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Molecular Identification of Mumps Virus Genotypes from Clinical Samples: Standardized Method of Analysis

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A sensitive nested reverse transcription-PCR assay, targeting a short fragment of the gene encoding the small hydrophobic protein (SH gene), was developed to allow rapid characterization of mumps virus in clinical samples. The sensitivity and specificity of the assay were established using representative genotypes A, B, C, D, E, and F. Mumps virus RNA was characterized directly from cerebrospinal fluid (CSF) samples and in extracts of mumps virus isolates from patients with various clinical syndromes. Direct sequencing of products and subsequent phylogenetic analysis enabled genetic classification. A simple web-based system of sequence analysis was established. The study also allowed characterization of mumps virus strains from Argentina as part of a new subgenotype. This PCR assay for characterization of mumps infections coupled to a web-based analytical program provides a rapid method for identification of known and novel strains.

Mumps virus (MV), in the genus *Rubulavirus* and family *Paramyxoviridae*, typically causes a benign childhood infection. Nonetheless, cerebrospinal fluid pleocytosis has been detected in more than a half of all mumps virus infections, indicating that the virus is commonly neuroinvasive (4). The most frequent central nervous system complication of mumps infection is aseptic meningitis (6, 15). Since mumps virus is monotypic, vaccine from any strain should provide lifelong protection against subsequent infection. However, mumps outbreaks have not been completely eliminated even in vaccinated populations (5, 9, 32) and, in some cases, mumps virus neutralizing antibodies do not protect against reinfection with a heterologous mumps virus genotype (18). While all MV live attenuated vaccines have been associated with cases of aseptic meningitis or viral encephalitis, some vaccine-derived strains may be more neuroinvasive.

The single-stranded mumps virus genomic RNA contains seven genes encoding the nucleocapsid (N), phospho (P), membrane (M), fusion (F), small hydrophobic (SH), hemagglutinin-neurominidase (HN), and large (L) proteins (7, 8). The SH gene encodes a protein of 57 amino acids (24). Reduced or absent expression of the SH gene, with a concomitant reduction of SH protein, has been described for certain mumps virus strains (3, 24, 25). Lack of expression of the SH protein does not affect virus replication in vitro, but may modify virus pathogenesis in vivo (24). Phylogenetic comparison of the SH gene revealed the existence of 10 genotypes, designated A to L (2, 10–14, 20, 27–30, 33, 34). Confirmation of mumps viral infection is generally carried out by direct culture of virus from cerebrospinal fluid (CSF). This method is time-consuming, expensive, and unable to detect low titers of virus.

The most promising development in direct detection of virus in the central nervous system has been the application of PCR. In a retrospective study in Argentina, mumps virus RNA was detected by a mumps reverse transcription (RT)-nested PCR targeting the NP gene in 25% of 236 CSF samples from patients with a clinical diagnosis of aseptic meningitis or acute encephalitis (22). Mumps virus PCR is more sensitive than viral culture and may detect mumps virus in CSF when viral culture does not (22). Here we describe a consensus RT-nested PCR assay, targeting the SH gene, for characterization of mumps virus. A simple sequence analysis of amplified products readily distinguishes vaccine and wild-type infections and can be used to characterize circulating genotypes for epidemiological research.

MATERIALS AND METHODS

Virus strains. The prototype strain of mumps virus strain Jeryl Lynn was obtained from the American Type Culture Collection, was propagated in Vero cells, and stored at −70°C. Prototype strains of other paramyxoviruses, such as measles virus; canine distemper virus; respiratory syncytial virus A and B; and human parainfluenza viruses 1, 2, 3, and 4, were also obtained from the American Type Culture Collection and used to test assay specificity. Aliquots of each virus were prepared and stored frozen at −70°C.

Clinical specimens. Clinical specimens were obtained from the specimen collections at the Diagnostic Microbiology Service (Centro Nacional de Microbiología, ISCIII, Spain) and the Neurovirosis Division (INEI, Instituto C. Malbrán, Argentina). Specimens consisted of two virus isolates and 23 clinical samples (16 CSF, 7 sera, and 1 saliva). Clinical data for the samples and patients is shown in Table 1.

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TABLE 1. Clinical data for the specimens presented in this study[a]

Sample	Date	Location	Type	Syndrome	Age of patient	Sex	Evolution time
528	1998	Argentina, Buenos Aires	Urine	AM	4 yr	M	1 day
M421	1998	Argentina, Mendoza	CSF	AM	ND	M	ND
SF58	1995	Argentina, Santa Fe	CSF	AM	5 yr	M	1 day
M380	1998	Argentina, Mendoza	CSF	AM	11 yr	M	ND
SF47	1995	Argentina, Santa Fe	CSF	AM	5 yr	M	4 days
M420	1998	Argentina, Mendoza	CSF	AM	7 yr	M	ND
M341	1998	Argentina, Buenos Aires	CSF	AM	ND	F	ND
47-98	1998	Argentina, Salta	CSF	AM	30 days	M	2 days
M5	1994	Argentina, Santa Fe	CSF	AM	4 yr	M	1 days
527	1998	Argentina, Buenos Aires	CSF	AM	10 yr	F	3 days
297	1998	Argentina, Mendoza	CSF	AM	ND	M	ND
LP42	1995	Argentina, La Pampa	CSF	AM	5 yr	F	4 days
538	1999	Argentina, Rio Negro	CSF	AM	8 yr	M	1 day
215	1997	Argentina, Santa Fe	CSF	AM	ND	M	ND
SF52	1995	Argentina, Santa Fe	CSF	AM	4 yr	F	1 day
21-98	1998	Argentina, Salta	CSF	AM	4 yr	M	ND
V090003	1998	Spain, Gran Canaria	CSF	AM	ND	M	ND
V0012	1998	Spain, Gran Canaria	CSF	AM	ND	M	ND
LP4	1994	Argentina, Santa Fe	CSF	Encephalitis	3 yr	M	1 day
LP71	1996	Argentina, La Pampa	CSF	AM	3 yr	M	5 days
570	1995	Argentina, Chubut	CSF	AM	4 yr	M	ND
SF65	1996	Argentina, Santa Fe	CSF	Encephalitis	1 yr	M	1 day

[a] AM, aseptic meningitis; ND, no data; F, female; M, male.

Oligonucleotides. Degenerate oligonucleotides for use in this study were designed using computer-assisted analysis of the genomic RNA sequences of those mumps virus serotypes that have been fully or partially sequenced in genomic regions which encode SH proteins (EMBL and GenBank databases; MACAW version 2.0.5 software, 1995, National Center for Biotechnology Information, Bethesda, Maryland). Table 1 shows the sequences and the respective primer positions in mumps virus strain Jeryl Lynn. Degenerate positions are R (A/g), Y (T/C), W (T/A), S (C/g), M (A/C), and K (T/g). Deoxyinosine residues are indicated by letter I.

Synthetic standards. To test the reactivity of the selected primer pairs against mumps virus genotypes that were not available as clinical or laboratory isolates, we synthesized the SH genomic region using overlapping PCR. Eighty nucleotides of the target sequences were designed to overlap each other by 25 bp. Two artificial 25-nucleotide sequences were added to the 5′ and 3′ ends of the region selected. Equimolar amounts of these oligonucleotides were mixed, and the full-length product was generated in an extension reaction. Following amplification by PCR using primers complementary to the artificial 25-nucleotide flanking sequences, the product was cloned into vector pGEM-T Easy (Invitrogen, Carlsbad, CA). Serial dilutions of linearized plasmid were used to optimize the assay. Thereafter, RNA standards were generated by in vitro transcription of linearized plasmid DNA (mMESSAGE mMACHINE T7; Ambion, Austin, TX) and used to determine assay sensitivity.

RNA extraction and nested RT-PCR from clinical samples. Nucleic acids from virus isolates and clinical samples were precipitated as previously described (5a). After processing, the dried pellet was dissolved in 10 μl of water for immediate use. Five μl of RNA was added to 45 μl of RT-PCR mixture comprising components for reverse transcription and PCR steps (Access RT-PCR System; Promega Co., Madison, WI). The first reaction mixtures contain avian myeloblastosis virus (AMV)/Tfl 5× reaction buffer, 400 mM each deoxynucleoside triphosphate (dNTP), 2 mM Mg_2SO_4, 5 units of AMV reverse transcriptase, 5 units of Tfl DNA polymerase, and 10 pmol of each of the mumps SH sense (Mumps-SH-1S) and mumps antisense (Mumps-SH-1AS) primers. Samples, isolated virus, and controls were subjected to an initial cycle for reverse transcription at 42°C for 45 min, forty cycles (94°C for 1 min, 50°C for 1 min and 68°C for 1 min), and a final incubation at 68°C for 5 min.

Nested PCR amplifications were performed using a reaction mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 200 μM each dNTP, 2.5 mM $MgCl_2$, 2.5 units of Taq polymerase (Amplitaq; Perkin-Elmer Cetus, Norwalk) and 10 pmol of Mumps-SH-2A and Mumps-SH-2S degenerate primers. Nested PCR assays were subjected to an initial cycle of 94°C for 2 min and thirty cycles (94°C for 1 min, 50°C for 1 min and 72°C for 1 min) followed by a final incubation at 72°C for 5 min. Amplifications were carried out in a PTC-200 (Peltier Thermal Cycler, MJ Research, Inc., MA) utilizing thin-walled reaction tubes. Upon completion of nested PCR assays, 5 μl of each reaction mixture was analyzed by 2% agarose gel electrophoresis. Amplification products were purified by isopropanol precipitation and sequenced with standard sequencing methods.

Mumps virus sequence database. A mumps virus sequence database was constructed by extracting sequences from the National Center for Biotechnology Information GenBank. Each sequence was identified by name, place of isolation or detection, year, and genotype. Previously described genotypes were taken from their corresponding references.

A manual search was carried out for all the sequences in GenBank encompassing the targets of our selected primers. Genetic characterization was performed on a total data set of 375 mumps virus sequences. The database is accessible at http://www.greeneidlab.columbia.edu

Sequence analysis of amplified products. Original sequence data were first analyzed by the CHROMAS software (version 1.3, McCarthy 1996; School of Biomolecular and Biomedical Science, Faculty of Science and Technology, Griffith University, Brisbane, Queensland, Australia), and forward and reverse sequence data of each sample were aligned with the program EDITSEQ (DNASTAR Inc., Software, Madison, Wisconsin). The consensus sequence was compared and aligned to other samples or DNA database sequences using the program CLUSTAL X (version 1.83). The MEGA package (version 2.1) was used to produce phylogenetic trees using neighbor joining as the method to reconstruct the phylogeny and Kimura two-parameter as the nucleotide substitution calculation method. The statistical significance of a particular tree topology was evaluated by bootstrap resampling of the sequences 1,000 times.

Pairwise comparisons of the mumps virus database were done by global alignment using the Needleman Wunsch (17) algorithm, implemented by a program from EMBOSS, the European Molecular Biology Open Software Suite (23). Z-scores were calculated to test the significance of each pairwise alignment by Monte Carlo simulation on the shuffled sequences. Statistical analysis was conducted with the SPSS statistical package (SPSS Software, Chicago, Ill.). An automated program to perform the same analysis is available at http://www.greeneidlab.columbia.edu.

Nucleotide sequence accession number. The GenBank accession numbers of the nucleotide sequences determined in this study are AY735412 to AY735441.

TABLE 2. Sequence alignment of primers and mumps virus strain sequences available on genomic databases

Primer (positions)[a]	Sequence[b]	
Mumps SH-2S .... 5′	AATGAATMTCMIRGGGTCGTAACGTCTC 3′	
external sense	AATGAATCTCCTGGGGTCGTAACGTCTC	(35)
(6621–6648)	AATGAATCTCCTAGGGTCGTAACGTCTC	(35)
	AATGAATCTCATGGGGTCGTAACGTCTC	(23)
	AATGAATCTCCCGGGGTCGTAACGTCTC	(13)
	AATGAATCTCCTGGGGTCGTAACGTCTC	(12)
	AATGAATCTCCTGGGGTCGTAACGTCTC	(12)
	AATGAATATCCTGGGGTCGTAACGTCTC	(5)
	AATGAATCTCCTAGGGTCGTAACGTCTC	(3)
	AATGAATCTCCTGGGGTCG**G**AACGTCTC	(2)
	AATGAATCTCCTAGGGTCGTAACGTCTC	(1)
	AATGAATCCCCTAGGGTCGTAACGTCTC	(1)
	AATGAATCTCCTAGGGTCGTAAC**T**TCTC	(1)
	AATGAATCTCCTGGGGTC**C**TAACGTCTC	(1)
	AATGAATCTCCAGGGGTCGT**T**ACGTCTC	(1)
Mumps SH-1S .... 5′	GTSACCCTGCIGTIGSACTATG 3′	
internal sense	GTGACCCTGCCGTTGCACTATG	(120)
(6249–6271)	GTGACCCTGCCGTAGCACTATG	(9)
	GTGACCCTGCCGTCGCACTATG	(4)
	GTGACCCTGCCGTA**A**CACTATG	(4)
	GT**C**ACCCTGCTGTTGCACTATG	(2)
	GTGACCCTGCCGTTGAACTATG	(1)
	GTGACCCTGCCGTTGGACTATG	(1)
	GTGACCC**C**GCCGTTGCACTATG	(1)
	GTGACCCTGCGGTTGCACTATG	(1)
Mumps SH-2AS ... 5′	GRAAGATCYYYARIYIGGRCRAGTCC 3′	
external	GAAAGATCTCCAGTTAGGACAAGTC**T**	(21)
antisense	GAAAGATCTCCAGCTGGGACAAGTCC	(19)
(6441–6466)	GGAAGATCTCCAGTTAGGACAAGTCC	(17)
	GAAAGATATCCAGTTAGGACAAGTC**T**	(12)
	GAAAGATCCCCAGTTAGGACAAGTCC	(10)
	GAAAGATCTCCAGTTGGGACAAGTCC	(10)
	GAAAGATCTCCAGTTAGGACGAGTC**T**	(9)
	GAAAGATCTCCAGATAGGACAAGTCC	(6)
	GAAAGATCTCCAACCCGGACAAGTCC	(5)
	GAAAGATCCTCAGTTAGGGCAAGTCC	(4)
	GAAAGATCTCCAGTTAGGACGAGTCC	(4)
	GATAGATCTCCA**T**TTAGGACAAGTCC	(4)
	GAAAGATCCCCAATTAGGACAAGTCC	(3)
	GAAAGATCTCCAGTTTG**A**ACA**C**GTCC	(3)
	GAAAGATCTCCAATTAGGACAAGTCC	(2)
	GAAAGATCTCCAGTTAGGACAAGTCC	(2)
	GGAAGATCTCCAGTTAGGACA**C**GTCC	(1)
	GGAAGATCCCCAGGTGGGACAAGTCC	(1)
	GAAAGATCTCCAGTTAGGGCAAGTCC	(1)
	GAAAGATCCCTAGTTAGGACAAGTC**T**	(1)
	GAA**C**GATCTCCAGTTAGGACAAGTC**T**	(1)
	GATAGATCTCCA**T**TTAGGACA**G**GTCC	(1)
	GATAGATCTCTA**T**TTAGGACAAGTCC	(1)
	GAAAGATCTCCA**T**CTAGGACAAATCC	(1)
	GAAAG**G**TCTCCAGCTGGGA**A**AAGTCC	(1)
	GAAAGATCTCCAGCTGGGAAAAGTCC	(1)
	GAAAGATCTCCAGC**G**GGGA**A**AAGTCC	(1)
	GAAAGATCTCCAGCCCGGACAAGTCC	(1)
	GA**G**AGATCTCCAGCCAGGACAAGTCC	(1)
Mumps SH-1AS.... 5′	GATCAMYCACTCTAGAAAGATCYCYAR 3′	
internal	GATCACTCACTCTAGAAAGATCTCCAG	(54)
antisense	GATCACCCACTCTAGAAAGATCTCCAG	(16)
(6427–6453)	GATCACTCACTCTAGAAAGAT**A**TCCAG	(12)
	GATCACTCACCCTAGGAAGATCTCCAG	(10)
	GATCACTCACTCTAGGAAGATCTCCAG	(8)
	GATCACTCACTCTAGAAAGATCTCCAA	(7)
	GATCACTCATTCTAGA**T**AGATCTCCA**T**	(5)
	GATCAATCACTCTAGAAAGATCCCCAG	(5)
	GATCAATCACTCTAGAAAGATCC**T**CAG	(4)
	GATCACTCACTCTAGAAAGATCCCCAA	(4)
	GATCACTCACTCTA**A**GAAGATCCCCAG	(1)
	GATCACTCACTCTAGAAAGATCTCTAG	(1)
	GATCACTCACTCTAGAAAGATCTCTAG	(1)
	GATCACCCACTCTAGAAAGATCCCTAG	(1)
	GAT**A**ACCCACTCTAGAAAGATCTCCAG	(1)
	GATCACCCACTCTAGAAA**A**ATCTCCAG	(1)
	GATCATTCACT**T**TAGAAAGATCTCCAG	(1)
	GATCACCCACT**T**TAGAAAGATCTCCAG	(1)
	GATCATC**A**ACTCTAGAAAGATCTCCAG	(1)
	GATCACTCACTCTAGAACGATCTCCAG	(1)
	GA**C**CACCCACTCTAGAACGATCTCCAG	(1)
	GATCACTCACTCTAGAAAG**G**TCTCCAG	(1)
	GATCACTCACTCTAGA*G*AGATCTCCAG	(1)
	GATCACTCA**T**TCTAGA**T**AGATCTCTA**T**	(1)
	GATCACTCACTCTAGAAAGATCTCCA**T**	(1)
	GATCACTCAC**C**CTAGAAAGATCTCCAG	(1)

[a] Positions refer to reference strain NC_002200.
[b] Pattern of sequence primer binding site followed by its frequency (in parentheses). The observed mismatches are in boldface type.

RESULTS

Primer design and PCR sensitivity. Alignment of mumps virus nucleotide sequences spanning the SH gene revealed the variability between strains. Thus, primers were designed to complement all possible codon combinations by incorporating mixed base residues or deoxyinosine residues at the degenerated codon positions (Table 2).

The Jeryl Lynn mumps virus RNA and the synthetic RNA of genotypes B, C, D, E, and F were reverse transcribed and amplified using primers for the first reaction. The first round amplified products were visualized on agarose gels establishing the limits of detection of the first reaction to 3 50% tissue culture infective doses ($TCID_{50}$) or 5,000 copies of each mumps virus genotype reference strain. The nested PCR assay increases the sensitivity of the assay to a threshold for detection of 0.03 $TCID_{50}$ or 5 to 50 copies of each standard.

Specificity of RT-nested PCR assays was examined using a panel consisting of dilutions equivalent to 10 $TCID_{50}$ of prototype strains of the following paramyxoviruses: measles virus; canine distemper virus; respiratory syncytial viruses A and B; and human parainfluenza viruses 1, 2, 3, and 4. All were negative in the assay.

Analysis of MV-positive clinical samples. Isolates of MV from clinical samples obtained through years 1995 to 2001 and

samples of CSF previously positive for mumps virus RNA were amplified and directly sequenced in both directions. All samples previously positive for mumps virus RNA (diagnostic assay) were positive for the SH RT-nested PCR assay.

Phylogenetic analysis of clinical samples and generation of a complete mumps virus SH database. A database was constructed comprising all published SH gene sequences and used to analyze the classification scheme and phylogenetically compare the sequences obtained by sequencing. The trees obtained by analysis of representative strains of all genotypes and our unknown sample sequences allowed quick and easy differentiation of the corresponding genotype (Fig. 1).

Seven sequences from Spain were analyzed and confirmed to be from genogroup H, as previously published by Montes et al. (16). Some clinical samples were also shown to be vaccine-associated cases of viral encephalitis or aseptic meningitis. Those cases appeared to be associated with the subcomponent J15 of the Jeryl Lynn strain. Whether these in fact represent cases of vaccine-induced disease remains to be determined. Finally, all the remaining sequences from mumps cases from Argentina belonged to the genotype D. This is the first description of mumps virus sequences from South America.

However, the generation of a database comprising all sequence information available for the region selected allowed us to delineate some classification problems; these problems were mainly related to the use of limited sets of available sequences for phylogenetic analysis.

In our analysis, group G and the strains that belong to this group, reported by Inou et al. (10) and Jin et al. (11), could be further differentiated as members of two different subgenotypes, G1 and G2, at the nucleotide (Fig. 2a) and amino acid (Table 3) levels. The subgrouping for selected sequences of genotype H by Utz et al. (30) could be applied to all remaining known sequences of the genotype allowing the subclassification of the strains into two groups, namely H1 and H2, and characterizing the Spanish isolates in the H2 subgenotype (Fig. 2b). The subdivision is also supported at the amino acid level (Table 3).

The subgroupings proposed for genotype C by Utz et al. (30) and Tecle et al. (28) do not seem to be significant when all sequences available where used (data not shown) although both could be supported by amino acid variation (Table 3).

Genotype D described in 1997 was a highly heterogeneous group that included strains of the actually recognized genotypes D, K, and H. A subset of those sequences reported by Orvell et al. (20) were only included for analysis later by Wu et al. (33). They were not included in any posterior analysis. We observed that they form a separate group to the actual genotype D (group D1 in the tree) (Fig. 2d), forming the group D2. The new proposed group seems to be an ancestor of the sequences of genotype K described originally by Tecle et al. (26) and also sequences of genotype L. Indeed, one of the old sequences (SE V28) belongs to genotype K. Genotypes D1, D2, K, L, and H are different at the amino acid level (Table 3).

Finally, although the topology of the tree is the same when all published MV sequences are included in the analysis, bootstrap values are consistently lower and sometimes not significant for some groups (data not shown). Some strain sequences confounded the analysis due to sequence dissimilarity and were classified as outliers. The strains RW (X63708), ge2-87 (reconstructed from the publication) (2), Tay-UK50 (AF142774), MP93-N (AB003415), and UK02-19 (AY380077) are in this list. The strains Tay-UK50 and UK02-19 have been reported previously as outliers (10, 12, 14) or proposed as a new genotype (12), respectively. The strain RW has been initially presented in group B5 in the initial classification of mumps virus SH genotypes (2) and after that was reported as genotype D (20, 26) or lately as an outlier (10, 30). The strain MP93-N was reported as a member of genotype D (10, 26, 33). However, in our analysis when we include all other strains of mumps virus, it is clearly separated at the base of the group D. In the previous publications, the strain also appears as a distant member of the group. We proposed this strain as a new outlier. Finally, the postvaccinal strain ge2-87, originally reported as the unique member of group B4 (2) and then reported in group D (33) and not included in any other analysis, thereafter is proposed here as being another outlier. Interestingly, most of the outliers seem to be related to genotype D. We found that their exclusion from the phylogenetic analysis resulted in improved bootstrapping values for other groups.

Sequence analysis results. Pairwise sequence analysis using Needleman Wunsch global alignment was carried out on the 200-bp SH fragment. An all-against-all sequence comparison was made to evaluate the possibility of using sequence similarity to classify genotypes. Significant sequence similarity was observed when comparing sequences within the same genotype. This was evaluated by analysis of variance between groups, comparing the scores of sequence comparisons within genotypes to comparisons between genotypes (Fig. 3) Each group was significant to the $P < 0.001$ level. Genotypes with only one member sequence were excluded from the analysis. Previously untyped sequences were assigned to the group with the greatest similarity. All unknowns were classified correctly into their genotypic group (Fig. 4).

DISCUSSION

We present a molecular method for genotyping of mumps viruses. Although it is not the first method proposed for mumps virus genotyping (1, 11, 27, 33), it is the first to be assessed as a diagnostic methodology with respect to sensitivity for analysis of a broad range of mumps virus genotypes. The method was applied mainly to CSF samples, where genotypic information is particularly important, given the significance of strain type as a determinant of neuroinvasiveness and the need to detect vaccine-derived disease.

The use of pairwise comparison to classify sequences has been used for enteroviruses (19, 21) and potyvirus (31). Multiple alignment and rigorous phylogenetic methods are preferable to establish exact lineages of sequence strains, discover recombination events, and positively identify regions; however, pairwise comparison can substitute if only a high level of taxonomic classification is desired. Here, we show that mumps virus genotypes can be classified using the sequence of SH gene region amplified by the PCR. The advantages of pairwise comparison for classification are speed, simplicity, and availability. The database and classification scheme provide a repository

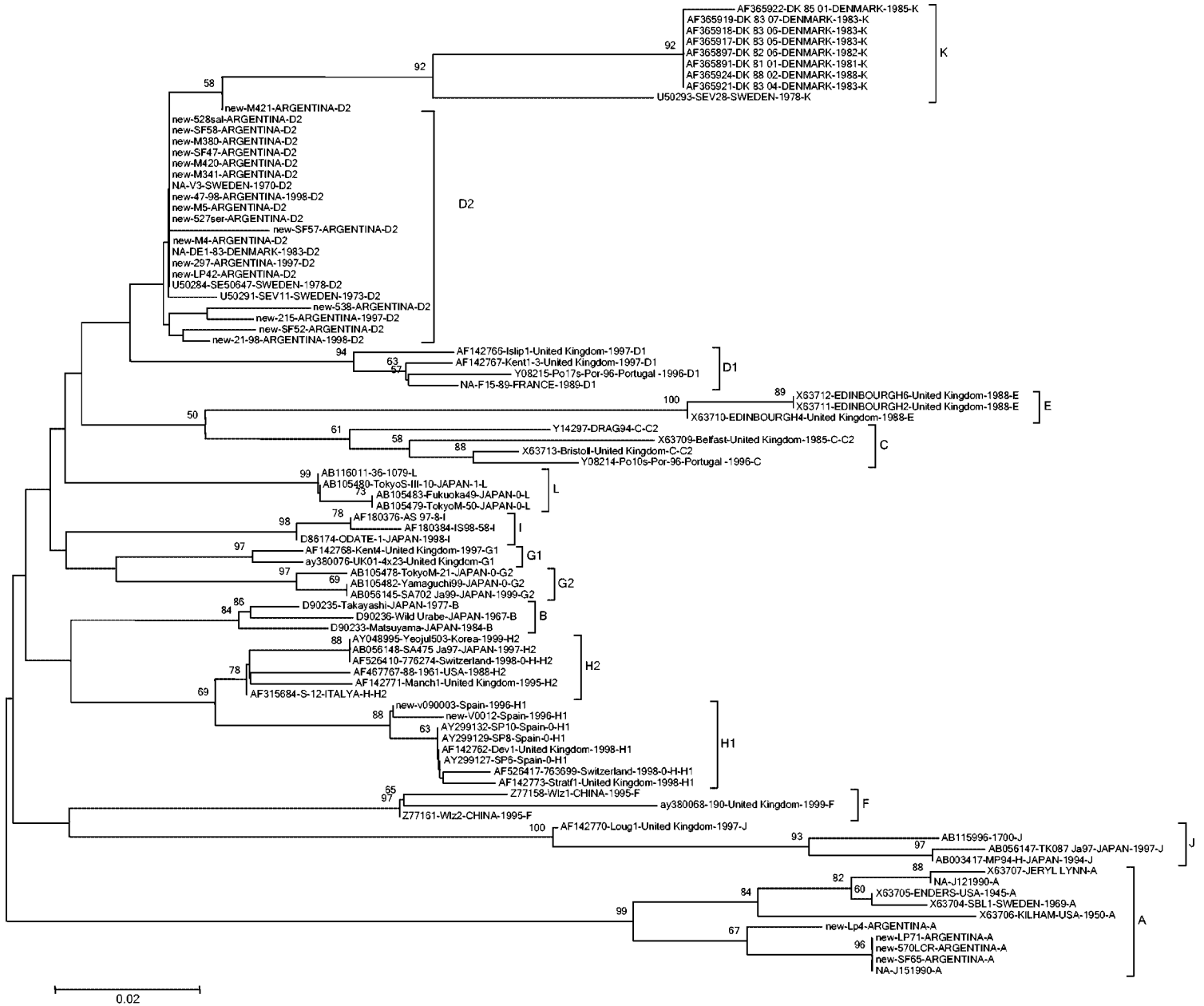

FIG. 1. The set of nucleotide sequences was aligned with Clustal W. Phylogenetic analysis was performed using the Kimura two-parameter model as a model of nucleotide substitution and using the neighbor-joining method to reconstruct the phylogenetic tree (MEGA version 2.1 software package). The statistical significance of the phylogenies constructed was estimated by bootstrap analysis with 1,000 pseudoreplicate data sets. For clarity, only a subset of the database is shown.

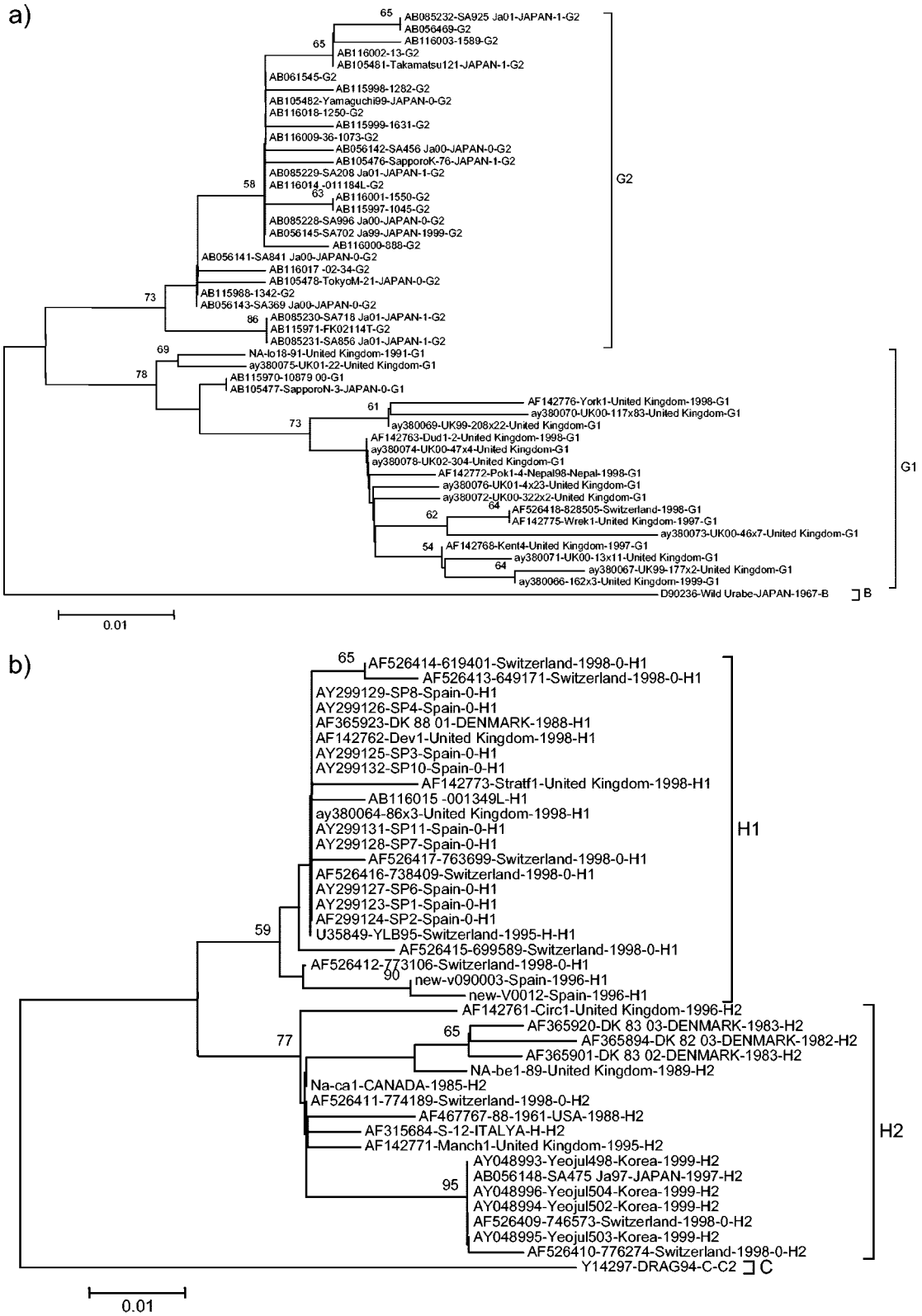

FIG. 2. (a) Phylogenetic tree of a genotype G strain; genotype B sequences were included as the outgroup. (b) Phylogenetic tree of genotype H strains; genotype C sequences were included as the outgroup. (c) Phylogenetic tree of genotype D and K strains, genotype F sequences were included as the outgroup. All available sequences of the corresponding genotype were included in the trees. The analysis method was the same as that reported for Fig. 1.

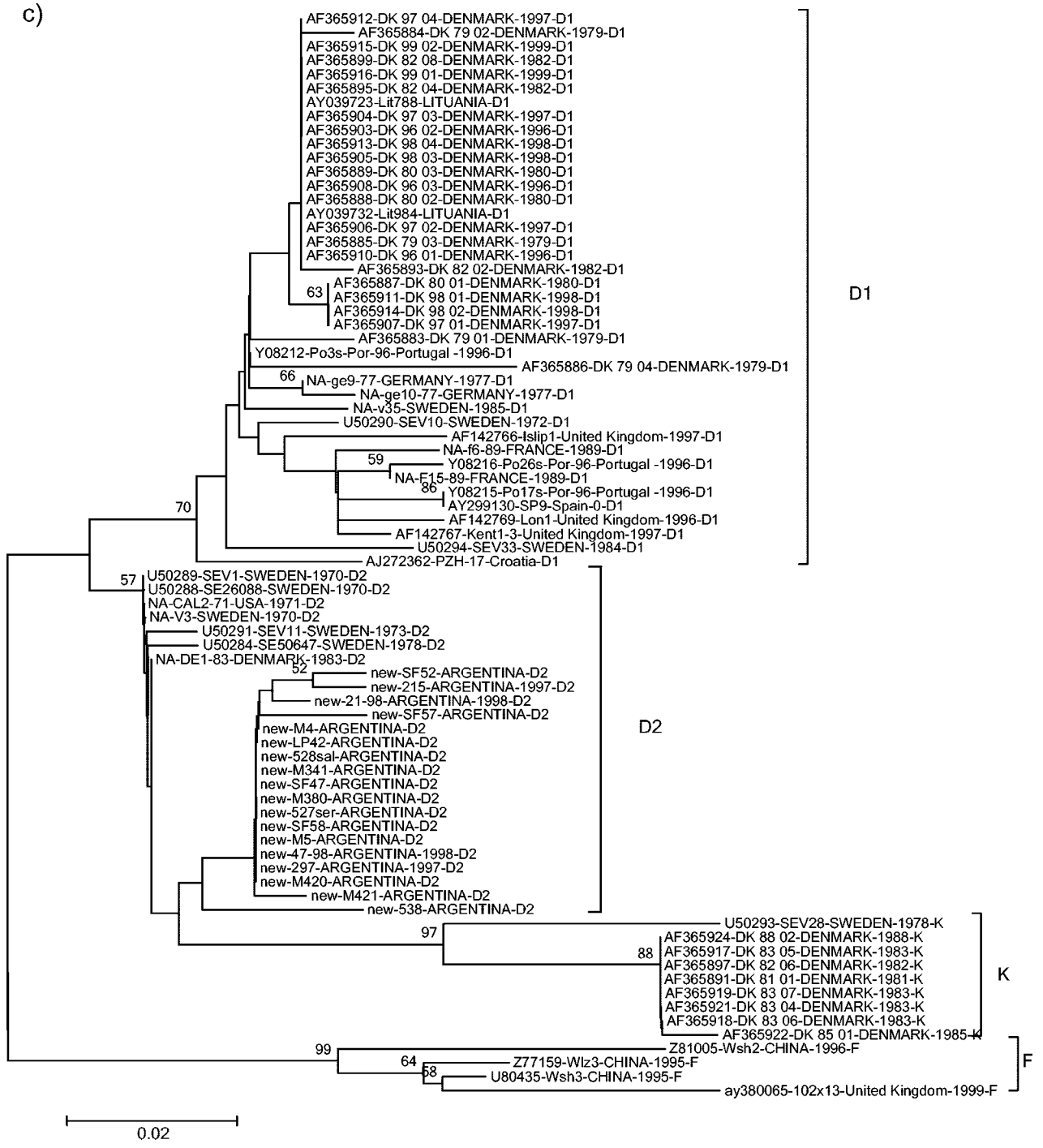

FIG. 2—*Continued*.

for sequences, complementing efforts in tracking mumps virus genotype distribution. A website has been deployed wherein clinical labs can post their sequences (www.greeneidlab.columbia.edu.) and location and circumstances of isolation or detection. The laboratory would instantly receive a report detailing the genotype, date, and location of the most similar sequence isolate in the database. New genotypes can readily be identified because the classification scheme will fail to relate them to any described group.

In conclusion, we propose a sensitive RT-nested PCR-based method for the molecular identification of mumps infections. The method has been tested using a broad range of genotypes and clinical samples. Major advantages of this approach are that cell culture is not needed, only a short fragment is re-

TABLE 3. Alignment of deduced amino acid sequences of clusters of mumps virus SH protein sequences[a]

Description	Accession no.	Proposed genotype	Amino acid sequence
Genotypes G1 and G2			
Dud1 2, United Kingdom, 1998	AF142763	G1	MPAIQPPLYL TFLLLILLYL IITLYVWIIL TVTYKTAVRH AALYQRSFFH WSFDHSL
Takamatsul21, Japan	AB105481	G2	MPAIQPPLYL TFLLLILLYL IITLYVWI**T**L TITYKT**V**VRH AALYQRS**Y**FH WSFDHSP
Genotypes H1 and H2			
Dev1 UK98, United Kingdom, 1998	AF142762	H1	MPAIQPPLHL TFLLLILLYL IITFYVWITS TITYKAAVRH ATLYQRSFFR WSFDHPL
circ1 UK96, United Kingdom, 1996	AF142761	H2	MPAIQPPLHL TFLLLILLYL IIT**L**YVWIT**L** TINYK**T**AVRH ATLYQRSFFR WSFDHPL
Genotype C			
Belfast, United Kingdom, 1985	X63709	C	MPAIQPPLYL TFLLLILLYR IITLYVWVVS TITYKTAVRH AALYQRSLFR WSFDHSL
Litl035, Lithuania	AY039722	C	MPAIQPPLYL TFLLLILLYL IITLYVWV**A**S TITYKTAVRH AALYQRS**S**FR WSFDHSL
689567, Switzerland, 1998	AF526404	C	MPAIQPPLH**P** TFLLLILLYL IITLYVWVVS TITYKTAVRH AALYQRSLFR WSFDHSL
Genotypes D1, D2 and K			
Po3s Por 96, Portugal, 1996	Y08212	D1	MPAIQPPLYL TSLLLILLYL IITLYVWIIL TITYKTAVRH EALYQRSFSR WSFDHSL
DE1 83, Denmark, 1983		D2	MPAIQPPLYL T**F**LLLILLYL IITLYVWIIL TITYKTAVRH **A**AL**H**QRSF**F**R WSFDHSL
DK 88 02, Denmark, 1988	AF365924	K	MPAIQ**L**PLY**P** TFLLLTLLYL ITTLYVW**T**I**S** TITYKT**T**VRH **A**AL**H**QRSF**F**R WSFDHSL

[a] Different signature sequences were observed between strains of groups G1 and G2; H1 and H2; and D1, D2, and K. The variations observed in two clusters of sequences from Switzerland and Lithuania for group C were included. The boldface positions represent signature amino acid positions that are observed in all members of the cluster and absent in all members of the outgroup.

quired for sequencing, and a "sequence similarity-based" software is established that facilitates the rapid acquisition of results. This approach constitutes a useful tool for both the diagnosis and the epidemiological research on mumps infections in endemic and nonendemic areas. We have detected vaccine-associated disease, associated the sequences obtained with known genetic groups, and characterized strains of mumps virus circulating in South America. The use of an

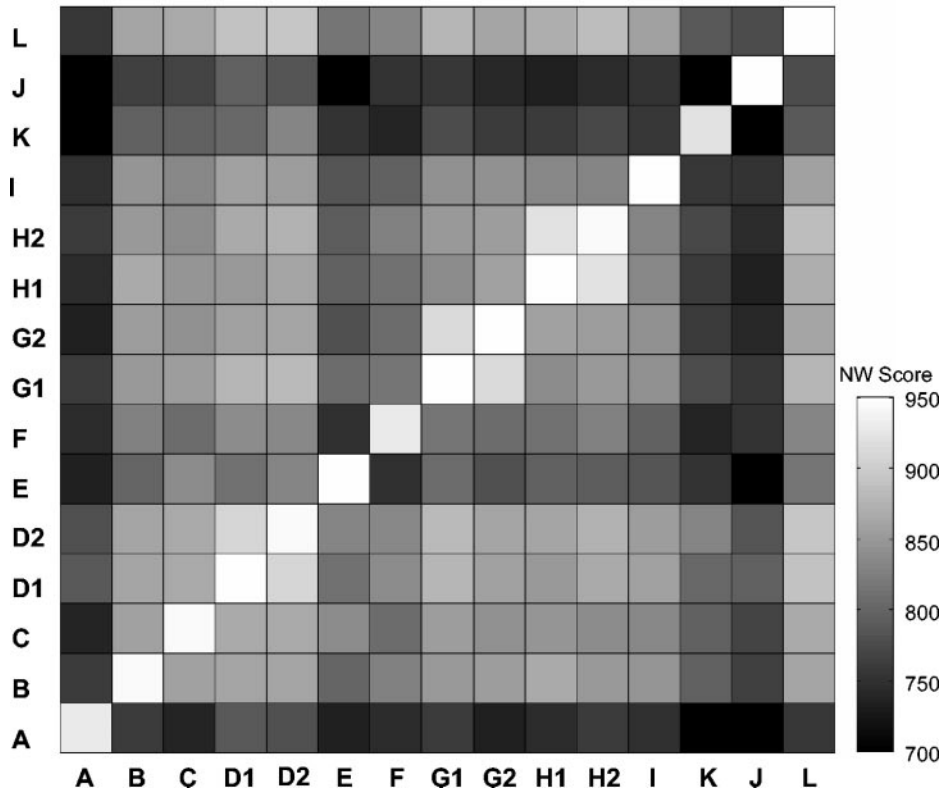

FIG. 3. Pairwise analysis of all available sequences of mumps virus SH against themselves is represented in the graph. Pairwise nucleotide alignment between all members of the database was done. The average score between members of each genotype is plotted in the graph. The different colors represent the Needleman Wunch scores obtained for each genotype comparison.

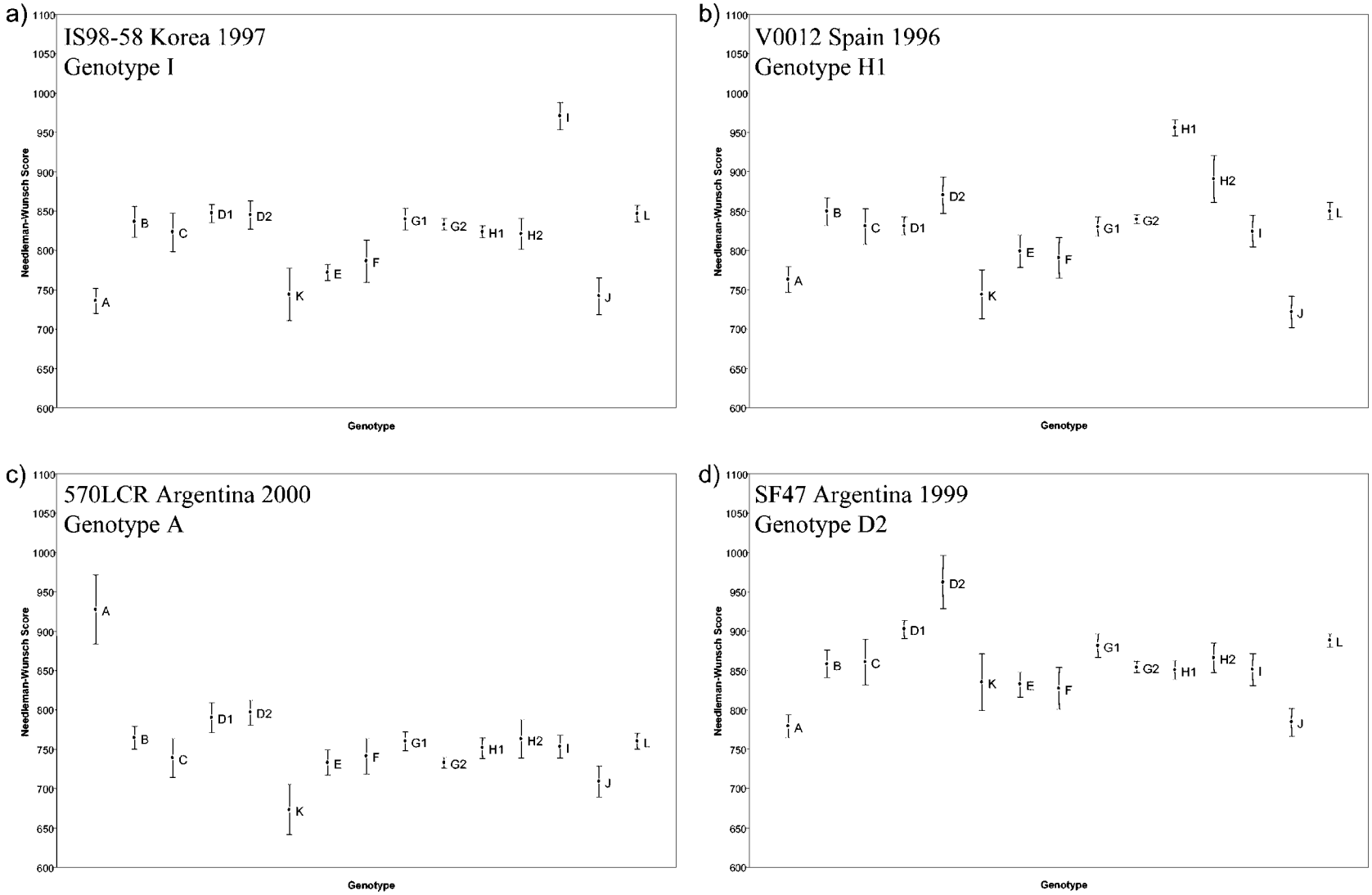

FIG. 4. Pairwise analysis of four mumps virus strains randomly chosen from the database. Samples are 570LCR-Argentina, genotype A; SF47-Argentina, genotype D2; V0012-Spain-1996, genotype H1; and IS98-58-KOREA-1997 (GenBank accession number AF180384). A pairwise Needleman Wunch score for the test sequence against all other members of the database was determined. The average score (with the corresponding standard deviation) against members of each genotype is plotted in the graph.

extended database of all available sequences of the mumps virus SH gene has also allowed us to reassess some of the previous classification attempts and propose or reevaluate some groupings.

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