MV infection allows replication of HHV 6B in the PBMCs of subjects that stimulates specific lymphocyte responses. Mobilized CMI may have suppressed viral reactivation under limited level characterizing the combination of DNA synthesis with possibly a production of immediate-early and/or early viral proteins but without specific antibody responses or virion formation. This novel type of reactivation might be named "abortive" reactivation.

In the present study HHV 6B specific lymphocyte proliferation activity was low in the acute phase and increased subsequently, peaking around day 16 and declining 5 weeks after the onset. In contrast, lymphocyte proliferation activities to PHA increased progressively from the acute to the convalescent phase of measles peaking in the third samples, supporting the view that lower lymphocyte proliferation activities to PHA in the acute stage of measles may reflect depressed CMI caused by MV infection while the increased activities of lymphoproliferative responses in the second and the third samples imply recovery of CMI from the influence of the MV infection. Taken together, even under the depressed immune function resulting from measles virus infection, HHV 6B specific CMI developed in a characteristic pattern composed of elevation and waning resembling that observed in acute varicella [Kumagai et al., 1980]. This pattern might reflect the fact that HHV 6B reactivation during the course of acute measles, and the time course characteristics represent HHV 6B specific T cell mobilization to antigenic re-exposure. To support interpretation of the results in measles patients, the kinetics of responses in lymphocyte proliferation assay to both HHV 6B and PHA were observed in a group of influenza patients and of a healthy population as a control group. Lymphoproliferative responses in both groups as control populations did not show any statistically significant changes in activities during the observation period, substantiating the view that the changes in lymphocyte activities in measles patients are specifically elicited by and directed against reactivated HHV 6B.

Regarding this specific point, one might argue that the present study gave no convincing evidence of HHV 6B reactivation, as there was no increase in antibody titer, and the virus could not be isolated. Also, HHV 6B DNA in PBMCs of healthy subjects without signs of HHV 6B reactivation has been documented [Cuende et al., 1994; Alvarez-Lafuente et al., 2002; Caserta et al., 2004]. Indeed, until now, HHV 6B reactivation was known to be associated with specific antibody elevation and positive virus isolation. However, even in a severe immune suppression induced by immunosuppressive therapy after organ transplantation, only some of the recipients show such a "typical" or "complete" type reactivation [Ohashi et al., 2008]. Even in the previous study, only a few subjects (3/42) revealed positive virus isolation and only two showed elevated antibody titers. Thus, in measles induced immunosuppression, there might be an "abortive" or "incomplete" type subclinical reactivation which is characterized by the presence of specific CMI response and DNA synthesis in PBMCs with lack of virus isolation and antibody rise. HHV 6B is reactivated in the latently infected macrophages and lymphocytes. Viral immediate early and early antigens synthesized to stimulate T-cell response and DNA is synthesized to be detected as DNA in the mononuclear cells. In early reactivation in measles subjects, sensitized T cells acting against immediate early antigens may suppress further replication cycles of HHV 6B and virion formation might be suppressed rendering any increase in the neutralizing antibody titer undetectable. In this regard, antibody determination to immediate early (IE) and early antigen (EA) was carried out additionally (data not shown) [Mori et al., 2000], showing that some patients responded to IE and EA and others did not, thus, the results could not explain this discrepancy. Differences of the major recognizing antigens between lymphocytes and antibodies against immediate early, early, and late glycoprotein antigens may present but not detected by this assay using sera. Furthermore, in our previous study, the CMI response was shown to be more sensitive than the humoral immune response in detecting host immune function in the subjects with varicella [Kumagai et al., 1999]. One possible interpretation is based upon the data composed of three consecutive specimens obtained at different stages of acute measles virus infection. Accordingly, the dynamic fluctuations in quantity of viral DNA in 17 out of 22 in subjects in a short period of time in combination with a statistically significant wax and wane pattern of elicitation of virus specific CMI response strongly support the view presented above. The mode of reactivation of various herpes viruses differ from each other. In HSV infections, even clinically apparent recurrence occurred without significant changes in the levels of virus-specific antibodies [Zweerink and Stanton, 1981; Zweerink and Corey, 1982; Kahlon et al., 1986]. In fact, measles impairs the immune response, but the duration of immunosuppression is shorter than that in organ transplant patients. Measles does not induce HSV or CMV diseases. Thus, this novel form of subclinical reactivation response might be unique to measles.

In addition, the influenza population data raises another interesting consideration. The cause of reactivation of HHV 6B in subjects with acute measles remains unknown. MV is known to suppress cell-mediated immunity, but it also causes marked imbalance of cytokine profiles during the course of the disease suggesting that cytokine excess might be involved in deranging the immune system. Of interest is whether the same level of HHV 6B reactivation in association with subsequent elevated activities of HHV 6B specific CMI occur in subjects with other viral infections that are not known to cause immunosuppression, but do invoke hypercytokinemia, influenza being an example. The present data suggest

that the level of hypercytokinemia induced by influenza virus infection was insufficient to provoke HHV 6B reactivation and did not affect mobilization of HHV 6B specific lymphocytes. However, such a conclusion is beyond the scope of this article because cytokine profiling was not part of the protocol mainly because of sample volume constraints.

The present study is limited by its small sample size. However, the observed temporal characteristics of HHV 6B specific lymphocyte proliferation responses as well as those to PHA were statistically robust, and this distinctive and unique approach to compare specific lymphoproliferative activities in three consecutive specimens minimized this limitation.

In conclusion, the dynamic fluctuations in specific lymphocyte proliferation activities observed in this study seem to reflect the process of internal re-exposure of the CMI system to HHV 6B antigen, involving both reactivation and suppression. The present data expands understanding of the HHV 6B-host interaction.

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Humoral Immune Response to Influenza A(H1N1)pdm2009 in Patients with Natural Infection and in Vaccine Recipients in the 2009 Pandemic

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Abstract

The 2009 pandemic H1N1 mainly affected adolescents and children, and most of the elderly in Japan escaped clinical illness. To clarify the role of humoral immunity in the infection, the time kinetics of hemagglutination inhibition (HI), neutralization (NT), and IgG subclass antibody response directed against influenza A(H1N1)pdm2009 were analyzed in three consecutive specimens obtained from 51 young adults and children (group 1) who contracted pandemic influenza and from 74 pediatric clinic employees (group 2) inoculated with pandemic monovalent vaccine. In group 1 patients, 6 and 30 patients had lower HI and NT antibody in the acute phase respectively. Thereafter, HI and NT antibody titers increased fourfold or more in 50 patients with peak response in the third specimens obtained four weeks after the onset. IgG1 in 45 patients, IgG3 in 18 patients, and IgG4 in 29 patients showed elevated responses. Forty (54%) and 70 (95%) subjects in group 2 had positive HI and NT antibodies in the prevaccination samples, with increased antibody responses in the follow-up peaking in the second specimens. Forty of those vaccinated had increased IgG1 responses peaking in the third specimens, whereas elevated IgG3 was observed in 22 recipients with the highest level in the second samples. IgG4 did not show any increase in subjects in group 2. A few participants showed an IgG2 response in both groups. An immunologically naive population contracted influenza with apparent clinical symptoms. However, already primed subjects through subclinical infection elicited the unique pattern of IgG subclass responses by vaccination, which differed from those of naive populations.

Introduction

In 2009, a newly emerged influenza A(H1N1)pdm2009 virus caused a worldwide pandemic. In spite of pessimistic concerns that there was no herd immunity, H1N1 mainly affected adolescents and children, while the majority of the elderly escaped clinical illness (4,5,9,12,16,19,24). Hardelid *et al.* pointed out that the same age distribution was observed in other pandemics such as those in 1918, 1957, and 1968 (12). In addition, many middle-aged and elderly people acquired antibodies directed against 2009pdm influenza virus without any clinical symptoms (3,4). In Japan, we also observed a similar distribution of subjects naturally infected in 2009. However, the cause of this skewed age distribution of the new pandemic influenza remains obscure.

Although many authors describe an important role of specific cell-mediated immunity (CMI) in protection (10), actual studies of specific CMI in humans is quite difficult in

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the clinical setting mainly because of sample volume constraints, especially in those with a primary immune response to influenza who are generally small children and infants. Also, it is virtually impossible to handle short-lived samples such as lymphocytes in the clinic during the heavy workload created by a pandemic. However, it is established that induced subclass IgG composition specific to antigen reflects a balance of Th1/Th2 activation in animal models (14), suggesting that it is worthwhile trying to trace the imprint of Th1/Th2 activities occurring in subjects with pandemic influenza or post-vaccination by studying IgG subclass responses to pandemic influenza.

In linking data obtained in humans with that from mice, we need to consider the confusing historical background regarding IgG subclass nomenclature. Human IgG subclasses have been identified according to the time of their discovery, but for the mouse, IgG subclasses reflect their electrophoretic mobility (22). Functional difference between mouse IgG subclasses and their similarly named human fractions are not yet fully documented. In mice, these responses correlate with IgG2a, IgG2b, and IgG3, regulated by the production of IFN-γ (21), while in humans such interrelationships are not yet well defined. IgG1 is the most abundant subtype, making up more than 50% of total IgG, and IgG4 is the smallest component. Human IgG1 reflects not only Th1 cytokine response but also Th2 cytokine activation. IgG4 subclass switch depend on IL-4 and IL-13, which are considered the Th2 response (2,18).

Primary antibody responses require T-cell help through functionally different Th1 and Th2 cytokines secreted by antigen-specific CD4+ T-lymphocytes. Therefore, the analysis for IgG subclass responses after infection or vaccination may provide additional information in terms of CD4+ T-cell effector functions, which might be supposed to reflect the balance of Th1 and Th2 activation provided by the antigenic stimulus of the novel influenza pandemic. Thus, our aim in the present study was twofold: first, to clarify the role of humoral immunity in the infection of 2009 pandemic; second, to evaluate the induced subclass IgG composition. To achieve these goals, we determined the time kinetics of hemagglutination inhibition (HI), neutralization (NT), and IgG subclass antibody response directed against influenza A/California/07/2009(H1N1) in patients with natural infection and subjects immunized with pandemic monovalent vaccine.

Materials and Methods

Study subjects

The study groups consisted of 51 healthy young adults and children (24 females) with a mean age of 7.6 years (range 0.5–25 years), who contracted pandemic influenza caused by influenza A(H1N1)pdm2009, and 74 pediatric clinic employees (63 females) with a mean age of 38.5 years (range 19–72 years) inoculated with one dose of pandemic vaccine. The vaccine formulation used in this study was monovalent HA split product of influenza A(H1N1)pdm09 virus made by Japanese vaccine manufacturers. Diagnosis of influenza was made by rapid test (ImmunoAceFlu®; Tauns, Izunokuni, Japan) followed by virus isolation, polymerase chain reaction (PCR), and seroconversion with HI and NT antibody determination. A detailed history was taken from pandemic vaccine recipients as to symptoms suggesting influenza such as fever, cough, rhinorrhea, headache, diarrhea, muscle pain, and arthralgia. None had had any of these symptoms during the past year. Three consecutive peripheral blood specimens (acute phases, 2 weeks and 4 weeks after the onset of illness in patients with influenza and both prior to and 2 and 4 weeks after vaccination in recipients) were obtained. Blood sampling in patients with natural infection was based on the first day of fever designated as day 0, and the first specimens were collected after 2 ± 2 days of the acute illness. Patients were recruited from September 16 through October 20 followed by an additional two samplings. Regarding pandemic vaccine recipients, the first specimens were obtained on the day of immunization, which extended from October 26 through November 21. Two late samplings were included. This study was approved by the ethical committee of Sapporo Medical University and by the separate institutions of the authors. Informed consent was obtained from the subjects after full explanation, and consent was also obtained from all children aged 6 years or more.

Influenza virus and antigen preparation used for antibody assay

A/California/07/2009 virus purified whole virion was prepared by means of sucrose density gradient centrifugation for HI test. HA split vaccine bulk material of influenza A(H1N1)pdm2009 was used to determine IgG subclass antibody titer, which was adjusted to 333 ng/mL of HA protein in phosphate-buffered saline (PBS) as antigen.

Determination of HI and NT antibody specific to influenza A/California/07/2009 virus

The serum samples were stored at −35°C until testing. The HI test was performed by a standard microtiter assay with human type O erythrocytes after removal of nonspecific inhibitors with receptor-destroying enzyme and of cold agglutinins by hemadsorption at 4°C. All specimens were tested on the same day using identical reagents.

Determination of NT antibody titers was carried out by the microneutralization (MN) assay (17). In brief, serially diluted sera (1:10 to 1:1280) in Dulbecco's minimal essential medium (DMEM) were prepared in a 96-well microplate. An equal volume of virus fluid (50 FFU/25 μL) was added and incubated at 37°C for 60 min in a 5% CO_2 incubator. As a positive control, viruses were mixed with an equal volume of DMEM without serum. After incubation, 50 μL of the NT mixture was inoculated onto a monolayer of MDCK cells in a 96-well microplate. After incubation for 16–20 h, cells were fixed with absolute ethanol and reacted with mouse monoclonal antibody against influenza A virus nucleoprotein (NP; clone C43) (17), followed by incubation with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG antibody (A2304; Sigma). Cells expressing viral antigens were visualized by 3,3′-diaminobenzidine tetrahydrochloride hydrate (D5637; Sigma). The MN titer was indicated by the reciprocal value of the maximum dilution at which the number of foci showed 50% reduction compared with the positive control.

In both the HI and NT tests, positive cutoff was set at 1:10. Thus, an antibody level $<1:10$ was designated as negative. The criterion for seroconversion was defined as elevation of antibody level by fourfold or more. To investigate antibody

responses in detail, we classified them into three categories: no elevation; antibody level unchanged or less than the first specimen; twofold increase and fourfold ore more elevation.

Determination of IgG subclass antibodies

We performed quantitative enzyme immunoassay (EIA) to detect IgG subclass antibodies against influenza A/California/07/2009 strain. Wells of a 96-well plate were coated with virus antigen, 33 ng/100 μL of split vaccine materials calibrated to the amount of HA protein for 16 h at room temperature. After four washes with PBS containing 0.05%Tween 20, wells were coated with Blocking One (Nacalai Tesque) for 45 min at room temperature. Serial twofold dilutions of serum samples were incubated in 100 μL/well for 60 min at room temperature, starting at 1:200 for IgG1, IgG2, and IgG4, and at 1:20 for IgG3. Secondary antibodies were added at 1:3,000 dilution for anti-human IgG1, at 1:1,000 dilution for IgG2, at 1:2,000 dilution for IgG3, and 1:2,000 dilution for IgG4 (Invitrogen). The plate was incubated for 60 min at room temperature. The wells were washed, and 100 μL of enzyme substrate was added, which was prepared with 40 mg of O-phenylenediamine (Wako Junyaku) in 100 mL of Na-citrate buffer, with 20 μL of 30% H_2O_2 added just before use. After 20 min incubation, 50 μL of 4N-H_2SO_4 was added, and optical density was measured at 450/630 nm. EIA antibody titers were expressed as the reciprocal dilution of 100×2^n that gave twofold value of optical density in the negative control well for each serum sample, derived from regression curve analysis with three dilution point plotting.

Statistical analysis

Consistency in HI, NT, and IgG subclass antibody directed against A/California/07/2009 virus at three points after onset of influenza and of pre- and post-vaccination was examined by using a nonparametric repeated-measures analysis of variance (Friedman's test) followed by Dunn's test as post-test employing StatFlex software v6. For calculation of geometric mean titer (GMT) estimates, a titer of < 10 was assigned a value of 5, and a titer of ≥ 8 in HI, NT test, and that of 9 in IgG subclass determination were assigned a value of 9 and 10 respectively.

Results

Time course characteristics of the antibody response specific to pandemic H1N1 2009 in naturally infected subjects was profiled (Fig. 1). GMTs determined in the first, second and third specimens with 95% confidence intervals (Cis) were 5.4 [5.1, 5.8], 12.4 [8.6, 17.8], 120.4 [94.1, 154.1] in HI, and 8.7 [7.4, 10.2], 44.7 [28.1, 71.1], 407.9 [312.6, 532.1] in NT, respectively. Statistically significant antibody elevations were observed (Friedman's test, $p < 0.0001$), and Dunn's test confirmed that peak antibody responses occurred in the third specimens ($p < 0.001$), implying that a primary immune response accompanied natural infection. More specifically, 6 of 51 patients had low-level HI, and 30 of the 51 low-level NT antibody in the first specimens, and 50 showed a fourfold increase or more in HI and NT antibody titers in the follow-up. The remaining patient had HI antibody of 1:10 in the first specimen followed by elevation to 1:20 in the second specimen. They also had NT antibody of 1:40 in the first specimen and did not show any increase. Virus isolation and PCR were positive in this patient.

IgG subclass antibody GMTs in three consecutive specimens obtained from patients were: for IgG1, 273.1 [198.0, 376.6], 737.3 [475.5, 1143.0], 1788.2 [1270.0, 2517.8]; for IgG3, 33.4 [22.4, 49.8], 39.8 [27.1, 58.3], 50.2 [33.7, 74.9]; and for IgG4, 476.6 [322.1, 705.2], 914.2 [548.8, 1522.9], 1780.1 [927.3, 3417.2]. IgG1, IgG3, and IgG4 antibody levels rose significantly ($p < 0.0001$ for IgG1; $p = 0.0045$ for IgG3; $p < 0.0001$ for IgG4) and peaked in the third specimen ($p < 0.05$ for IgG1; $p < 0.002$ for IgG3; $p < 0.001$ for IgG4). Only three cases developed IgG2 antibody at a very low level. Among 51 patients, initial specimens had some level of IgG1 (16 patients), IgG3 (15), and IgG4 (25) antibody activity, and 9, 2, and 21 of the above-mentioned cases exhibited a subsequent rise of fourfold or more. Twofold elevations were also observed in two cases for IgG1, three for IgG3, and four for IgG4. Five cases for IgG1 and 10 for IgG3 did not show any increase. IgG1 in 34 cases, IgG3 in 13 cases, and IgG4 in four cases were negative in the first samples but eventually manifested a fourfold or more rise in 32, 12, and 4 patients. The remaining two patients for IgG1 and one for IgG3 showed a twofold increase. No elevated response for IgG1, IgG2, IgG3, or IgG4 was observed in 1, 48, 23, and 22 subjects, respectively, throughout natural infection.

GMTs in HI and NT in pre- and post-vaccination serial specimens were 11.3 [8.9, 14.4], 70.1 [50.8, 96.9], 62.8 [45.4, 86.7], and 38.1 [28.2, 51.5], 329.0 [231.6, 467.4], 304.8 [215.6, 430.9], respectively (Fig. 2). Post-vaccination antibody titers were significantly higher than prevaccination ($p < 0.0001$) titers for both HI and NT. Antibody titers peaked in the second specimens, then declined in the third ($p < 0.001$). Of those vaccinated, 11 of 74 exhibited prevaccination HI antibody of $\geq 1{:}40$, three of whom subsequently manifested fourfold or more increases. Four participants showed only a twofold rise thereafter, and the remaining four showed no elevation. Twenty-nine had prevaccination HI antibody of 1:10 to 1:20, and subsequent antibody increases were observed in all but two cases. Among these, 24 had fourfold or more increases, and three showed a twofold increase. Thirty-four had no HI antibody in prevaccination samples, and 23 of those showed fourfold elevation or more, 11 a twofold HI antibody rise, and two had no HI antibody response. Regarding NT antibody, 42 had a prevaccination titer of $\geq 1{:}40$, and 29 of those showed fourfold or more increases thereafter. The remaining nine revealed a twofold rise, and four did not exhibit any increased antibody response. Twenty-eight had positive NT antibodies of 1:10 to 1:20 in prevaccination samples, and 22 of those developed antibody rises of fourfold or more, four showed twofold increases, and two revealed no elevation. The remaining four did not have NT antibody before vaccination but developed a fourfold or more antibody response.

Regarding IgG subclass antibody developments in vaccine recipients, GMTs of IgG1 in consecutive three specimens were 1085.4 [827.4, 1423.7], 1970.0 [1541.7, 2517.3], 2020.2 [1587.3, 2571.1]; those of IgG3 were 88.4 [56.5, 138.5], 134.2 [88.5, 203.5], 128.8 [81.2, 204.6]; and those of IgG4 were 172.3 [129.0, 230.1], 172.2 [128.9, 230.0], 171.5 [127.7, 230.4]. IgG2 was found in only six subjects. IgG1 and IgG3 antibodies levels were significantly elevated ($p < 0.0001$ for IgG1; $p = 0.0001$ for IgG3), and the peak IgG1 responses occurred in the third samples ($p < 0.05$), whereas the levels of IgG3 titers

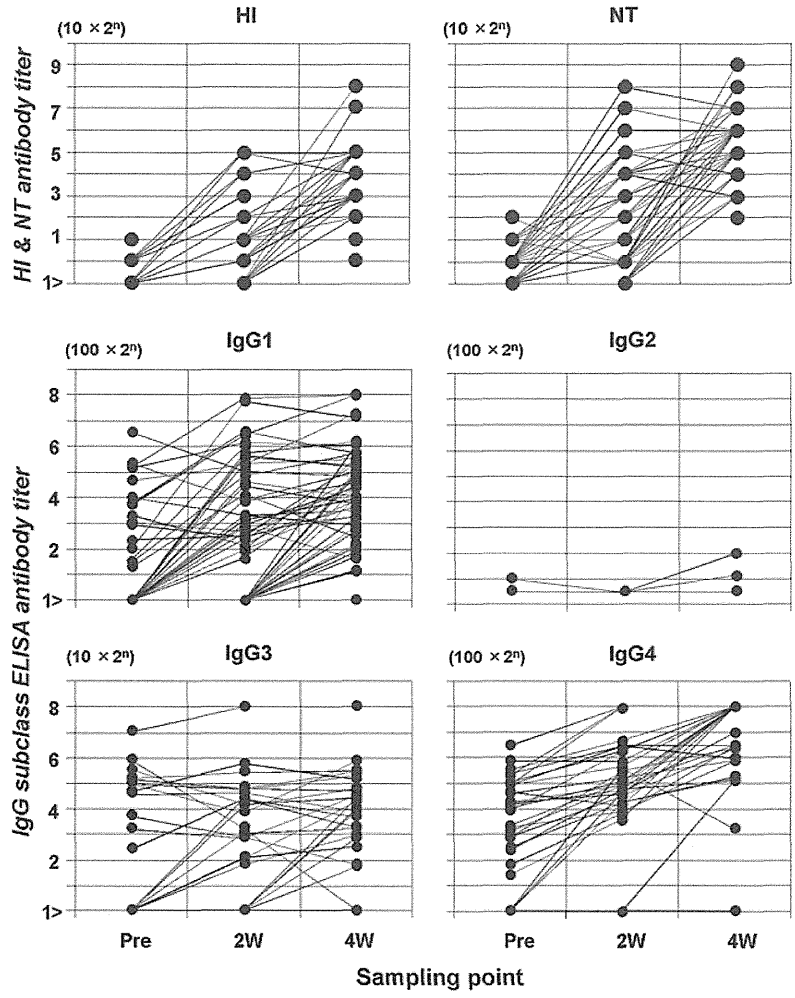

FIG. 1. Hemagglutination inhibition, neutralization, and IgG subclass antibody titers specific to A/California/07/2009(H1N1) in group 1 patients with natural infection. Black dot shows antibody titer. Abscissa in sampling point after onset of illness. Ordinate in serum dilution.

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

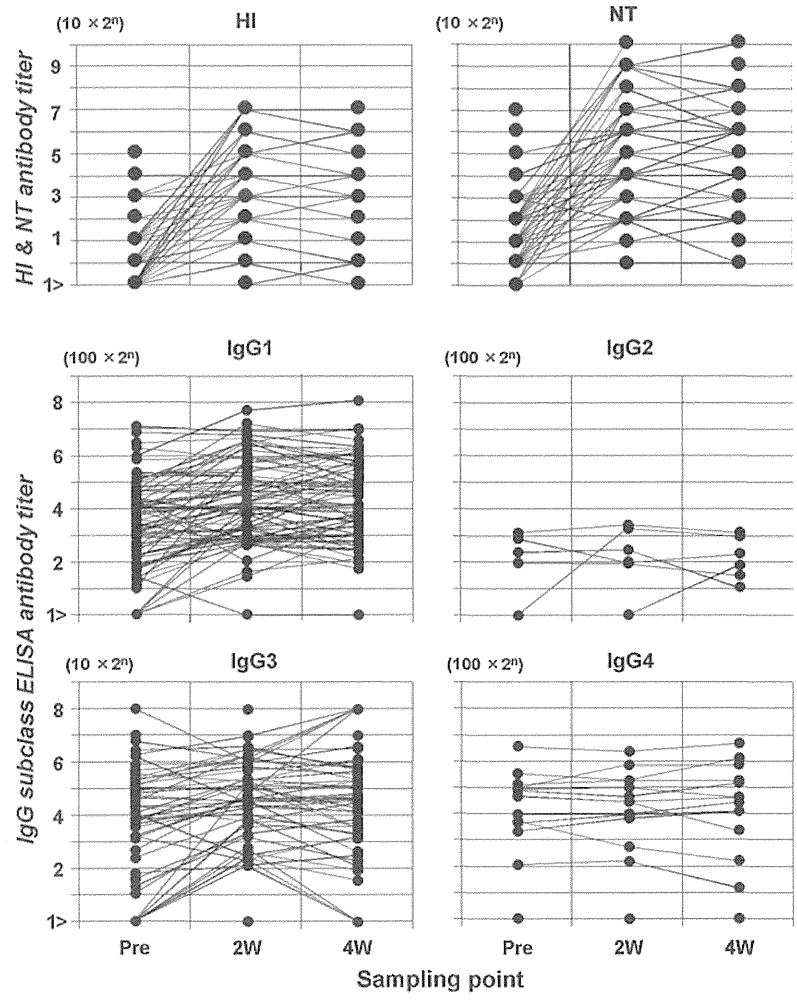

FIG. 2. Hemagglutination inhibition, neutralization, and IgG subclass antibody titers specific to A/California/07/2009(H1N1) in group 2 vaccine recipients. Black dot represents antibody titer. Abscissa in sampling point before and after vaccination. Ordinate in serum dilution.

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 Fcγ 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.

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Author Disclosure Statement

No competing financial interests exist.

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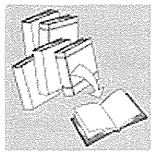

Genomic diversity of mumps virus and global distribution of the 12 genotypes

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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.

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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.

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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 15384 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

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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)	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.

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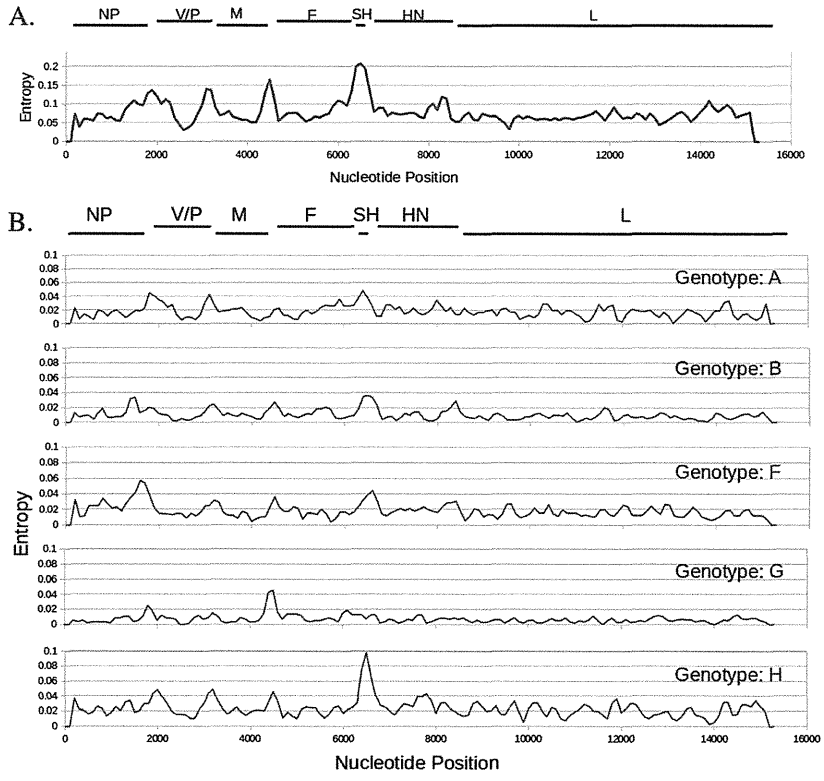

Figure 1. A. Plot showing average entropy relative to nucleotide position within the Mumps virus genome. Entropy was calculated using 73 MuV genomes and averaged within a window of 200 nucleotides with a step of 100 nucleotides. The positions of protein coding regions within the MuV genome are indicated 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

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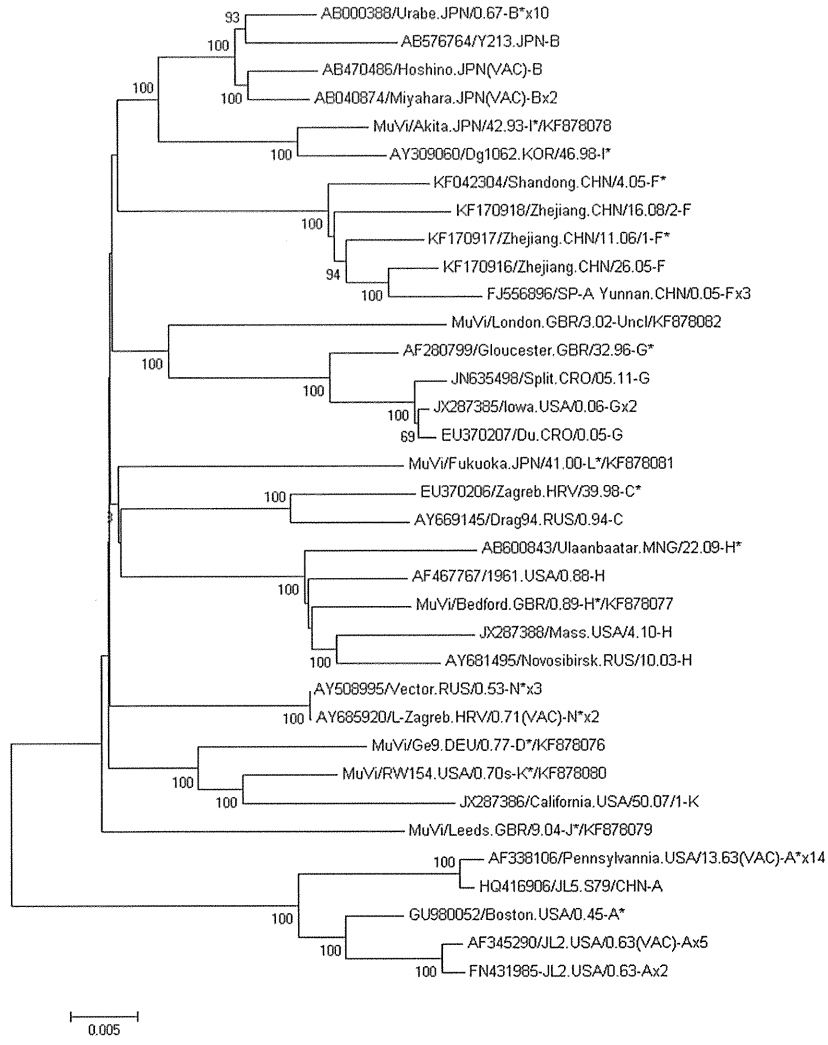

Figure 2. Phylogenetic tree of 12 MuV genotypes based on 73 genomes. The un-rooted trees were drawn using the neighbour-joining method of MEGA 6.06 program. The parameter employed was P-distant model, and the robustness of the internal branches was determined by 1000 bootstrap replications. The horizontal 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

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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.

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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.

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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)

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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

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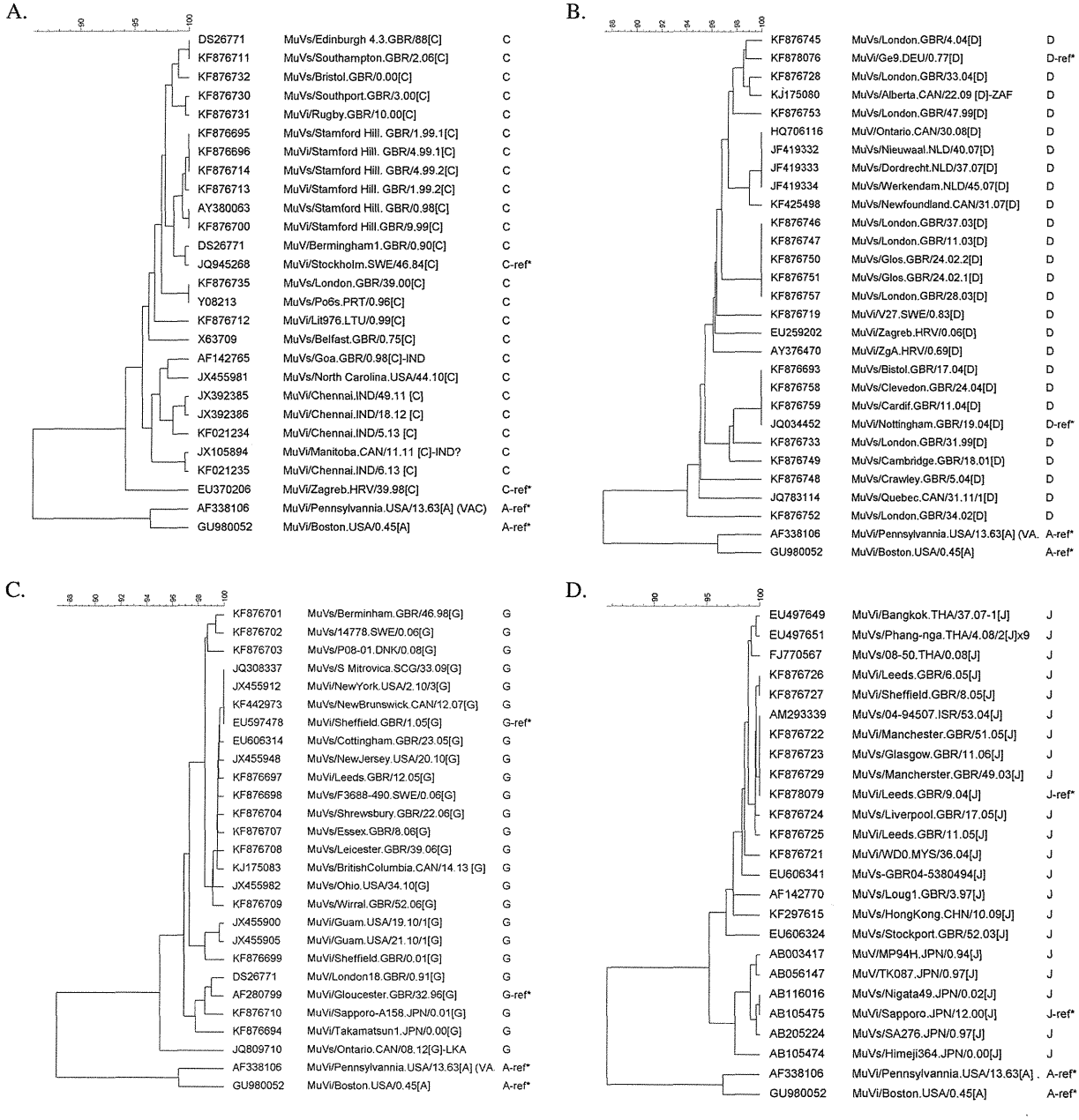

Figure 3. A–D. Phylogenetic trees drawn based on SH gene using BioNumerics v6.1. The representatives and newly generated sequences were included, respectively, for genotype C (Figure 3A); D (Figure 3B); G (Figure 3C) and J (Figure 3D) strains

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

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