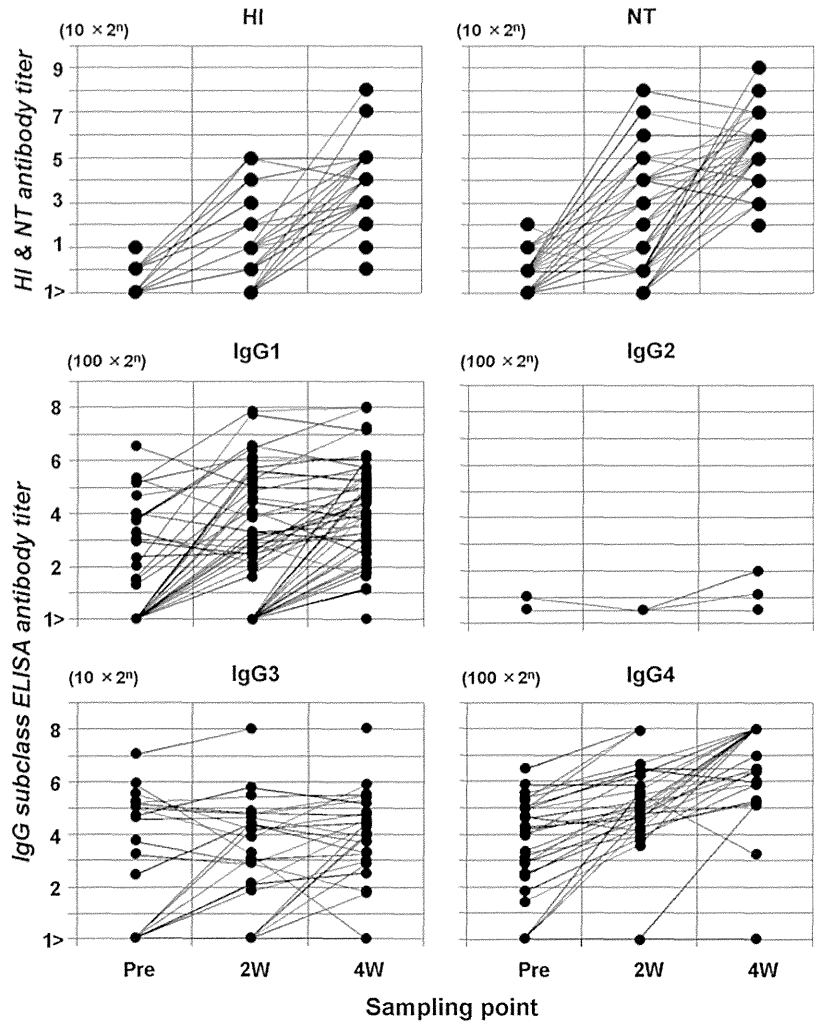


**FIG. 1.** Hemagglutination inhibition, neutralization, and IgG subclass antibody titers specific to A/California/07/2009(H1N1) in group 1 patients with natural infection. Black dot shows antibody titer. Abscissa in sampling point after onset of illness. Ordinate in serum dilution.

peaked in the second samples ($p<0.001$). IgG1 was positive in prevaccination specimens in 70 subjects, and 19 and 17 of those showed fourfold or more and twofold increases thereafter, respectively. Four recipients had no IgG1 antibody in the first specimens and showed a fourfold or more rise in the follow-up. No IgG1 antibody rise was observed in 34 patients who had positive antibody prevaccination. In terms of IgG3 in vaccine recipients, 49 had positive prevaccination samples, and 5 and 10 of those showed fourfold or more and twofold rises thereafter. Seven recipients had no IgG3 in the first specimens and acquired IgG3 fourfold or more. Thirty-four who had positive IgG3 in the first samples did not show any elevation thereafter. Eighteen recipients did not have IgG3 in any of three serial specimens. Only 13 recipients had positive IgG4 in prevaccination samples and showed no elevation in the follow-up. Sixty-one subjects did not have any IgG4 antibody in serial three specimens.

## Discussion

Our main purpose in the present study was to determine the time kinetics of the development of HI and NT antibody to influenza A(H1N1)pdm2009, in order to clarify the role of the humoral immune response to newly appearing influenza subtypes. We observed a fourfold increase in HI antibody titers with the peak titer appearing 4 weeks after infection in almost all patients with natural infection, suggesting that a primary immune response was elicited during the course of pandemic influenza. A fourfold rise in antibody response does not necessarily imply a primary immune response if it occurred after contracting seasonal influenza. However, influenza A(H1N1)pdm2009 was a newly emerged reassortant subtype influenza virus, which did not strongly cross-react to any other subtypes, including even A/USSR/90/77 (H1N1) (1). Thus, it is likely that a fourfold elevation in antibody response to this particular strain strongly suggests a primary infection. A distinctive and unique point of the present study was the detailed time kinetic observation of antibody development using three consecutive specimens obtained from subjects with natural infection and those who were vaccinated.

Regarding antibody development in pandemic vaccine recipients, 40 (54%) and 70 (95%) of 74 subjects had positive HI and NT antibodies, respectively, in prevaccination specimens. These antibodies were assumed to be obtained through asymptomatic infection, which is supported by the

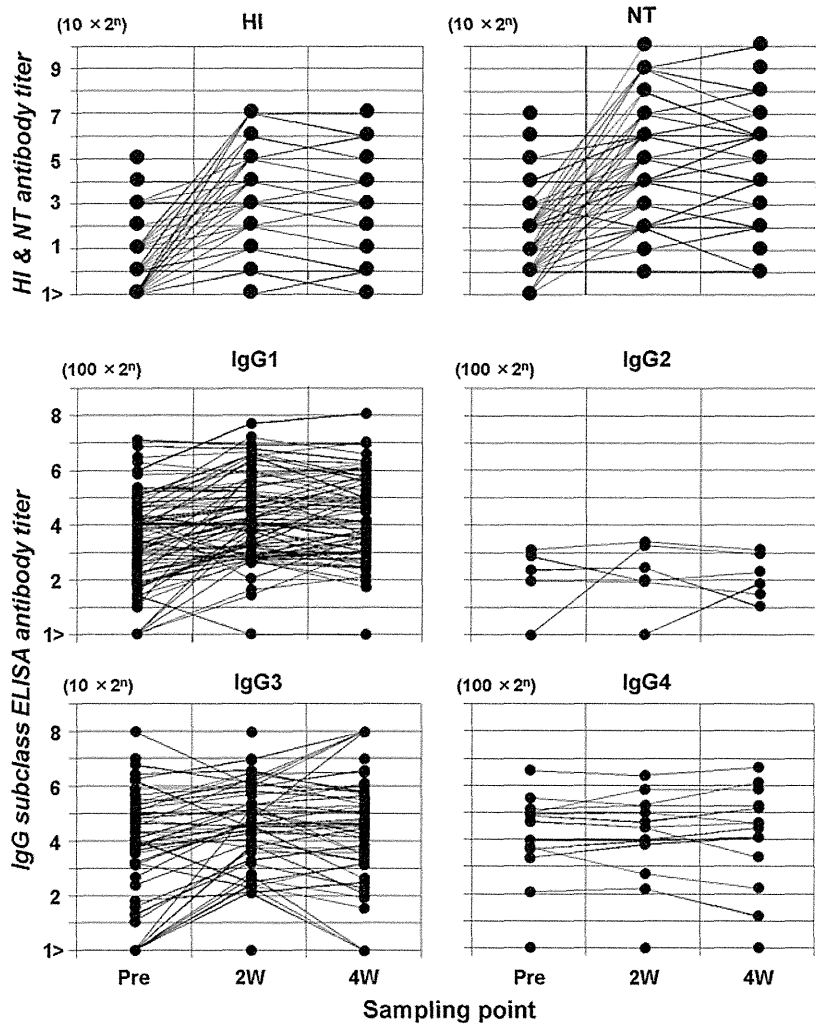


**FIG. 2.** Hemagglutination inhibition, neutralization, and IgG subclass antibody titers specific to A/California/07/2009(H1N1) in group 2 vaccine recipients. Black dot represents antibody titer. Abscissa in sampling point before and after vaccination. Ordinate in serum dilution.

fact that all pediatric employees enrolled in this study were free of symptoms of influenza during the pandemic in Japan. The percentage of positive HI is similar to other reports (3,4). However, the number of subjects with positive NT antibodies was remarkable. At least in part, this is because these subjects were employees of pediatric clinics where they were exposed to massive amounts of live viruses of pandemic influenza during a 6 month period. A time kinetic study showed that their antibody levels peaked in the second specimens, implying that the secondary immune responses were elicited by the subsequent pandemic vaccination.

The discrepancy in kinetic pattern of antibody development between HI and NT may depend on a fundamental difference in immunologic characteristics between the two antibodies. The mechanism of action of underlying antibodies in the interaction between antibody and cellular components in the human immune system is poorly understood. Thus, we need to interpret these data cautiously, and further characterization of the antibodies would be valuable. In this respect, we need to remember that influenza A(H1N1)pdm2009 virus was a natural reassortant strain with North America and Eurasian swine lineages originated from avian or human viruses (9).

With regard to this point, our study provides another set of unique data concerning specific IgG subclass responses to influenza A(H1N1)pdm 2009. Our observations are the first data to provide serial three point time kinetics of the development of IgG subclass antibodies specific to influenza A(H1N1)pdm 2009. IgG1, IgG3, and IgG4 antibodies were developed in natural infections, and peak responses were found in the third specimen. The majority (88%) of subjects with natural infection experienced IgG1 antibody elevation, whereas 18 and 29 showed a rise in IgG3 and IgG4, respectively. In vaccine recipients, peak IgG1 responses were found in the third specimens, whereas those of IgG 3 were found in the second samples, which may be important in explaining the findings that the peak responses in HI and NT appeared in the second specimens (25). IgG4 did not change in the follow-up, while IgG2 did not develop in most subjects with either natural infection or vaccinations.

Developmental patterns or characteristics of these specific IgG subclass antibodies in humans are so far quite distinct

from those observed in mice (11,15). In mice, IgG1 is closely related to the activities of HI, but that is no longer the case in the humans in our study. However, caution must be used in interpreting these data because affinity of Fc$\gamma$ receptors is totally different between humans and mice.

In an earlier study, Garçon *et al.* for the first time reported high levels of IgG1 antibodies with lower amounts of IgG2 and IgG3 detected after immunization with different vaccine formulations, including cold-adapted attenuated live influenza vaccine intranasal, trivalent inactivated, and purified HA conjugated to diphtheria toxoids (8). In natural infection with H3N2, IgG1 levels increased 18-fold after infection, and the other IgG subclasses increased five- to eightfold. The levels of IgG1 and IgG3 increased after immunization with live cold-adapted vaccines intranasally, and inactivated vaccine induced IgG1, IgG2, and IgG3 subclasses (13). IgG subclass responses were different between the vaccine formulations, and also the increased levels of IgG1 differed by the serological status before vaccination. Stepanova *et al.* observed the different responses according to the vaccine formulations and age (23). IgG1 and IgG4 responses were detected only in young adults immunized with live influenza vaccine and, contrarily, inactivated vaccine induced IgG1 and IgG3 in young adults and IgG1 alone in elderly. Recently, similar results were obtained by Frasca *et al.* in a study conducted in 2011–2012, after two seasons had elapsed following the 2009 pandemic, showing IgG subclass antibody responses to H1N1pdm2009 vaccination characterized by robust IgG1 and IgG3 elevation in young adults with lesser IgG3 response in older people basically in the manner of a secondary immune response (7). IgG2 and IgG4 antibody levels were indiscernible in their cases. Interestingly, surprisingly broad IgG subclass antibody responses including igG2 were reported by Yam *et al.*, even in naive infants and young children. However, they carried out their study in the 2009–2010 season with AS03-adjuvanted H1N1pdm2009 vaccine, making direct comparison with our data difficult, although the finding of early development of IgG3 was interesting (25).

Although IgG2 was the second most abundant next to IgG1 in general, an extremely poor or no IgG2 response was also observed in both natural infection and vaccination in the present study. There have been several reports on prominent IgG2 responses after immunization with *Streptococcus pneumoniae* and *Haemophilus influenzae*, and these bacterial polysaccharides were potent stimulators of IgG2 response with different responses by age, regulated by Th1 cytokines (20). Divergent IgG2 responses were observed between several reports, with repeated infection or immunological maturation presumably influencing the response (6).

A potential weakness of the present study is its small sample size, which particularly can lead to statistical errors (i.e., not finding a difference that actually exists in the population). However, a high level of statistical significance for the time kinetics of antibody development was observed, even with this small cohort, suggesting that another statistical error (i.e., finding differences where nonexist) is unlikely and that the results are robust enough to demonstrate that influenza A(H1N1)pdm 2009 virus infection induced a unique pattern of antibody development in the human population and also to characterize the configuration of IgG subclass antibody generation.

In conclusion, an immunologically naive population contracted influenza with apparent clinical symptoms with a primary immune response in humoral immunity. However, some poorly understood immune mechanisms existed and worked to modify host experience to a subclinical infection during the pandemic period. Difference observed in the time kinetic patterns of IgG subclass antibody responses might reflect a hitherto unrecognized component of influenza immunity, which poses an attractive research question relevant to the development of the next generation influenza vaccine.

### Acknowledgments

We are indebted to Professor Peter M. Olley (Professor Emeritus of Pediatrics, University of Alberta at Edmonton) for his invaluable help in revising the manuscript.

### Author Disclosure Statement

No competing financial interests exist.

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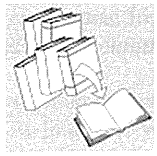
Rev. Med. Virol.
Published online in Wiley Online Library
(wileyonlinelibrary.com)
DOI: 10.1002/rmv.1819



REVIEW



# Genomic diversity of mumps virus and global distribution of the 12 genotypes

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## SUMMARY

The WHO recently proposed an updated nomenclature for mumps virus (MuV). WHO currently recognizes 12 genotypes of MuV, assigned letters from A to N (excluding E and M), which are based on the nucleotide sequences of small hydrophobic (SH) and haemagglutinin-neuraminidase (HN) genes. A total of 66 MuV genomes are available in GenBank, representing eight of the 12 genotypes. To complete this dataset, whole genomes of seven isolates representing six genotypes (D, H, I, J, K and L) and one unclassified strain were sequenced. SH and HN genes of other representative strains were also sequenced. The degree of genetic divergence, predicted amino acid substitutions in the HN and fusion (F) proteins and geographic distributions of MuV strains were analysed based on the updated dataset. Nucleotide heterogeneity between genotypes reached 20% within the SH gene, with a maximum of 9% within the HN gene. The geographic and chronologic distributions of the 12 genotypes were summarised. This review contributes to our understanding of strain diversity for wild type MuV, and the results support the current WHO nomenclature. © 2014 Crown copyright. *Reviews in Medical Virology* © 2014 John Wiley & Sons, Ltd.

Received: 15 July 2014; Revised: 14 October 2014; Accepted: 15 October 2014

## GENERAL INTRODUCTION OF MUMPS AND MUMPS VIRUS

Medical historians believe that documentation of a clinical illness consistent with mumps dates back to Greco-Roman times. The first description of a mild epidemic illness with swelling near the ears, and occasionally with painful swelling of one or both testes, is accredited to Hippocrates in the 5th century BC. The name mumps may be derived from the old English verb meaning "to sulk" or from the Scottish verb meaning "to speak indistinctly" [1,2]. In 1934, Johnson and Goodpasture demonstrated that mumps was caused by a virus present in saliva of infected patients [3].

Mumps is a highly contagious, vaccine preventable disease caused by the mumps virus (MuV). In the absence of vaccination, annual incidences of mumps range from 100 to 1000/100 000 of the general population, with epidemic peaks every 2 to 5 years usually during winter and spring in temperate countries and throughout the year in the tropics. Since the 1980s, over 60% of WHO member states have incorporated mumps vaccination into their national immunisation programmes

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***Abbreviations used***
*AFRO, WHO Regional Office for Africa; AFRO, WHO Regional Office for America; BRA, Brazil; CAN, Canada; CHN, China; C, terminal/terminus, the carboxyl-terminus; C/T to T/C, cytosine/thymine to thymine/cytosine; DNK, Denmark; DEU, Germany; ESP, Spain; EMOR, WHO Regional Office for the Eastern Mediterranean; EURO, WHO Regional Office for Europe; GB, GenBank; GBR, United Kingdom; HRV, Croatia; JL, Jeryl Lynn; JPN, Japan; IRL, Ireland; KOR, Republic Korea; LUX, Luxembourg; MMR, measles–mumps–rubella; MuV, mumps virus; NCR, noncoding region; NLD, Netherlands; Nt, nucleotides; PHE, Public Health England; RUS, Russian Federation; SEARO, WHO Regional Office for South-East Asia; SH, small hydrophobic; SWE, Sweden; THA, Thailand; TTO, Trinidad and Tobago; TUR, Turkey; WPRO, WHO Regional Office for the Western Pacific.*

which effectively reduced the incidence of mumps and the serious complications associated with mumps infection [4–7].

The clinical diagnosis of mumps is not difficult in populations with low vaccination coverage, but is more difficult in areas with high vaccination coverage and lower incidence rates, where laboratory confirmation becomes increasingly important. Generally, laboratory confirmation is based on detection of mumps-specific IgM antibodies in serum or oral fluid specimens with previous infection demonstrated by the presence of mumps specific IgG in serum. Detection of mumps viral RNA by RT-PCR is often essential since mumps-IgM may be undetectable in early samples (collected less than 3 days after symptom onset mumps) and in samples from previously vaccinated individuals [6,8,9].

MuV belongs to the genus *Rubulavirus* of the family *Paramyxoviridae*. The genome is composed of non-segmented single stranded RNA of 15 384 nucleotides (nt), which encodes two surface glycoproteins, fusion (F) and haemagglutinin-neuraminidase (HN); four core proteins, nucleoprotein (NP), virion/phospho (V/P), matrix (M) and large protein (L); and the putatively membrane associated small hydrophobic (SH) protein [10]. MuV is serologically monotypic; however, distinct lineages of wild-type viruses are co-circulating globally. The characterisation of MuV diversity is based from studying nt sequences of its most variable gene, SH. An updated mumps nomenclature in 2012 was proposed by WHO consisting of 12 genotypes based on both SH and HN sequences [6].

Increased transmissions and recent outbreaks [11,12] have increased interest in MuV genotyping as a means to map transmission pathways [6] and identify vaccine-associated cases. This paper provides an overview of the genetic divergence in MuV strains from recently accumulated data. The whole genomes of seven isolates representing six genotypes and one unclassified strain have been sequenced to generate a complete dataset including at least one genomic sequence for each assigned genotype. In addition, 11 HN and 67 SH genes were sequenced to fill gaps in the datasets. The results confirm the proposed nomenclature [6] and contribute to mumps virologic surveillance by increasing our understanding of MuV strain diversity and the global distribution of mumps genotypes.

## MUV ISOLATES AND SEQUENCE DATA ANALYSIS

Over 40 isolates were shipped from several countries to the Virus Reference Department, Public Health England (PHE) in London. Each virus was passaged on Vero cells to prepare RNA for sequence analysis. MuV growth was confirmed by both the characteristic CPE and PCR detection according to standard operational procedures [13,14]. The entire MuV genome was generated in 26 overlapping fragments. The HN and SH genes were amplified in three and one PCR reactions, respectively, according to previous publications [8,15–17]. More than 1250 sequences were analysed including newly generated data and sequences downloaded from GenBank (whole genomes, HN, F and SH sequences). Divergences between sequences were calculated and phylogenetic analyses were performed using BioNumerics 6.1, DNAstar and MEGA6.06 software. The viruses sequenced for whole genome and HN gene were mainly based on the availability of the isolates, although ideally more strains should be sequenced, preferably every 5–10 years for all genotypes. Majority sequence data of the SH gene were generated directly from clinical specimens. GenBank accession numbers (two letters and 6 numbers) are indicated as appropriate.

## DIVERSITY OF MUV GENOMES

Seventy-three whole genomes (Table 1) were analysed including genotypes A (22), B (14), C (2), F (7), G (9), H (4), I (2), K (1) and N (5) from GenBank with seven additional isolates sequenced, one each for genotypes D, H, I, J, K, L and an unclassified strain (KF878076–KF878082). Sixteen were MuV genotyping reference strains [6]. The greatest intra-genomic diversity was found within the SH gene, confirming previous observations (Figure 1A). However, for genotype G strains the greatest diversity was located in the noncoding region (NCR) between the M and F genes, and for genotype F the greatest diversity was located in the NCR between the NP and V/P genes (Figure 1B). The greatest inter-genomic nucleotide sequence difference was 7% between genotypes A and F based on the whole genome sequences (Table 2, Supplement 1 and Figure 2).

Comparisons with the novel MuV-like bat virus sequenced from an African bat spleen in 2009 (HQ660095) [18] showed that the lengths of its coded proteins were identical to MuV, but it shared only 46–46.3% nt identity with the 73 MuV

 
DOI: 10.1002/rmv

**Table 1. Mumps genotype reference strains and those with full genome sequence for analysis (73 sequences)**

| Genotype (no. seqs) | *Reference strain × no. of identical sequences | GenBank accession number: Full genome (identical sequences) | GenBank accession number: SH/HN of ref strain |
|---|---|---|---|
| **A** (22) | *MuVi/Boston.USA/0.45[A] | GU980052 | |
| | *MuVi/Pennsylvannia.USA/13.63[A] (VAC)x13 | AF338106 (BD293023, EA500331–2, AX081133, DI021804, DI064912, AX081123, FJ211584–6, BD293022, AF201473) | |
| | MuVi/JL2.USA/0.63(VAC)-Ax5 | AF345290 (EA500333,BD293024, DI035997, HQ416907) | |
| | MuVi/JL2.USA/0.63-Ax2 | FN431985 (AX081134) | |
| | MuVi//JL5.S79/CHN-A | HQ416906 | |
| **B** (14) | *MuVi/Urabe.JPN/0.67[B]x10 | AB000388 (AB000386–7, AF314558–62, FJ375177–8) | JQ945269/JQ946041 |
| | *MuVi/Himeji.JPN/24.00[B] | – | |
| | MuVi/Y213.JPN/0.0[B] | AB576764 | |
| | MuVi/Miyahara.JPN/vac[B] x2 | AB040874 (NC002200) | |
| | Muvi/Hoshino.JPN/vac[B] | Ab470486 | |
| **C** (2) | *MuVi/Zagreb.HRV/39.98[C] | EU370206 | JQ945268/JQ999999 |
| | *MuVi/Stockholm.SWE/46.84[C] | – | |
| | MuVi/Drag94.RUS/0.94[C] | AY669145 | |
| **D** (1) | *MuVi/Ge9.DEU/0.77[D] | *KF878076* | JQ945275/JQ946039 |
| | *MuVi/Nottingham.GBR/19.04[D] | – | JQ034452/JQ034464 |
| **F** (7) | *MuVi/Shandong.CHN/4.05[F] | KF042304 | |
| | *MuVi/Zhejiang.CHN/11.06/1[F] | KF170917 | |
| | MuVi/SP-A.Yunnan.CHN/0.05-Fx3 | FJ556896 (EU884413, DQ649478) | |
| | MuVi/Zhejiang.CHN/16.08/2-F | KF170918 | |
| | MuVi/Zhejiang.CHN/26.05-F | KF17091 | |
| **G** (9) | *MuVi/Gloucester.GBR/32.96[G] | AF280799 | EU597478/JQ946046 |
| | *MuVi/Sheffield.GBR/1.05[G] | – | |
| | MuVi/Split.CRO/05.11[G]x5 | JN635498 (JX287387, JX287389–91) | |

*(Continues)*


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**Table 1. (Continued)**

| Genotype (no. seqs) | *Reference strain × no. of identical sequences | GenBank accession number: Full genome (identical sequences) | GenBank accession number: SH/HN of ref strain |
|---|---|---|---|
| | MuVi/Iowa.USA/0.06-Gx2 | JX287385 (JN012242) | |
| | MuVi/Du.CRO/0.05-G | EU370207 | |
| **H** (5) | *MuVi/Bedford.GBR/0.89[H] | *KF878077* | JQ945273/JQ946035 |
| | *MuVi/Ulaanbaatar.MNG/22.09[H] | AB600843 | |
| | MuVi//1961.USA/0.88[H] | AF467767 | |
| | MuVi/Mass.USA/4.10[H] | JX287388 | |
| | MuVi/Novosibirsk.RUS/10.03[H] | AY681495 | |
| **I** (3) | *MuVi/Akita.JPN/42.93[I]x2 | *KF878078* (AB600942) | JQ945274/JQ946037 |
| | *MuVi/Dg1062.KOR/46.98[I] | AY309060 | |
| **J** (1) | *MuVi/Leeds.GBR/9.04[J] | *KF878079* | JQ945271/JQ946033 |
| | *MuVi/Sapporo.JPN/12.00[J] | – | AB105475/JQ946044 |
| **K** (2) | *MuVi/RW154.USA/0.70s[K] | *KF878080* | JQ945276/JQ946040 |
| | *MuVi/Stockholm.SWE/26.83[K] | – | JQ945270/JQ946045 |
| | MuVi/California.USA/50.07/1-K | JX287386 | |
| **L** (1) | *MuVi/Fukuoka.JPN/41.00[L] | *KF878081* | AB105483/JQ946036 |
| | *MuVi/Tokyo.JPN/6.01[L] | – | AB105480/JQ946043 |
| **N** (5) | *MuVi/Vector.RUS/0.53[N] (VAC)x3 | AY508995 (JF727651–2) | |
| | *MuVi/L-Zagreb.HRV/0.71[N] (VAC)x2 | AY685920 (AY685921) | |
| **Unclassified** | MuVi/Taylor.GBR/0.50s | – | AF142774/JQ946042 |
| | MuVi/Tokyo.JPN/0.93 | – | AB003415/AB003415 |
| | MuVi/London.GBR/3.02 | *KF878082* | AY380077/JQ946038 |

Notes: Italic: sequenced in this study;
*Reference strains.




*Rev. Med. Virol.* DOI: 10.1002/rmv

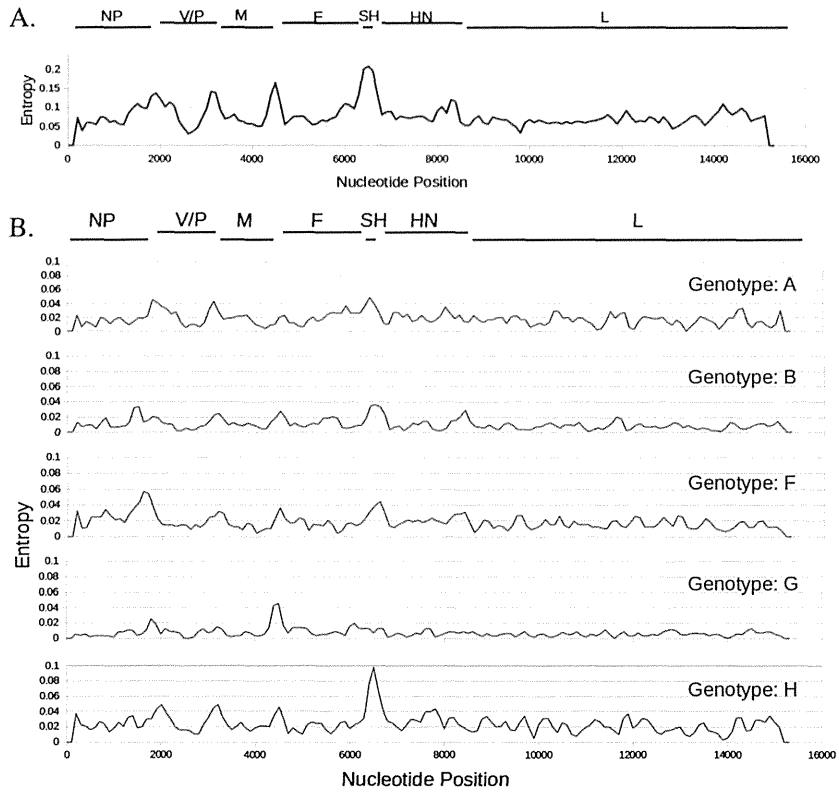


Figure 1. A. Plot showing average entropy relative to nucleotide position within the Mumps virus genome. Entropy was calculated using 73 MuV genomes and averaged within a window of 200 nucleotides with a step of 100 nucleotides. The positions of protein coding regions within the MuV genome are indicated at the top of the figure. B. Plots showing average intra-genotype entropy relative to nucleotide position for sequences belonging to one of five mumps virus genotypes. Average entropy was calculated within windows of 200 nucleotides using a step of 100 nucleotides. The five genotypes shown were selected because there were five or more unique whole genome sequences per genotype, within GenBank. The positions of coding regions within the MuV genome are shown at the top

genomes. Two of nine N-linked glycosylation sites (N-X-T/N-X-S) at aa12–15 and aa400–402 in the HN protein were absent in the bat-virus. Based on the F and HN genes, 85.5–88.7% and 82.1–83.4% aa similarity was identified (Supplement 2).

## Variations in the SH gene

The SH gene is considered the most variable gene in the MuV genome, and the SH sequence is recommended for genotyping [6]. The evolutionary divergence based on 120 representative SH sequences, including the reference strains and 67 newly generated sequences from historic strains (KF876693–KF876759), was estimated to be 17.9% between genotype A and H (Table 2, Supplement 1).

Unusual SH protein sequences were found in 14 genotype G strains detected in UK [19] and a C strain in India (KC429766), due to a mutation (T to C) that abolished the C-terminal stop codon, or a C to T mutation that resulted in the generation of an internal stop codon. Identical sequences of these atypical SH genes were detected from

**Table 2. Estimates of evolutionary divergence (min–max %) between 12 MuV genotypes based on 73 whole genomes, and 120 SH, 95 HN and 98F gene sequences**

| Diversity | Complete genome | SH gene | HN gene | F gene |
|---|---|---|---|---|
| Intra-genotypic | Up to 2.7 | Up to 9.6 | Up to 3.7 | Up to 2.9 |
| Inter-genotypic | 2.2–7.0 | 3.8–17.9 | 2.1–8.6 | 2.1–6.7 |

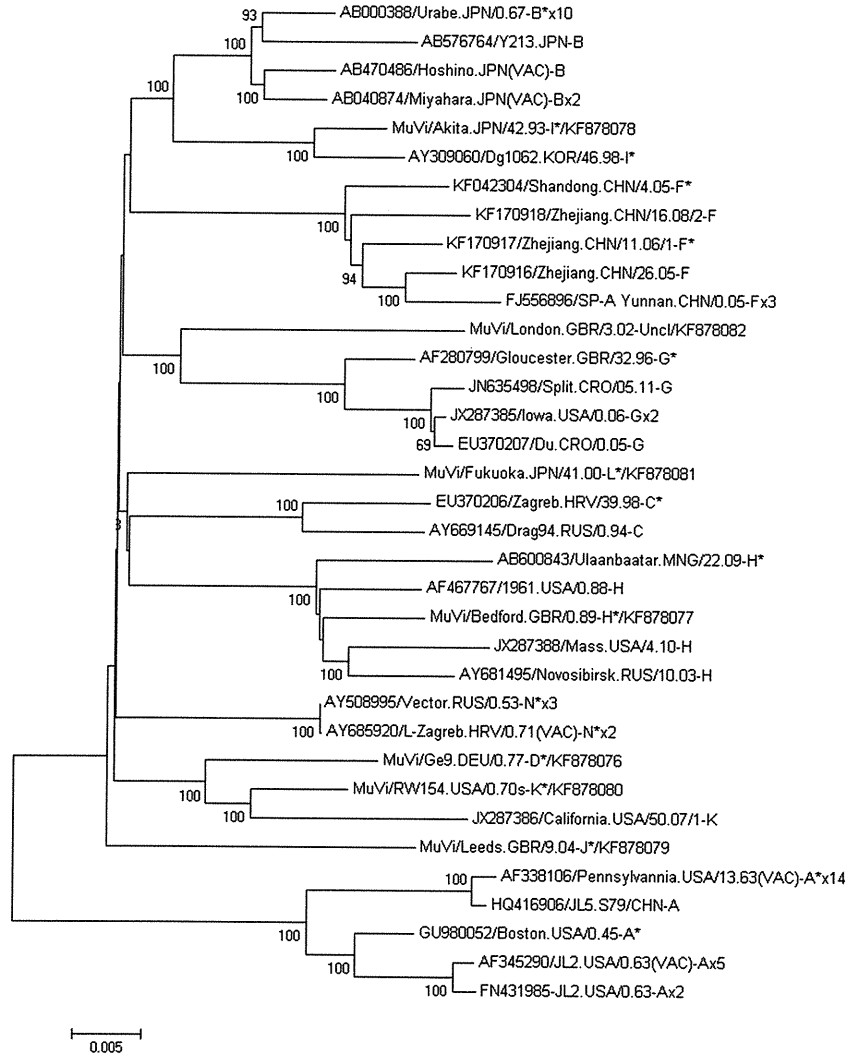


**Figure 2. Phylogenetic tree of 12 MuV genotypes based on 73 genomes. The un-rooted trees were drawn using the neighbour-joining method of MEGA 6.06 program. The parameter employed was P-distant model, and the robustness of the internal branches was determined by 1000 bootstrap replications. The horizontal length of the bar denotes percentage difference between sequences (see scale at bottom), and the bootstrap numbers (%) are given at each node. (*) assigned reference strains; (-uncl) Not yet classified**

multiple patients, suggesting that these viruses were infectious and supporting the hypothesis that the SH gene is not essential for MuV replication [20]. Although the SH protein has been ruled out as a virulence factor in animal models [21], further studies might be necessary to assess the impact of the altered termination of SH protein.

## Variations in the surface proteins (HN and F gene)

The nucleotide divergence between the 12 genotypes based on 95 HN and 98F gene sequences (KF864460–KF864470, KF878076–KF878082) is summarised in Table 2 (Supplement 1). Up to 8.6% difference between genotypes A and H (HN gene) and 6.7% between genotype A and an unclassified isolate (KF878082) (F gene) was identified.

The HN protein is the major target of humoral immunity. Nine N-linked glycosylation sites within the HN protein play important roles in maintaining the structure and antigenic properties of the extracellular domains [22]. An alignment of 95 HN sequences showed variations at these locations (Supplementary 2). An absence of the aa12–15 site occurred mainly in wild-type strains, including three C strains isolated in Sweden, Russia and UK

between 1984 and 1998, most (13) of the G strains except for the earliest (1996), three J strains isolated in Japan, UK and Malaysia and one unclassified strain isolated in UK (2002). Interestingly, an absence of a second site, aa400–402, occurred in the three J strains, and in the bat-virus sequence. Further analysis of all available HN sequences (11) of genotype J identified only two of the eight J strains detected in Singapore had the aa400–402 site (AF448532–3) [23]. In addition, the aa400–402 site was absent in two D (JQ034464 and KF864462), two H (AB600843 and KF864470) and one unclassified strain (JQ946042). The aa464–466 site was absent from many vaccine strains, including 21 genotype A sequences which were either minor (JL2) or major (JL5) components in Jeryl Lynn (JL) vaccine strain. Only the earliest wild-type A isolate (GU980052) [24] had all nine sites. The aa464–466 site was also absent in three B strains sequenced from either the Urabe vaccine or post vaccination clinical isolates (AF314559, AF314562 and FJ375177), two F strains originating from the same isolate (SP) and heavily passaged in cell culture for vaccine development (FJ556896 and EU884413) [25,26] and one K strain known as RW which has been broadly used for laboratory study (KF878080). The data suggests that the N-linked glycosylation sites were affected during passage in cell culture or transmission.

The aa265–288, aa329–340 and aa352–360 markers of the HN protein have been shown to be antigenic, and mutations at these locations may reduce cross neutralisation between strains [27–34]. Two or three substitutions at residues aa265–288 occurred in all 22 genotype A sequences and one or two occurred randomly in other strains (one B, five C, three D, two H, one J and two L) (Supplement 2). Substitution at aa336 (S336L) within the marker aa329–340 was present in all JL5 A strains, three D, two G, one J, five K and one unclassified (AB003415) strain. Eleven other strains had a random substitution at this aa329–340 marker. At marker aa352–360, D356E and Q354P were present in only JL2 A sequences and the earliest wild-type A isolate (GU980052).

The S466N substitution resulted in decreased receptor binding (haemagglutination) and release (neuraminidase) activities in the rat model [10]. This mutation occurred only in one JL2 sequence (FN431985). Mutations found in a recent wild-type virus (genotype G) at three regions, aa113–130, aa375–403 and aa440–443, were responsible for escape from neutralisation with sera from guinea pigs immunised with three vaccine strains [35]. The alignment shows that there are genotype-specific aa residues in these potential antigenic epitopes. However, the neutralisation process is a complex mechanism probably involving other MuV proteins such as fusion. Little information is available on cellular immunity in contrast to B cell epitopes. The impact of these mutations on T cell mediated immunity requires further study.

Changes in neurovirulence and fusion activity are associated with mutations in the HN protein such as K335R [31]. This mutation occurred in two genotype I strains isolated from a mumps outbreak with a high incidence of aseptic meningitis in Japan (AB600942 and KF878078) [36,37] and a third strain, genotype C, from a previously vaccinated 8-year-old child in Russia, without neurological symptoms (AY669145, Dr AP Agafonov, personal communication). The strains from these cases further demonstrate that multiple changes can influence MuV neurovirulence and neuroattenuation [38], and the observation that the development of complications is reduced in secondary cases following previous vaccination [4,7].

Four N-linked glycosylation sites in the F protein are present in all 98 sequences (Supplementary 3). Past reports have shown that nt271/aa91 in the F gene plays a significant role in viral pathogenesis; the nt271G cDNA clone was more fusogenic in vitro but less neurovirulent in vivo than the nt271A cDNA from a clinical isolate [21]. Amongst the 98F sequences, nt271A occurred only in the earliest isolate (GU980052), causing an aa change from alanine to threonine (A91T). The S195F substitution was previously reported to be associated with a change in neurovirulence and fusion activity [31,39,40]. This occurred randomly in most of the genotype B, one C, three F, two G, one I and five N sequences analysed. Leucine at aa383 of the F gene, which is responsible for fusogenicity of wild-type MuV in B95a cells was found in all strains except for the Hoshino vaccine (AB470486). The alignment is in agreement with previous reports [41,42] that the structure and antigenicity of the F protein among strains of different genotypes are well conserved both within and between genotypes over a long period of time. Phylogenetic analysis of the F gene of these strains resulted in similar clusters as SH and HN [6] indicating that aa substitutions located in antigenic regions in the MuV genome might be genotype specific.

 
DOI: 10.1002/rmv

## GLOBAL DISTRIBUTION OF MUV GENOTYPES

Though virologic surveillance for mumps virus is only routinely performed in a few countries, more systematic sequencing has greatly assisted the tracking of MuV transmission and the knowledge of its geographic distribution (Table 3, Figure 3A–D). The MuV strains described are named following the WHO recommendation [6] indicating the location (city and country by ISO3 code), the onset/collection date (week number and year) and genotype assigned.

### Genotype A and B

Genotypes A and B strains mainly represent earlier isolates following the first isolation in 1945; some were attenuated and used as vaccines. The JL strain isolated in the 1960s was subsequently developed as a vaccine and licensed in 1967 [43,44]. The vaccine is a mixture of two closely related viruses (JL2 and JL5) and widely used as the mumps component in the MMR vaccine [45]. The SBL-1 strain was isolated in 1969 in Sweden, and circulated within the country until 1996 [22] without being detected in other parts of the world. Wild-genotype A strains have not been detected from the 1990s when modern molecular technologies became available for MuV genotyping. Two A strains isolated in Canada and Germany were obtained from post vaccinated patients in the 1980s [46]. Genotype A viruses reported in Argentina [47] and Spain [48] were confirmed based on limited sequence data (150–200 nt) from the SH gene and appear to be associated with the major-component, JL5, of the JL vaccine. Recently, two cases associated with recent MMR vaccination were identified in UK, with the JL5 strain sequenced from the clinical specimens. There is no evidence of transmission of MuV vaccine strains within the population.

Genotype B viruses were mainly found in Japan with the first isolate, Urabe Am9, discovered in 1967 which led to the development and subsequent licensing of Urabe AM9 in Japan, Belgium, France and Italy as a vaccine. However, the vaccine was shown to cause unacceptably high rates of CNS complications in vaccinees [48–50] leading to its withdrawal in the early 1990s [5,48]. Other genotype B viruses, including Hoshino, Torii, Miyahara and Himeji were also found in Japan during the 1980s–1990s [51]. Genotype B viruses have been gradually replaced by genotype D, G, H, I, J and L viruses in Japan [52]. Two B strains detected in UK were obtained from post vaccination cases [46] before Urabe AM9 was replaced by the JL strain. Genotype B viruses of unknown sources were detected in Israel in 2004 (AM293338) and Hong Kong in 2009 (KF031046) that were identical to Urabe AM9 (AB000388) based on the SH gene.

### Genotype C and E

Genotype C strains have been identified around the world, mainly in Europe beginning in 1975 (MuVs/Belfast.GBR/0.75[C]) [46,48,53–56]. Genotype C was predominant in UK before mumps vaccination was introduced in 1988 [46]. During 1998–1999 two outbreaks in UK were caused by genotype C [8,16,57]. One index case (AF142765) was a schoolboy who developed mumps after returning from holiday in Goa, India. Genotype C has since only been found in sporadic cases in UK [19]. Interestingly, an identical SH gene sequence was detected in unrelated cases: MuVs/London.GBR/39.00[C] and MuVs/Po6s.PRT/0.96[C] which caused an outbreak in Portugal 4 years earlier. MuVs/Southampton.GBR/2.06[C] SH gene was identical to MuVs/Edinburgh 4.3.GBR/88[C] detected 18 years later (Figure 3A). Recently, a C strain detected in a Canadian patient (MuVi/Manitoba.CAN/11.11 [C]) who returned from a trip to India, Kenya and Dubai was closely related to MuVi/Chennai.IND/6.13[C] isolated in India [58]. The geographic distribution of genotype C viruses remains unclear.

Genotype E strains, previously referred to as Ed2/UK88 (X63711) and Ed4/UK88 (X63710), were genetically closely related to the C strains, Ed2.2/UK88 and Ed4.3/UK88. These E sequences were possible laboratory contaminants [46], and no similar E strains have since been detected. This has led to the decision by WHO to exclude genotype E and its reference strains from the MuV nomenclature [6].

### Genotype D, K and M

Genotype D strains were first described in Croatia in 1969 [59]. They have since been detected in West European countries including Germany [46], Denmark [28], Sweden (MuVi/V27.SWE/0.83 [D]), Portugal [53], UK [8,16] and again in Croatia (MuVi/Zagreb.HRV/0.06[D]). Strain MuVs/Glos.GBR/24.02.1[D] detected in four individuals had 12-nt differences from the reference strain MuVi/Nottingham.GBR/19.04[D] in the SH gene.

 
DOI: 10.1002/rmv

**Table 3. Global distribution of MuV wild-type genotypes: up to 2013 (44 countries)**

| WHO region | ISO3 | Country name | MuV genotype/year detected (*) |
|---|---|---|---|
| AFR | ETH | Ethiopia | G/13(CAN) |
| | ZAF | South Africa | D/09 (CAN), H/12(CAN) |
| AMR | ARG | Argentina | K/94-98 |
| | BRA | Brazil | K/07(CAN) |
| | CAN | Canada | A/88, C/85,88,11-13; D/07 (imported-Africa), 08 (imported-NLD), 09 (imported-ZAF), 11 (imported-M East); F/11-12 (imported-CHN), G/05-13; H/07 (imported-SDN), 08 (imported-PHL), 11-13 (imported-PHL, ZAF); K/07(imported-BRA), 09,12-13 (imported-VNM) |
| | DOM | Donimican Republic | H/05 (SWE) |
| | USA | United States of America | A/45,50,63-91; C/08-10; D/09; G/06-10; K/70s,07,08,10; H/88, 06-10 |
| EMR | IRN | Iran (Islamic Republic of) | H/86 |
| | SDN | Sudan | H/07 (CAN) |
| | PAK | Pakistan | G/10(CAN) |
| EUR | DIH | Bosnia and Herzegovina | G/00-11 |
| | BLR | Belarus | H/01-03 |
| | DEU | Germany | A/87,90; C/87,90,92,93; D/77; N/87; G/05, 10 |
| | DNK | Denmark | Unclassified/64; D/79-99; K/81-88; H/88; G/08 |
| | FRA | France | D/89; C/90 |
| | SCG | Serbia and Montenegro | G/09; H/09 |
| | ESP | Spain | C/99,06; D/00,01,06-07; G/05-07; H/96,00-02,05-06, 10; J/03 |
| | GBR | United Kingdom | B/89,90;C/75,80s,90,98-00,04,06; D/96,97,99, 01-04; F/99; G/96-13; H/88,95-96,98,00-04; K/99, 02; J/97,03-06 |
| | HRV | Croatia | C/98; D69,06; G5/05 |
| | IRL | Ireland | G/05-06,08; J/05 |
| | ISR | Israel | B-Urabe/04, H/03-05; J/04; G/05 |
| | LTU | Lithuania | C/98-99; D/99 |
| | MDA | Republic of Moldova | G/07-08 |
| | MKD | The former Yugoslav Republic of Macedonia | G/08-09 |
| | NLD | Netherlands | F/04; G/04-05, 09-12; D/07-08 (CAN) |
| | PRT | Portugal | C/96; D/96 |
| | RUS | Russian Federation | N/53; C/94, 02-04; H/02-04 |
| | SWE | Sweden | A/69,85-93; C/84; D/83; K/71,83; G/06,10 |
| | SWZ | Switzerland | A/74; C/92, 98-00; H/95,98-00 |
| | TUR | Turkey | H/05-07 |
| | BGD | Bangladesh | C/08 (USA) |
| SEAR | IND | India | |

*(Continues)*

**Table 3. (Continued)**

| WHO region | ISO3 | Country name | MuV genotype/year detected (*) |
|---|---|---|---|
| | | | C/98(GBR), 08 (USA), 11-13, G/08(CAN),12, 13(CAN) |
| | THA | Thailand | J/07-08; G/08 |
| | LKA | Sri Lanka | G/09(CAN), 12(CAN) |
| | AUS | Australia | J/07-08 |
| WPR | CHN | China | F/95, 01-12 (11-12/CAN); J/09 (CHN-HK), G/09-11 (CHN-HK); H/11(CHN-HK) |
| | JPN | Japan | B/63-95,97-00; D/93; G/97, 99-05; H/97; I/93; J/94,97,99,02; L/00-02; Unclassified/93 |
| | MNG | Mongolia | H/09; F/11 |
| | MYS | Malaysia | J/04 (GBR) |
| | KOR | Republic of Korea | I/97-01; H/98-01, 07-10, F/07-10 |
| | PHL | Philippines | G/10(CAN); H/07-08(CAN), 11-13(CAN) |
| | SGP | Singapore | J/99-00; G/99-00 |
| | VNM | Vietnam | K/12-13(CAN) |
| | LAO | Lao People's Democratic Republic | G/11 |

*ISO3 country code: where the virus detected had an epidemic link to the country.

The D strain isolated from an orthodox low-vaccination community in the Netherlands (MuVs/Dordrecht.NLD/37.07[D]) spread to Canada where the identical strain was detected (MuVs/Ontario.CAN/30.08/1[D]). Although the data suggest that genotype D had been circulating in Europe, the latest two D strains were linked to recent travel to the Middle East and South Africa (detected in Canada: MuVs/Ontario.CAN/4.11/1 [D] and MuVs/Alberta.CAN/22.09/1[D], respectively) (Figure 3B).

Only four K isolates were available for sequencing of the HN gene or whole genome. Analysis based on either the SH or HN gene shows that K strains are most closely related to D; however, they always cluster separately in phylogenetic trees. The K strains were mainly found in Sweden and Denmark during the 1970s–1980s with sporadic occurrences in USA (MuVi/RW154.USA/0.70s[K], KF878080) and UK (MuVs/Hereford.GBR/20.02[K], AY380079). During the parotitis epidemic in São Paulo, Brazil, 18 MuV strains were identified and classified as genotype M based on SH sequences [60] and the criteria proposed in 2005 [61]. Analysis based on the SH gene (HN not available) with all K strains shows these M sequences (EU069917–30) should belong to genotype K. A similar virus with identical SH gene, MuV/California.USA/50.07/1 (JX287386) was confirmed as genotype K based on both SH and HN alignments. Subsequently, genotype M was excluded from the MuV nomenclature [6]. Evidence suggests that genotype K is still circulating with the latest K variant, MuVs/Ontario.CAN/52.12[K] (KF212191/2), detected in Canada and imported from Viet Nam. Divergence rates between this K strain and other K variants ranged from 5.1% to 8.1% based on SH gene and 1.2–3.7% based on HN gene, and all five K strains contain the same aa336 substitution that JL5 strains do (Supplement 2).

## Genotype G, H and J

The strain MuVs/London18.GBR/0.91(Lo18), previously assigned as genotype B2 [46], is close to the reference strain MuVi/Gloucester.GBR/32.96[G], and may represent the earliest genotype G detected (Figure 3C). Genotype G viruses were

 
DOI: 10.1002/rmv

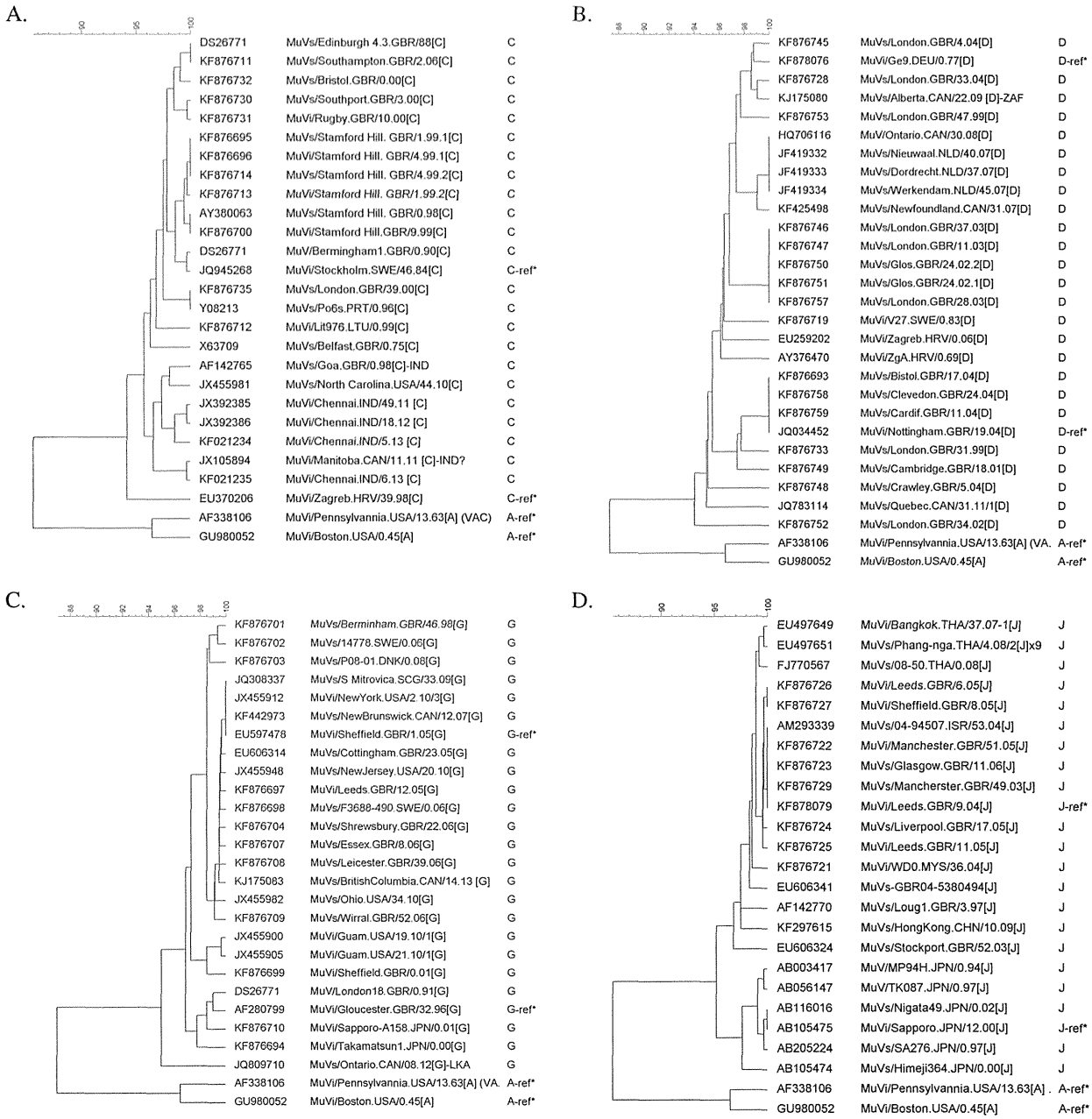


**Figure 3. A–D. Phylogenetic trees drawn based on SH gene using BioNumerics v6.1. The representatives and newly generated sequences were included, respectively, for genotype C (Figure 3A); D (Figure 3B); G (Figure 3C) and J (Figure 3D) strains**

initially detected sporadically in UK (1996) [16]. A small outbreak occurred in a group of Gurkhas in the British Army in 1998 after their arrival in UK from Nepal, the source of the infection [16,62]. Since then, a dozen G variants with point mutations in the SH gene were detected with two strains becoming predominant in UK [63]. One variant (MuVi/Sheffield.GBR/0.01[G]) circulated since 1998 and disappeared after 2005. The second, identified in 2000 (MuVi/Sheffield.GBR/1.05[G]), continues to circulate within UK and other European countries including the Netherlands [64], Croatia [55], Ireland [65], Israel [66], Germany [67], Republic of Moldova [68], the

 

former Yugoslav Republic of Macedonia [69], Bosnia & Herzegovina [70], Spain [48], Sweden (MuVs/14778.SWD/0.06[G]), Serbia & Montenegro (MuVs/S Mitrovica.SCG/33.09[G]) and Denmark (MuVs/P08-01.DNK/0.08[G]). In the mumps resurgence in USA and Canada 90% of viruses were identified as genotype G [71,72], which was continually detected up to 2010 in USA (MuVi/Texas.USA/40.10/1[G]) and 2013 in Canada (MuVs/British Columbia.CAN/14.13[G]). G variants were also identified in Asian countries including Japan [51], Thailand (FJ770566) and China–Hong Kong (KF031049, KF031055, etc.). A strain detected in Canada (JQ809710) was imported from Sri Lanka (Table 3). The phylogenetic tree (Figure 3D) of the SH gene sequences shows the two G strains detected in Serbia, MuVs/S Mitrovica.SCG/33.09[G] and MuVs/Kuzmin.SCG/33.09[G] were identical to two detected four years earlier in UK, MuVi/Sheffield.GBR/1.05[G] and MuVs/Cottingham.GBR/23.05[G], respectively. In contrast to other genotypes, the SH gene is not the most variable region in G strains (Figure 2), suggesting that further studies are needed to determine the divergence and evolution of this genotype.

The earliest genotype H, MuVi/S-12.IRN/0.86[H] (AF315684) was found in Iran. Subsequently, sporadic detections with divergences have occurred in the following countries: Canada [8,46], UK MuVi/Bedford.GBR/0.89[H](KF878077) [8], Japan [52] and Serbia (MuVs/Sabac.SCG/9.09[H], (JQ308338). Outbreaks caused by H strains were reported in Switzerland [73], Republic of Korea [74], Spain [75], Belarus [54], Russia [76]; Israel [77], Turkey [78] and Mongolia [79]. In addition, H virus was found in a Swedish patient returning from the Dominican Republic (MuVs/SE171-82.SWE/8.05[H], JQ034432) and in Canada imported from Sudan (MuVi/Calgary.CAN/30.07[H], JN687468/9), Philippines (MuVs/Alberta.CAN/28.11/1[H], JQ783112; MuVs/BritishColumbia.CAN/07.12[H], JQ809709) and South Africa (MuVs/BritishColumbia.CAN/01.12[H], JQ783116). Genotype H has the most intra-genotypic divergence amongst the 12 genotypes, up to 9.6% and 3.4% based on the SH and the HN gene, respectively. In contrast, an identical SH sequence was found in MuV detected in Denmark (MuVs/V88-14555.DNK/0.88[H]), UK (MuVs/Watford.GBR/5.03[H]) and Serbia (MuVs/Sabac.SCG/9.09[H]) over a period of 20 years, suggesting existence of some MuV strains with permitted genetic fitness, at least in the SH gene.

Genotype J was mainly reported in Japan [52,80], Singapore [23], UK (EU606324, KF878079, KF876722–7) and Thailand (EU497649–57). Figure 3D shows Japanese strains form into a separate cluster within genotype J, with a maximum divergence of up to 8.3% between the Japanese cluster (MuVs/Himeji364.JPN/0.00) and the other J cluster (MuVs/Stockport.GBR/52.03). Retrospectively, an unclassified strain [16], MuVs/Loug1.GBR/3.97[J] was the first J found in UK. Sporadic cases due to genotype J were also identified in Spain [48], Malaysia (MuVi/WD0.MYS/36.04[J]) and Ireland [81]. Genotype G was also simultaneously circulating within these countries.

## Genotype F, I and L

Genotype F, I and L appear to have limited circulation. Genotype F has been predominant in China since its discovery in 1995 [82] and was also detected in the neighbouring countries of Republic of Korea (MuVi/Incheon.KOR/16.08/22[F]) and Mongolia (MuVs/Umnugobi.MNG/11.11/1[F]). F strains have been found elsewhere, but only occasionally: 14 confirmed cases from a UK boarding school outbreak in 1999 [8]; a single incident within a Chinese restaurant in the Netherlands (MuVi/Tiel.NLD/50.04[F]); and four cases in Canada, three of which were imported from China. One Canadian strain imported from China (MuVs/Ontario.CAN/04.12[F]) was identical to an F strain detected in Hong Kong (MuVs/HongKong.CHN/12.09[F]). Multiple F variants with point mutations have circulated simultaneously in China for over 15 years [83] without a sustained predominant strain, in contrast to genotype G in UK. However, like genotype G, the SH gene is not the most variable region in F strains (Figure 2). Any possible relationship between the frequency of genetic variations and population size/density should be considered and investigated further.

Genotype I was initially found in Japan in 1993 [84], then predominantly within Republic of Korea from 1997 to 2001 [85]. Genotype L has similar restricted distribution with detection in Japan only from 2000 to 2002 [51] (AB116011). In contrast to genotype A, B and N, genotype I and L have never been used as vaccines, and there were no similar substitutions at those antigenic markers in the HN

 
DOI: 10.1002/rmv

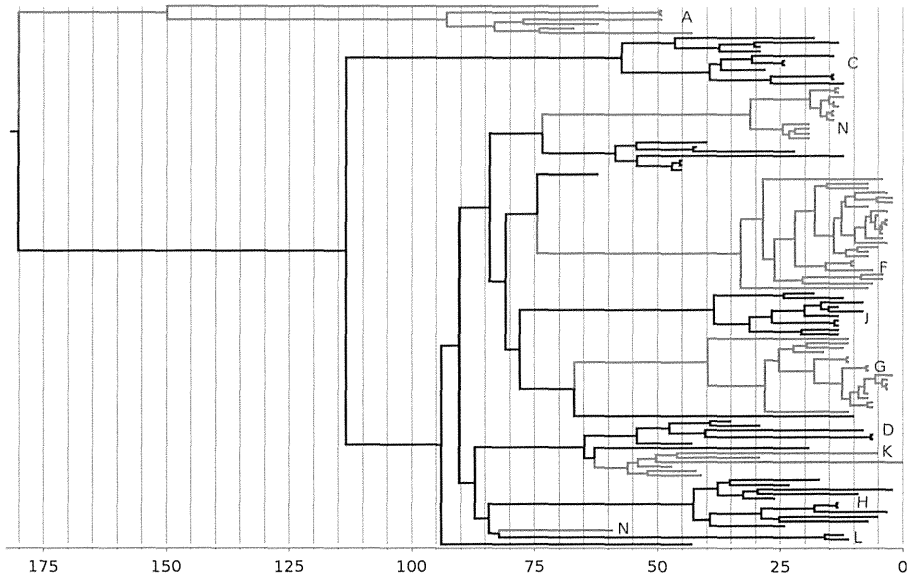


Figure 4. BEAST phylogeny of 119 MuV HN sequences. The timescale across the bottom of the phylogeny represents years since the date of the last sequence (2012). The genotypes are coloured black or grey and labelled for clarity

and F genes as occurred in genotype A, B and N strains. Whether genotype I and L remain active or will reappear is currently unknown.

## Genotype N and unclassified variants

Leningrad-3 strain was isolated in the Former Soviet Union in 1953 and developed as a vaccine. It has been used in the national immunisation programme since 1980 and was further attenuated in Croatia by adaptation to chick embryo fibroblasts. This new strain, designated as Leningrad–Zagreb, has been used for vaccine production in Croatia and India, and administered to millions of children around the world. No genetically similar wild-type strain has been found over this period anywhere. These two vaccine strains were proposed as a potential new genotype in 2005 [61,86], and their continued broad spectrum usage has led to their being designated as genotype N [6].

Divergences of MuV variants MuVi/Taylor.GBR/0.50s, MuVi/Tokyo.JPN/0.93 and MuVi/London.GBR/3.02 [6] from the reference genotypes ranged from 5 to 16.5% and 2.3 to 7.7% based on the SH and HN genes, respectively, suggesting new genotypes. Inclusion of these unclassified sequences in the dataset for genotyping analysis would help with a more comprehensive analysis of genotype designation.

## EVOLUTION OF MUV GENOTYPES

Bayesian phylogeny was generated with BEAST software using 119 HN sequences representing the diversity of MuV genotypes. Sequences that were excluded from this analysis were partial HN gene sequences, sequences missing sample date and sequences known to be from viruses passaged in cell-culture (unlikely to represent MuV isolates found in the wild). The final exclusion criteria removed many cell-culture adapted viruses that have been used to generate MuV vaccines. The final sequence dataset contained sequences from isolates characterised in the 1950s to 2012. BEAST was run using the general time reversible (GTR) model of nucleotide substitution, a log normal relaxed molecular clock and a constant population model for 30 million iterations. A Maximum Clade Credibility (MCC) tree was generated using treeAnnotator with a 3000 tree burn-in.

Phylogenetic analysis of MuV HN sequences using BEAST (Figure 4) suggests that genotype A is distant from all of the other genotypes and that the common ancestor for genotype A may be older than the single common ancestor of the other genotypes. Genotype A has not been detected as a wild-type virus since the 1990s (Table 3). The majority of currently circulating mumps genotypes appear to have arisen between 60 and 100 years ago, with the exception of genotype C which

 
DOI: 10.1002/rmv

appears somewhat older (100–125 years ago). There are also examples of sequences from MuVs detected in the 1950s and 1960s that do not cluster within genotypes and presumably represent circulating strains for which there was limited sampling. The majority of clusters that have been observed and classified into genotypes do not appear to remain in circulation for extended time periods. It appears likely that regions with low vaccine coverage and limited (or non-existent) sampling seed outbreaks in regions with higher vaccine coverage and more detailed molecular surveillance, and this results in the observed pattern of genetically and temporally discreet clusters.

## SUMMARY

Much remains to be learned about the epidemiology of mumps and the distribution of different genotypes locally. Genotypes C, G, H, J and K were observed in the Western Hemisphere, whereas genotypes B, F, I and L predominate in Asia. Different genotypes can also co-circulate in the same country: multiple genotypes, B, G, I, J and L were found in Japan in the 1990s; C, D, G, H and J in the UK prior to the mumps resurgence of genotype G from 2004 to 2013. However, only six of the 12 genotypes have been circulating since 2010 including genotype G (52%), H (16%), C (12%), F (8%), K (8%) and D (4%) based on the detected in or linked with 25 countries (Table 3). Close monitoring of MuV distribution is needed in the vaccine era. Improvement in mumps surveillance based on advanced sequencing technologies will enable routine whole genome sequencing. The use of this data will facilitate both the understanding of mumps diversity and its use in analysing transmission chains. This review has contributed to the limited MuV data and highlighted the current state of knowledge.

## CONFLICT OF INTEREST

The authors have no competing interests.

## ACKNOWLEDGEMENTS

We would like to acknowledge Dr David WG Brown and the MMR team at PHE for their support. The authors also thank other colleagues (ISO3 country code) for their enthusiastic communications and contributions in molecular surveillances of mumps (alphabetical order): Alexandre. P Agafonov (RUS), Alper Akcali (TUR), Bettina Bankamp (USA), Rob van Binnendijk (NLD), Blenda E Böttiger (DNK), Michael Carr (IRL), Enrique Royuela Casamayor (ESP), Aili Cui (CHN), David Featherstone (WHO-HQ), Yan Feng (CHN), Todd Hatchette (CAN), Judith Huebschen (LUX), Youngmee Jee (WPRO-WHO), Malaiyan Jeevan (IND), Kari Johansen (SWE), Atsushi Kato (JPN); Minoru Kidokoro (JPN), Kisoon Kim (KOR), Janice Lo (CHN), Elena Lopareva (USA), Yiyu Lu (CHN), Annette Mankertz (DEU), Thangam Menon (IND), Sirima Pattamadilok (THA), Sabine Santibanez (DEU), Silke Schepelmann (GBR), Lottie Schloss (SWE), Alberto Severini (CAN), Sergey Shulga (RUS), Makoto Takeda (JPN), Sunil R Vaidya (IND), Wenbo Xu (CHN) and JeongSun Yang (KOR).

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