry J. Anderson

Division of Viral Diseases, National Center for Immunization and Respiratory Diseases, Centers for Disease Control and Prevention, Atlanta, Georgia 30333

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We have developed a set of reverse transcription-PCR assays for the detection and identification of known and novel paramyxoviruses in clinical specimens. Primers were designed from the conserved motifs of the polymerase *pol* gene sequences to detect members of the *Paramyxovirinae* or *Pneumovirinae* subfamily or groups of genera within the *Paramyxovirinae* subfamily. The consensus-degenerate hybrid oligonucleotide primer design and seminested or nested PCR assay design were used to enhance the breadth of reactivity and sensitivity of the respective assays. Using expressed RNA and 10-fold dilution series of virus-infected tissue culture isolates from different members of the family or genera, these assays were able to detect on average between 100 and 500 copies of template RNA. The assays were specific to the respective group of genera or subfamily viruses. This set of primers enhances our ability to look for novel viruses in outbreaks and diseases of unknown etiology.

The paramyxoviruses are ubiquitous pathogens and have been identified in a variety of hosts, including birds (chickens and turkeys), aquatic animals (salmon, whale, seal, dolphin, and porpoise), rodents (mice and rats), dogs, cats, sheep, reptiles (snake and lizards), horses, cattle, bats, pigs, simians, and humans. In humans, paramyxoviruses have been associated with a wide range of diseases, including croup, bronchiolitis, pneumonia, encephalitis, meningitis, parotitis, orchitis, spontaneous abortion, rash illnesses, and persistent infections (7, 10, 12, 16). Recently a variety of new members of the *Paramyxoviridae* family have been identified in animals, and some, most notably Nipah and Hendra viruses, have caused serious and sometimes fatal human infections (1, 2, 4). It is likely that there are additional, as yet unidentified members of this family and some will likely infect and cause disease in humans. Therefore, we believe the *Paramyxoviridae* constitute an important target for improved methods to detect novel viruses.

Detection of most paramyxoviruses has been routinely carried out by cell culture isolation, electron microscopy, antigen detection assays (immunofluorescence assays or enzyme immunoassays [EIA]), serologic assays, and genome-based assays, such as PCR assays. Each system has limitations. Traditional genome-based, antigen-based, and antibody-based assays usually are too specific to detect novel viruses. Cell culture isolation will allow detection only of viruses that grow and replicate in the culture system used and will require further characterization, usually by antigen- or genome-based assays. Electron microscopy requires a fairly high titer of virus for visualization and also requires further characterization.

To increase our ability to detect novel viruses, we chose to develop broadly reactive PCR assays. This strategy has been used very successfully to identify and characterize a number of novel human viruses, including severe acute respiratory syndrome coronavirus (5), hepatitis G virus (17), Sin Nombre virus (8), human retrovirus 5 (3), and novel animal viruses, such as the macaque gammaherpesvirus (15) and pig endogenous retrovirus (9). The present article describes the development of a set of PCR assays that should detect all known and novel paramyxoviruses. The primers for these assays were developed from highly conserved regions of the genome. We applied the consensus-degenerate hybrid oligonucleotide primer methodology (13, 14) in primer design and seminested PCRs to optimize the specificity and sensitivity of these broadly reactive assays.

MATERIALS AND METHODS

Virus and isolation of viral RNA. The reference viruses or viral RNA used in this study are listed in Table 1 and include three strains from the genus *Avulavirus*, two strains from the genus *Henipavirus*, five strains from the genus *Morbillivirus*, seven strains from the genus *Respirovirus*, five strains from the genus *Rubulavirus*, four strains from the genus *Pneumovirus*, three strains from the genus *Metapneumovirus* and three newly isolated unclassified paramyxoviruses. RNAs were extracted from 100 μl of supernatant fluid of virus-infected cells with the QIAamp viral RNA kit (Qiagen, Santa Clarita, CA) according to the manufacturer's instructions. The RNA was eluted from the column in 50 μl of RNase-free water. RNAs for the Hendra virus, Nipah virus, Menangle virus, and Fer-de-Lance virus (FDLV) strains were obtained from Paul Rota (Centers for Disease Control and Prevention) as extracts in Trizol, which were prepared according to the instructions provided by the commercial source (Invitrogen, Carlsbad, CA).

Broadly reactive oligonucleotide primer selection. Conserved amino acid sequences for the family, subfamily, and genera were selected from alignment of deduced L-protein-coding sequences recorded in GenBank (National Institutes of Health, Bethesda, MD). A total of 33 nonredundant paramyxovirus L-protein sequences were used and aligned using the Clustal W program. We selected highly conserved domains between 8 and 10 amino acids in length and back translated into degenerate nucleotide sequences to represent all possible codons for the corresponding amino acids. To minimize the number of primers, primers were designed with mixed degenerate bases restricted to between 9 and 12 nucleotides in the 3′ portion of the primers, and inosines (maximum of four) and consensus nucleotides were used for the remaining middle and 5′ portion of the

* Corresponding author. Mailing address: Gastroenteritis and Respiratory Viruses Laboratory Branch, 1600 Clifton Road, MS G18, Atlanta, GA 30333. Phone: (404) 639-1372. Fax: (404) 639-4005. E-mail: sot1@cdc.gov.

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TABLE 1. Reference viruses used in evaluating PCR in this work

Virus and classification	Abbreviation	Source
Paramyxovirinae		
Avulavirus		
Newcastle disease virus	NDV	Jack King, Qingzhong Yu
Avian paramyxovirus-2	APMV-2	Jack King, Qingzhong Yu
Avian paramyxovirus-3	APMV-3	Jack King, Qingzhong Yu
Henipavirus		
Hendra virus	Hendra	Paul Rota
Nipah virus	Nipah	Paul Rota
Morbillivirus		
Measles virus	MV	Paul Rota
Canine distemper virus	CDV	Randy Renshaw
Phocine distemper virus	PDV	Bert Rima
Dolphin morbillivirus	DMV	Bert Rima
Porpoise morbillivirus	PMV	Bert Rima
Respirovirus		
Bovine parainfluenza virus 1	BPIV1	Dean Erdman
Bovine parainfluenza virus 3	BPIV3	Randy Renshaw
Human parainfluenza virus 1	HPIV1	Dean Erdman
Human parainfluenza virus 3	HPIV3	Dean Erdman
Sendai virus	Sendai	Lela K. Riley
Unclassified *Respirovirus*		
Pacific salmon paramyxovirus	PSPV	Gael Kurath
Guinea pig parainfluenza virus 3	CaVPIV3	Lela K. Riley
Rubulavirus		
Mumps virus	Mumps	Paul Rota
Human parainfluenza virus 2	HPIV2	Dean Erdman
Canine parainfluenza virus 2	CaPIV2	Randy Renshaw
Simian virus 5	SV5	Richard W. Compans
Simian virus 41	SV41	Yasuhiko Ito
Unclassified *Paramyxovorinae*		
Fer-de-Lance virus	FDLV	Gael Kurath
Menangle virus	Menangle	Paul Rota
Salem virus	SalV	Randy Renshaw
Pneumovirinae		
Metapneumovirus		
Avian metapneumovirus type C	AMPV-C	Jack King, Qingzhong Yu
Human metapneumovirus 75	HMPV75	Dean Erdman
Human metapneumovirus 83	HMPV83	Dean Erdman
Pneumovirus		
Bovine respiratory syncytial virus	BRSV	Randy Renshaw
Human respiratory syncytial virus A	hRSV[a] A	Dean Erdman
Human respiratory syncytial virus B	hRSV B	Dean Erdman
Pneumonia virus of mice	PVM	Joseph Domachowske

[a] hRSV, human RSV.

primers. In addition, primers were designed to achieve similar reaction conditions and an amplicon size between 200 and 500 bp. To minimize the potential for nonspecific cross-reactivity, a Blastn search analysis of GenBank was performed for similarities with known sequences. The primers were synthesized at the Biotechnology Core Facility, Division of Scientific Resources of the Centers for Disease Control and Prevention (CDC).

RT-PCR and nested RT-PCR amplification. To maximize sensitivity and specificity, we nested or seminested PCR assays and optimized reaction conditions, including primer concentration, magnesium (Mg^{2+}) concentration, and thermal cycling temperatures and profiles. For the first PCR in the seminested assay, we used the SuperScript III One-Step reverse transcription-PCR (RT-PCR) kit (Invitrogen, Carlsbad, CA). The optimized PCR mixtures contained 50 pmol each of forward and reverse primers, 1× buffer with a final concentration of 2.0 mM $MgSO_4$ and 200 μM (each) deoxynucleoside triphosphates, 20 U of RNase inhibitor, a 5-μl aliquot of RNA/DNA extracts, and 1 U of SuperScript III RT/Platinum *Taq* mix. Water was then added to achieve a final volume of 50 μl. The RT-PCR mixture was sequentially incubated at 60°C for 1 min for denaturing, 44 to 50°C for 30 min (for RT), 94°C for 2 min (for hot start), and then 40 cycles at 94°C for 15 s, 48 to 50°C for 30 s, 72°C for 30 s, and a final extension at 72°C for 7 min. For the second amplification in the seminested PCR assay, we used 1× buffer (Platinum *Taq* kit; Invitrogen), 2 mM $MgCl_2$, 200 μM (each) of deoxynucleoside triphosphates, 50 pmol (each) of forward and reverse primers, 1 U Platinum *Taq*, one 2-μl aliquot from the first reaction, and water to achieve a final volume of 50 μl. The mixture was first heated to 94°C for 2 min. The cycling conditions were 40 cycles with the same conditions as for the first amplification: 94°C for 15 s, primer annealing at 48 to 50°C for 30 s, and 72°C for 30 s. A final extension was carried out at 72°C for 7 min. The final nested or seminested PCR products were visualized by UV light after electrophoresis on a 2% agarose gel containing 0.5 μg/ml ethidium bromide in 0.5× Tris-borate buffer (pH 8.0). A DNA VIII marker (Roche, Indianapolis, IN) was run in the gels to estimate amplicon size.

Specificity and sensitivity. Initial primer validation and selections were performed using one reference RNA template from the subfamily or genera for which the PCR assay was designed. Mumps virus RNA was used for the *Rubulavirus-Avulavirus* genus subgroup-specific primers and the *Paramyxovirinae* subfamily primers, Hendra RNA for the *Morbillivirus-Respirovirus-Henipavirus* genus subgroup-specific primers, and respiratory syncytial virus A RNA for the *Pneumovirinae* subfamily-specific primers. Following the initial screening, the selected primer pairs were then tested against the representative paramyxoviruses listed in Table 1. Finally, to test for unanticipated nonspecific reactivity, the PCR assays were tested against pooled nucleic acids of influenza A and B viruses, rhinoviruses, adenovirus, two distinct human coronaviruses, human coronavirus

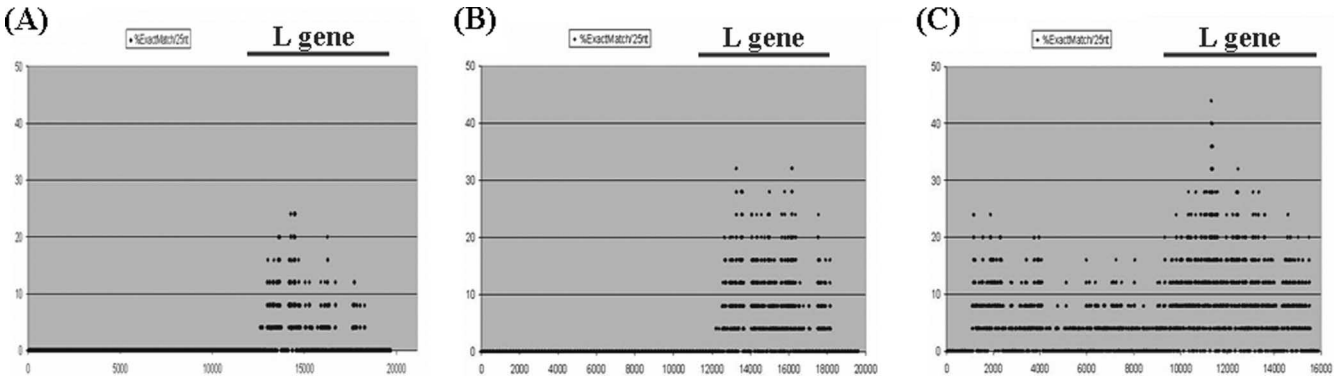

FIG. 1. Similarity plots of the aligned paramyxoviruses' genomes. The plots were obtained using an in-house program based on multiple alignments of viral genomes from 29 different strains in the *Paramyxoviridae* (A), 22 different strains in the *Paramyxovirinae* (B), and 7 different strains in the *Pneumovirinae* (C). The identity percentage score given on the *y* axis was calculated based on the exact-match percentage with a window of 25 nucleotide positions, and the window was progressively moved across the alignment in 1-nucleotide-position steps. The *x* axis shows the first position of the window in the multiple alignments of the viral genomes.

229E, and human coronavirus OC-43 and bacteria (*Chlamydia pneumoniae*, *Haemophilus influenzae*, *Streptococcus pneumoniae*, and *Mycoplasma pneumoniae*).

The sensitivities of the PCR assays were determined using two sources of RNA: RNA that was extracted from each dilution of a 10-fold dilution series of virus-infected cell culture with known infectivity titers (PFU) and serial dilutions of synthetic RNA that was transcribed in vitro from cloned genome fragments as previously described (18).

Sequencing. Amplicons from the final round of PCR were purified using the QIAquick PCR purification kit (Qiagen, Inc., Valencia, CA). Both strands of the amplicons were sequenced with a BigDye Terminators v1.1 ready reaction cycle sequencing kit on an ABI Prism 3100 automated sequencer (Applied Biosystems, Foster City, CA) using the corresponding PCR primers. The remaining reaction conditions were according to the manufacturer's instructions.

RESULTS

Development of consensus degenerate primers. To design family- or subfamily-specific primers, all full-length genome sequences were obtained from the *Paramyxoviridae* family viral sequences archived in the GenBank viral database. Viral sequences with more than 95% similarity were treated as identical; the resulting 29 nonredundant full-length genome sequences were used initially to design primers for this study. First, a multiple full-length genome sequence alignment was performed by using Clustal W and was scanned according to a program written in house to identify genes that have the most-conserved multiple regions from the broadest possible grouping in the family *Paramyxoviridae*. The plot (Fig. 1) showed that the RNA polymerase (L) coding sequences were the most conserved, and thus, this gene was selected as the locus for the broadly reacting PCR assays. For the final primer design, we used an additional four L-gene sequences available in GenBank for a total of 33 L-gene-specific nonredundant sequences. Since the subfamilies, *Paramyxovirinae* and *Pneumovirinae*, are so genetically distinct, primers were designed for each separately. The highly conserved amino acid regions a with minimal length of six amino acids were checked for possible presence in other nontarget organisms by a BLAST search to avoid nonspecific amplification in PCR. Primers were designed from these highly conserved regions using all codon possibilities for 9 to 12 bases at the 3′ end of the primer and consensus sequences or inosines at positions of fourfold degeneracy for a "consensus clamp" at the 5′ end. After comparative analysis including sizes of amplicons, the similarity of reaction kinetics, nonspecific cross-reactivity, and experimental evaluation, three consensus degenerate primers (two for the first PCR and the third for the second, seminested PCR) corresponding to the most-conserved motifs were selected as the pan-*Paramyxovirinae* primers and three consensus degenerate primers corresponding to the most-conserved motifs were selected as the pan-*Pneumovirinae* primers, as shown in Table 2. Since the subfamily *Paramyxovirinae* has greater diversity

TABLE 2. Consensus degenerate primers used for detection of paramyxoviruses

Primer name	Amino acid motif in RNA-dependent DNA polymerase	Targeted group
PAR-F1	GAA GGI TAT TGT CAI AAR NTN TGG AC	*Paramyxovirinae*
PAR-F2	GTT GCT TCA ATG GTT CAR GGN GAY AA	*Paramyxovirinae*
PAR-R	GCT GAA GTT ACI GGI TCI CCD ATR TTN C	*Paramyxovirinae*
RES-MOR-HEN-F1	TCI TTC TTT AGA ACI TTY GGN CAY CC	*Respirovirus, Morbillivirus, Henipavirus*
RES-MOR-HEN-F2	GCC ATA TTT TGT GGA ATA ATH ATH AAY GG	*Respirovirus, Morbillivirus, Henipavirus*
RES-MOR-HEN-R	CTC ATT TTG TAI GTC ATY TTN GCR AA	*Respirovirus, Morbillivirus, Henipavirus*
AVU-RUB-F1	GGT TAT CCT CAT TTI TTY GAR TGG ATH CA	*Avulavirus, Rubulavirus*
AVU-RUB-F2	ACA CTC TAT GTI GGI GAI CCN TTY AAY CC	*Avulavirus, Rubulavirus*
AVU-RUB-R	GCA ATT GCT TGA TTI TCI CCY TGN AC	*Avulavirus, Rubulavirus*
PNE-F1	GTG TAG GTA GIA TGT TYG CNA TGC ARC C	*Pneumovirinae*
PNE-F2	ACT GAT CTI AGY AAR TTY AAY CAR GC	*Pneumovirinae*
PNE-R	GTC CCA CAA ITT TTG RCA CCA NCC YTC	*Pneumovirinae*

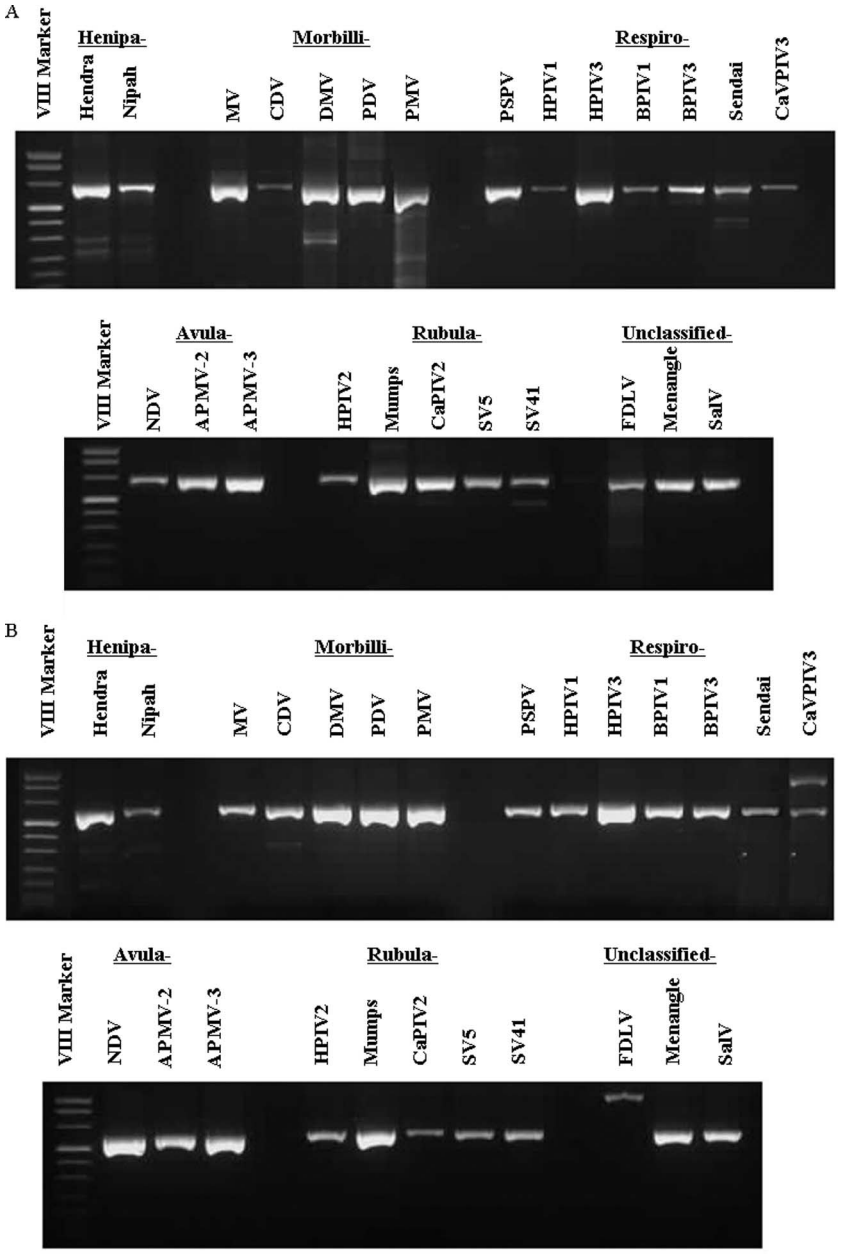

FIG. 2. Amplification of RNAs from 25 different viral members in the subfamily *Paramyxovirinae* by one-step RT-PCR using the pan-PAR-F1/PAR-R primer pair (A) or the pan-PAR-F2/PAR-R primer pair (B). Viral names are abbreviated as shown in Table 1.

among its members than the subfamily *Pneumovirinae*, we further divided the *Paramyxovirinae* into two subgroups of genera based on RNA polymerase gene relatedness, the *Morbillivirus-Respirovirus-Henipavirus* subgroup and the *Rubulavirus-Avulavirus* subgroup, and further developed primers to achieve less degeneracy and greater sensitivity, as noted in Table 2.

Standardization of optimized conditions. Different combinations of RT and PCR steps including one tube/one step, one tube/two steps, and two tubes/two steps, have a significant effect on the outcome of the assays. By comparative testing, we observed that a one-tube/one-step RT-PCR protocol was not only more convenient and more sensitive (data not shown) but also minimized risk of handling cross-contamination. RT-PCRs were optimized for band intensity and low background by evaluating combinations of primer concentration, Mg^{2+} concentration, and annealing temperatures.

Broad reactivity of consensus degenerate primers. As shown in Fig. 2A and B, all of the 25 reference viruses representing five genera from the *Paramyxovirinae* subfamily were successfully detected by pan-*Paramyxovirinae* primers. As shown in Fig. 3 and 4, members of the two groups of genera within this subfamily were also detected by the appropriate subgroup-specific primers. The observed variation in amplicon intensity was probably due at least in part to differences in amount of template RNA for the respective viruses. No amplicons were detected for the PCR assays against the pooled "other respiratory pathogen genomes" or the negative controls (see Fig. 6). Two previously unclassified paramyxoviruses, FDLV and Sa-

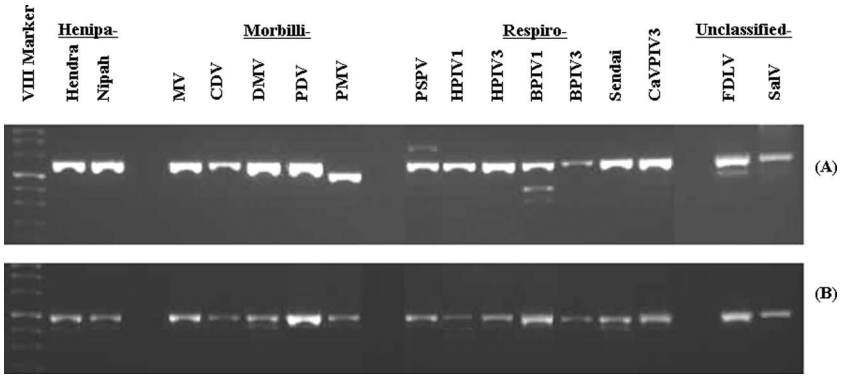

FIG. 3. Amplification of RNAs from 14 different viral members in the genera of *Henipavirus*, *Morbillivirus*, and *Respirovirus* and two unclassified viral members in the subfamily *Paramyxovirinae* by one-step RT-PCR using the pan RES-MOR-HEN-F1/RES-MOR-HEN-R primer pair (A) or the pan RES-MOR-HEN-F2/RES-MOR-HEN-R primer pair (B). Each viral name is abbreviated as shown in Table 1.

lem virus (SalV), were amplified only by the *Morbillivirus-Respirovirus-Henipavirus* genus subgroup-specific primers and Menangle virus only by the *Rubulavirus-Avulavirus* genus subgroup-specific primers. These results suggest that FDLV and SalV strains are more closely related to the *Morbillivirus-Respirovirus-Henipavirus* subgroup and the Menanagle strain to the *Rubulavirus-Avulavirus* subgroup.

As shown in Fig. 5, the *Pneumovirinae* seminested PCR assay detected all of the seven tested reference viruses, and the specificity of the amplification was confirmed by sequence studies. These primers did not amplify RNA from other common respiratory viruses or the negative controls (Fig. 6).

Sensitivity of consensus degenerate PCR assay. To test the sensitivity of the PCR assays, we used mumps virus RNA for both the *Paramyxovirinae* subfamily-specific primers and the *Rubulavirus-Avulavirus* genus subgroup-specific primers. Hendra viral RNA was used to evaluate the *Morbillivirus-Respirovirus-Henipavirus* genus subgroup-specific primers and respiratory syncytial virus RNA for the *Pneumovirinae* subfamily-specific primers. Generally, sensitivity was improved at least 10-fold with the addition of the nested or seminested step (Fig. 7). The same 10-fold serial dilutions of mumps virus RNA were used to compare the sensitivity between the *Paramyxovirinae* subfamily-specific RT-PCR and the *Rubulavirus-Avulavirus* genus subgroup-specific RT-PCR. As expected, the *Rubulavirus-Avulavirus* genus subgroup-specific primers with less degeneracy resulted in a 10-fold- to 100-fold-higher sensitivity than the *Paramyxovirinae* subfamily-specific primers. By using serial dilutions of RNA transcripts, the sensitivity limit was calculated as between 10 and 100 RNA copies for the *Rubulavirus-Avulavirus* subgroup-specific PCR, the *Morbillivirus-Respirovirus-Henipavirus* subgroup-specific PCR, and the *Pneumovirinae*

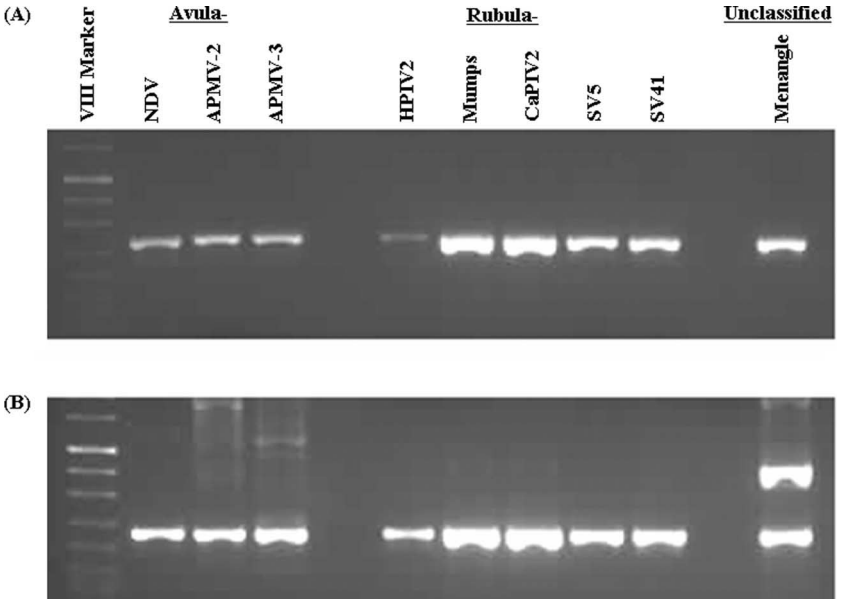

FIG. 4. Gel electrophoresis of amplification products of a one-step RT-PCR assay against RNA from eight different members of *Avulavirus* and *Rubulavirus* genera and one previously unclassified member of the subfamily *Paramyxovirinae*. The pan AVU-RUB-F1/AVU-RUB-R primer pair (A) or the pan AVU-RUB-F2/AVU-RUB-R primer pair (B) was used. Each virus gives an appropriately sized band and is identified by its abbreviation as shown in Table 1.

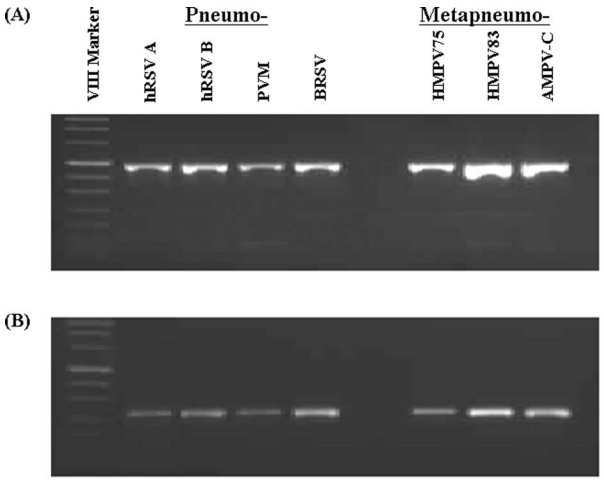

FIG. 5. Gel electrophoresis of amplification products of one-step RT-PCR assays against RNA from seven different members of the subfamily *Pneumovirinae*. (A) Pan PNE-F1/PNE-R primer pair. (B) Pan PNE-F2/PNE-R primer pair. Each virus gives an appropriately sized band and is identified by its abbreviation as shown in Table 1.

subfamily-specific PCR and between 500 and 1,000 copies for the *Paramyxovirinae* subfamily-specific PCR(data not shown).

Validation with clinical specimens. To validate further the specificity and sensitivity of each of these group-specific PCRs with clinical samples, we tested a panel of 14 blinded samples provided by Dean Erdman (CDC), consisting of paramyxovirus-containing clinical samples, non-paramyxovirus-containing clinical samples, and cell culture isolates in different dilutions, which had been tested by agent-specific assays. As noted in Table 3, the appropriate subfamily-specific or genus subgroup-specific PCR assays correctly identified each of the specimens with the correct specificity.

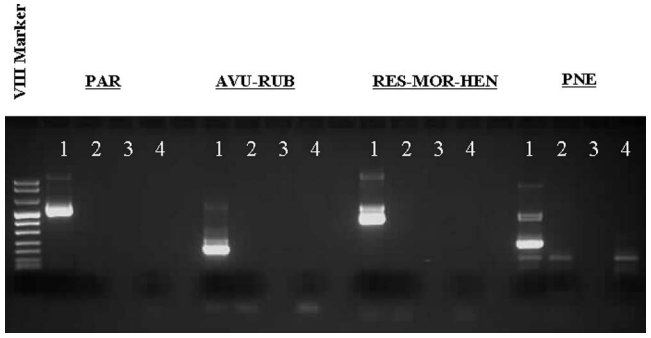

FIG. 6. Gel electrophoresis of amplification products with subfamily and genus group seminested RT-PCR assays showing the appropriately sized positive band for the positive control material for the respective RT-PCR assay and no appropriately sized band for negative-control material or a pool of RNA from other common respiratory pathogens, as described in Materials and Methods. The seminested RT-PCR assays are pan-PAR (*Paramyxovirinae* subfamily), pan RES-MOR-HEN (group of *Respirovirus*, *Morbillivirus*, and *Henipavirus* genera), pan-AVU-RUB (group of *Avulavirus* and *Rubulavirus* genera), and pan-PNE (*Pneumovirinae* subfamily). Lanes: 1, positive control; 2, negative control (water); 3, blank; 4, pool of RNA from other common respiratory pathogens.

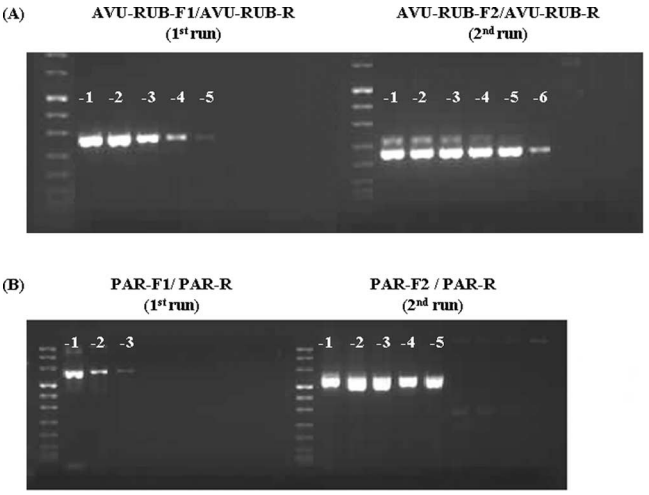

FIG. 7. Improved detection sensitivity by seminested RT-PCR and by genus subgroup primers with less degeneracy. (A) Seminested RT-PCR amplification of RNA extracted from 10-fold serial dilution of mumps virus stock using the pan-AVU-RUB-F1/AVU-RUB-R primer pair (1st run) and the pan-AVU-RUB-F2/AVU-RUB-R primer pair (2nd run). (B) Seminested RT-PCR amplification of RNA extracted from 10-fold serial dilution of mumps virus stock using the pan-PAR-F1/PAR-R primer pair (1st run) and the pan-PAR-F2/PAR-R primer pair (2nd run).

DISCUSSION

In this report, we describe successful development of a set of broadly reactive PCR assays for *Paramyxoviridae*. These assays were developed by identifying conserved sequences among members of various groups in the family *Paramyxoviridae*, using degenerate and inosine-containing primers to account for mismatches, a second, nested, PCR to improve sensitivity, and the consensus-degenerate hybrid oligonucleotide primer strategy (13) to improve sensitivity and specificity. In designing the broad-range primers, we first looked for conserved amino acid sequences in the RNA-dependent RNA polymerase protein coded by the L gene, the most conserved viral gene in the family *Paramyxoviridae*. The analysis of the RNA-dependent RNA polymerase proteins of paramyxoviruses indicated that they encompass three conserved domains (I, II, and III) separated by two nonconserved hinge regions, and strong selective constraints act against amino acid sequence changes in these three conserved domains (6, 11). They are predicted to be essential for the key functions of RNA binding, RNA replication, and protein kinase activity and may have retained the same structure, that of their putative common ancestor, as they have diverged in sequence. The invariance of these conserved sequences suggests that they may be ideal targets for the exploration of unidentified members in the *Paramyxoviridae*. The broad-range primers in this report were designed from the highly conserved domain I and II among paramyxovirus members.

The extensive variability within this family and the drop in sensitivity with increased primer degeneracy prevented us from developing a single assay for the family but instead led us to develop subfamily-specific and two genus subgroup PCR assays to achieve the desired level of sensitivity, <100 copies of RNA in the reaction mixture. The two genus subgroup assays took advantage of more closely related genera, i.e., a group of

TABLE 3. Validation with clinical specimens[a]

Specimen ID	PCR result				Sequence confirmation result	Blinded result
	Paramyxovirinae	Rubulavirus-Avulavirus subgroup	Respirovirus-Morbillivirus-Henipavirus subgroup	Pneumovirinae		
2005495091	Pos	Neg	Pos	Neg	HPIV3	HPIV3
2005495105	Pos	Neg	Pos	Neg	HPIV3	HPIV3
2005495106	Pos	Neg	Pos	Neg	HPIV3	HPIV3
2002023415	Neg	Neg	Neg	Pos	hRSV-B1	hRSV-B1[c]
2002023422	Neg	Neg	Neg	Pos	hRSV-B1	hRSV-B1
2002023428	Neg	Neg	Neg	Neg	Neg	FLU A
2002023434	Neg	Neg	Neg	Neg	Neg	FLU A
2002023436	Pos	Neg	Pos	Neg	HPIV3	HPIV3
2002023437	Pos	Neg	Pos	Neg	HPIV3	HPIV3
2002023644[b]	Neg	Neg	Neg	Neg	Neg	HMPV
2002029001	Neg	Neg	Neg	Pos	HMPV	HMPV
2002029002	Neg	Neg	Neg	Pos	HMPV	HMPV

[a] Pos, positive; Neg, negative. For other abbreviations, see Table 1.
[b] The specimen 2002023644 had a low viral load (<10 copies/5 μl template) which is below the detection limit of the pan-*Pneumovirinae* RT-PCR.
[c] hRSV-B1, human RSV-B1.

the *Rubulavirus* and *Avulavirus* genera and a group of the *Morbillivirus*, *Respirovirus*, and *Henipavirus* genera. The two genus subgroup assays reached the desired level of sensitivity, 10 and 100 copies, while the corresponding *Paramyxovirinae* subfamily assay achieved a sensitivity of 500 to 1,000 copies.

In summary, we have developed a set of seminested RT-PCR assays for detection of paramyxoviruses. The broad reactivity of these RT-PCR assays should allow us to detect known and novel members of the family *Paramyxoviridae* within genera described to date. The utility of these assays in discovery of novel members is supported by our ability to detect and classify eight recently isolated paramyxovirus species, *Porpoise morbillivirus*, *Pacific salmon paramyxovirus*, *Bovine parainfluenza virus 1*, *Guinea pig parainfluenza virus 3*, *Canine parainfluenza virus 2*, *Canine parainfluenza virus 3*, *Menangle virus*, and *Salem virus*, whose sequences were not available when the primers were designed. These pan-paramyxovirus PCR assays and similar assays for other viral families should enhance our ability to quickly identify, by virus family, subfamily, or genus, a wide range of novel viral pathogens and should enhance our ability to respond to and characterize outbreaks and diseases of unknown etiology.

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