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

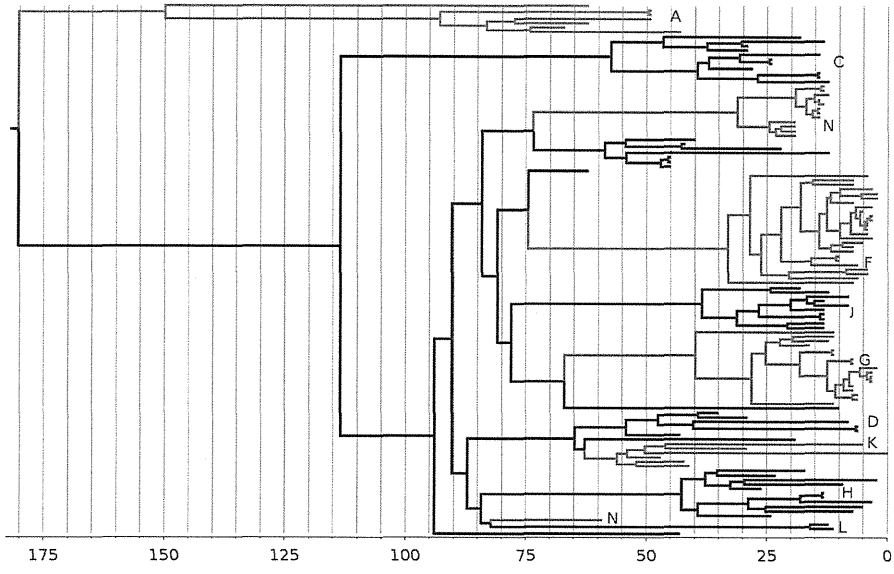

Figure 4. BEAST phylogeny of 119 MuV HN sequences. The timescale across the bottom of the phylogeny represents years since the date of the last sequence (2012). The genotypes are coloured black or grey and labelled for clarity

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).

REFERENCES

1. Gershon AA. Chickenpox, measles and mumps. In Infectious diseases of the fetus and newborn infant, Remington JS, Klein JO (eds). WB Saunders Company: Philadephia, 1995. 565–618.
2. Knowles W, Jin L. Rubulavirus: Mumps Virus. In Topley and Wilson-Microbiology and Microbial Infections, Mahy BWJ, ter Meulen V (eds). Hodder Arnold: London, 2005; 744–762.
3. Johnson CD, Goodpasture E. An investigation of the etiology of mumps. *J Exp Med* 1934; **59**(1):1–19.
4. Cui A, Brown DW, Xu W, Jin L. Genetic variation in the HN and SH genes of mumps viruses: a comparison of strains from mumps cases with and without neurological symptoms. *PLoS One* 2013; **8**(4):e61791.
5. WHO. Global status of mumps immunization and surveillance. *Weekly Epidemiological Record* 2005; **84**: 418–424.
6. WHO. Mumps virus nomenclature update: 2012. *Weekly Epidemiological Record* 2013; **87** [22]: 217–224.
7. Yung CF, Andrews N, Bukasa A, Brown KE, Ramsay M. Mumps complications and effects of mumps vaccination, England and Wales, 2002–2006. *Emerg Infect Dis* 2011; **17**(4): 661–667.
8. Jin L, Brown DW, Litton PA, White JM. Genetic diversity of mumps virus in oral fluid specimens: application to mumps epidemiological study. *J Infect Dis* 2004;**189** (6): 1001–1008.
9. Jin L, Vyse A, Brown DW. The role of RT-PCR assay of oral fluid for diagnosis and surveillance of measles, mumps and rubella. *Bull World Health Organ* 2002; **80**(1): 76–77.
10. Carbone KM, Rubin S. Mumps virus. In Fields Virology, Knipe DM, Howley PM (eds). Lippincott Williams & Wilkins: Philadelphia, 2007; 1527–1550.
11. Savage E, Ramsay M, White J, Beard S, Lawson H, Hunjan R, *et al.* Mumps outbreaks across England and Wales in 2004: observational study. *BMJ* 2005; **330**(7500): 1119–1120.

DOI: 10.1002/rmv

12. Peltola H, Kulkarni PS, Kapre SV, Paunio M, Jadhav SS, Dhere RM. Mumps outbreaks in Canada and the United States: time for new thinking on mumps vaccines. *Clin Infect Dis* 2007; **45**(4): 459–466.
13. Afzal MA, Dussupt V, Minor PD, Pipkin PA, Fleck R, Hockley DJ, *et al.* Assessment of mumps virus growth on various continuous cell lines by virological, immunological, molecular and morphological investigations. *J Virol Methods* 2005; **126** (1–2): 149–156.
14. Vaidya SR, Brown DW, Jin L, Samuel D, Andrews N, Brown KE. Development of a focus reduction neutralization test (FRNT) for detection of mumps virus neutralizing antibodies. *J Virol Methods* 2010; **163**(1): 153–156.
15. Jin L, Richards A, Brown DW. Development of a dual target-PCR for detection and characterization of measles virus in clinical specimens. *Mol Cell Probes* 1996; **10**: 191–200.
16. Jin L, Beard S, Brown DW. Genetic heterogeneity of mumps virus in the United Kingdom: identification of two new genotypes. *J Infect Dis* 1999; **180**(3): 829–833.
17. Jin L, Beard S, Hale A, Knowles W, Brown DW. The genomic sequence of a contemporary wild-type mumps virus strain. *Virus Res* 2000; **70**(1-2): 75–83.
18. Drexler JF, Corman VM, Muller MA, Maganga GD, Vallo P, Binger T, *et al.* Bats host major mammalian paramyxoviruses. *Nature Communications* 2012; **3**(796): 1–12.
19. Cui A, Myers R, Xu W, Jin L. Analysis of the genetic variability of the mumps SH gene in viruses circulating in the UK between 1996 and 2005. *Infect Genet Evol* 2009; **9**(1): 71–80.
20. Takeuchi K, Tanabayashi K, Hishiyama M, Yamada A. The mumps virus SH protein is a membrane protein and not essential for virus growth. *Virology* 1996; **225**(1): 156–162.
21. Malik T, Shegogue CW, Werner K, Ngo L, Sauder C, Zhang C, *et al.* Discrimination of mumps virus small hydrophobic gene deletion effects from gene translation effects on virus virulence. *J Virol* 2011; **85**(12): 6082–6085.
22. Tecle T, Johansson B, Jejcic A, Forsgren M, Orvell C. Characterization of three co-circulating genotypes of the small hydrophobic protein gene of mumps virus. *J Gen Virol* 1998; **79**(12): 2929–2937.
23. Lim CS, Chan KP, Goh KT, Chow VT. Hemagglutinin-neuraminidase sequence and phylogenetic analyses of mumps virus isolates from a vaccinated population in Singapore. *J Med Virol* 2003; **70**(2): 287–292.
24. Young DF, Galiano MC, Lemon K, Chen YH, Andrejeva J, Duprex WP, *et al.* Mumps virus Enders strain is sensitive to interferon (IFN) despite encoding a functional IFN antagonist. *J Gen Virol* 2009; **90**(11): 2731–2738.
25. Liang Y, Ma S, Yang Z, Liu L, Wang L, Wang J, *et al.* Immunogenicity and safety of a novel formalin-inactivated and alum-adjuvanted candidate subunit vaccine for mumps. *Vaccine* 2008; **26**(33): 4276–4283.
26. Liang Y, Ma S, Liu L, Zhao H, Wang L, Jiang L, *et al.* Identification and development of a promising novel mumps vaccine candidate strain. *Microbes Infect* 2010; **12** (14–15): 1178–1187.
27. Orvell C, Alsheikhly AR, Kalantari M, Johansson B. Characterization of genotype-specific epitopes of the HN protein of mumps virus. *J Gen Virol* 1997; **78**(12): 3187–3193.
28. Tecle T, Bottiger B, Orvell C, Johansson B. Characterization of two decades of temporal co-circulation of four mumps virus genotypes in Denmark: identification of a new genotype. *J Gen Virol* 2001; **82**(2675): 2680.
29. Rubin SA, Pletnikov M, Taffs R, Snoy PJ, Kobasa D, Brown EG, *et al.* Evaluation of a neonatal rat model for prediction of mumps virus neurovirulence in humans. *J Virol* 2000; **74**(5382): 5384.
30. Rubin SA, Pletnikov M, Li Z, Vanderzanden J, Mauldin J, Sauder C, *et al.* Changes in mumps virus gene sequence associated with variability in neurovirulent phenotype. *J Virol* 2003; **77**: 11616–11624.
31. Rafiefard F, Johansson B, Tecle T, Orvell C. Characterization of mumps virus strains with varying neurovirulence. *Scand J Infect Dis* 2005; **37**(5): 330–337.
32. Kulkarni-Kale U, Ojha J, Manjari GS, Deobagkar DD, Mallya AD, Dhere RM, *et al.* Mapping antigenic diversity and strain specificity of mumps virus: a bioinformatics approach. *Virology* 2007; **359**(2): 436–446.
33. Ivancic-Jelecki J, Santak M, Forcic D. Variability of hemagglutinin-neuraminidase and nucleocapsid protein of vaccine and wild-type mumps virus strains. *Infect Genet Evol* 2008; **8**(5): 603–613.
34. Shah D, Vidal S, Link MA, Rubin SA, Wright KE. Identification of genetic mutations associated with attenuation and changes in tropism of Urabe mumps virus. *J Med Virol* 2009; **81**(1): 130–138.
35. Šantak M, Lang-Balija M, Ivancic-Jelecki J, Košutic-Gulija T, Ljubin-Sternak S, Forcic D. Antigenic differences between vaccine and circulating wild-type mumps viruses decreases neutralization capacity of vaccine-induced antibodies. *Epidemiol Infect* 2013; **141**(6): 1298–1309.
36. Saito H, Takahashi Y, Harata S, Tanaka K, Sato H, Yamada A, *et al.* Cloning and characterization of the genomic RNA sequence of the mumps virus strain associated with a high incidence of aseptic meningitis. *Microbiol Immunol* 1998; **42**(2): 133–137.
37. Saika S, Kidokoro M, Ohkawa T, Aoki A, Suzuki K. Pathogenicity of mumps virus in the marmoset. *J Med Virol* 2002;**66** (1):115–122.
38. Sauder CJ, Zhang CX, Ngo L, Werner K, Duprex WP, Malik T, *et al.* Gene-specific contributions to mumps virus neurovirulence and neuroattenuation. *J Virol* 2011; **85**(14): 7059–7069.
39. Tanabayashi K, Takeuchi K, Okazaki K, Hishiyama M, Yamada A. Identification of an amino acid that defines the fusogenicity of mumps virus. *J Virol* 1993; **67**(5): 2928–2931.
40. Tanabayashi K, Takeuchi K, Hishiyama M, Yamada A. Effect on fusion induction of point mutations introduced into the F protein of mumps virus. *Virology* 1994; **204** (2): 851–853.
41. Tecle T, Johansson B, Yun Z, Orvell C. Antigenic and genetic characterization of the fusion (F) protein of mumps virus strains. *Arch Virol* 2000; **145**(6): 1199–1210.
42. Uchida K, Shinohara M, Shimada S, Segawa Y, Kimura K, Hoshino Y. Characterization of the F gene of contemporary mumps virus strains isolated in Japan. *Microbiol Immunol* 2003; **47**(2): 167–172.

43. Kilham L. Isolation of mumps virus from the blood of a patient. *Proc Soc Exp Biol Med* 1948; **69**(1): 99.
44. Young ML, Dickstein B, Weibel RE, Stokes J Jr, Buynak EB, Hilleman MR. Experiences with Jeryl Lynn strain live attenuated mumps virus vaccine in a pediatric outpatient clinic. *Pediatrics* 1967; **40**(5): 798–803.
45. Afzal MA, Pickford AR, Forsey T, Heath AB, Minor PD. The Jeryl Lynn vaccine strain of mumps virus is a mixture of two distinct isolates. *J Gen Virol* 1993;**74**(Pt 5): 917–920.
46. Afzal MA, Buchanan J, Heath AB, Minor PD. Clustering of mumps virus isolates by SH gene sequence only partially reflects geographical origin. *Arch Virol* 1997; **142** (2): 227–238.
47. Palacios G, Jabado O, Cisterna D, de Ory F, Renwick N, Echevarria JE, *et al.* Molecular identification of mumps virus genotypes from clinical samples: standardized method of analysis. *J Clin Microbiol* 2005; **43**(4): 1869–1878.
48. Echevarria JE, Castellanos A, Sanz JC, Perez C, Palacios G, Martinez M, *et al.* Circulation of mumps virus genotypes in Spain from 1996 to 2007. *J Clin Microbiol* 2010; **48**(4): 1245–1254.
49. Sugiura A, Yamada A. Aseptic meningitis as a complication of mumps vaccination. *Pediatr Infect Dis J* 1991; **10**(3): 209–213.
50. Kimura M, Kuno-Sakai H, Yamazaki S, Yamada A, Hishiyama M, Oya A, *et al.* Adverse events associated with MMR vaccines in Japan. *Acta Paediatr Jpn* 1996; **38**(3): 205–211.
51. Inou Y, Nakayama T, Yoshida N, Uejima H, Yuri K, Kamada M, *et al.* Molecular epidemiology of mumps virus in Japan and proposal of two new genotypes. *J Med Virol* 2004; **73**(1): 97–104.
52. Uchida K, Shinohara M, Shimada S, Segawa Y, Hoshino Y. Characterization of mumps virus isolated in saitama prefecture, Japan, by sequence analysis of the SH gene. *Microbiol Immunol* 2001; **45**(12): 851–855.
53. Afzal MA, Buchanan J, Dias JA, Cordeiro M, Bentley ML, Shorrock CA, *et al.* RT-PCR based diagnosis and molecular characterisation of mumps viruses derived from clinical specimens collected during the 1996 mumps outbreak in Portugal. *J Med Virol* 1997 Aug; **52**(4): 349–353.
54. Atrasheuskaya AV, Kulak MV, Rubin S, Ignatyev GM. Mumps vaccine failure investigation in Novosibirsk, Russia, 2002-2004. *Clin Microbiol Infect* 2007; **13**(7): 670–676.
55. Santak M, Kosutic-Gulija T, Tesovic G, Ljubin-Sternak S, Betica-Radic L, Forcic D. Mumps virus strains isolated in Croatia in 1998 and 2005: Genotyping and putative antigenic relatedness to vaccine strains. *J Med Virol* 2006; **78**(5): 638–643.
56. Tecle T, Mickiene A, Johansson B, Lindquist L, Orvell C. Molecular characterisation of two mumps virus genotypes circulating during an epidemic in Lithuania from 1998 to 2000. *Arch Virol* 2002; **147**(2): 243–253.
57. Van Den Bosch CA, Cohen B, Walters T, Jin L. Mumps outbreak confined to a religious community. *Euro Surveill* 2000; **5**(5): 58–60.
58. Vaidya SR, Chowdhury DT, Kumbhar NS, Tomar R, Kamble MB, Kazi MI. Circulation of TwoMumpsVirus Genotypes in an Unimmunized Population in India. *J Med Virol* 2013; **85**(8): 426–432.
59. Ivancic J, Forcic D, Gulija TK, Zgorelec R, Repalust L, Baricevic M, *et al.* Genetic characterization of a mumps virus isolate during passaging in the amniotic cavity of embryonated chicken eggs. *Virus Res* 2004; **99**(2): 121–129.
60. Santos CL, Ishida MA, Foster PG, Sallum MA, Borges DB, Correa KO, *et al.* Detection of a new mumps virus genotype during parotitis epidemic of 2006-2007 in the state of Sao Paulo, Brazil. *J Med Virol* 2008; **80**(2): 323–329.
61. Jin L, Rima B, Brown D, Orvell C, T Tecle T, Afzal M, *et al.* Proposal for genetic characterisation of wild-type mumps strains: preliminary standardisation of the nomenclature. *Arch Virol* 2005; **150**(1903): 1909.
62. Cohen BJ, Jin L, Brown DW, Kitson M. Infection with wild-type mumps virus in army recruits temporally associated with MMR vaccine. *Epidemiol Infect* 1999; **123**(2): 251–255.
63. Cohen C, White JM, Savage EJ, Glynn JR, Choi Y, Andrews N, *et al.* Vaccine effectiveness estimates, 2004-2005 mumps outbreak, England. *Emerg Infect Dis* 2007; **13**: 12–17.
64. Whelan J, van Binnendijk R, Greenland K, Fanoy E, Khargi M, Yap K, *et al.* Ongoing mumps outbreak in a student population with high vaccination coverage, Netherlands, 2010. *Euro Surveill* 2010; **15**(17): 19554.
65. Carr MJ, Moss E, Waters A, Dean J, Jin L, Coughlan S, *et al.* Molecular epidemiological evaluation of the recent resurgence in mumps virus infections in Ireland. *J Clin Microbiol* 2010; **48**(9): 3288–3294.
66. Anis E, Grotto I, Moerman L, Warshavsky B, Slater PE, Lev B. Mumps outbreak in Israel's highly vaccinated society: are two doses enough? *Epidemiol Infect* 2012; **140**(3): 439–446.
67. Otto W, Mankertz A, Santibanez S, Saygili H, Wenzel J, Jilg W, *et al.* Ongoing outbreak of mumps affecting adolescents and young adults in Bavaria, Germany, August to October 2010. *Euro Surveill* 2010; **15**(50): 19748.
68. Bernard H, Schwarz NG, Melnic A, Bucov V, Caterinciuc N, Pebody RG, *et al.* Mumps outbreak ongoing since October 2007 in the Republic of Moldova. *Euro Surveill* 2008; **13**(13): 8079.
69. Kuzmanovska G, Polozhani A, Mikik V, Stavridis K, Aleksoski B, Cvetanovska Z, *et al.* Mumps outbreak in the former Yugoslav Republic of Macedonia, January 2008-June 2009: epidemiology and control measures. *Euro Surveill* 2010; **15**(23): 19586.
70. Hukic M, Ravlija J, Dedeic Ljubovic A, Moro A, Arapcic S, Muller CP, *et al.* Ongoing large mumps outbreak in the Federation of Bosnia and Herzegovina, Bosnia and Herzegovina, December 2010 to July 2011. *Euro Surveill* 2011; **16**(35): 19959.
71. Watson-Creed G, Saunders A, Scott J, Lowe L, Pettipas J, Hatchette TF. Two successive outbreaks of mumps in Nova Scotia among vaccinated adolescents and young adults. *Canadian Medical Association Journal* 2006; **175**(5): 483–488.
72. Rota JS, Turner JC, Yost-Daljev MK, Freeman M, Toney DM, Meisel E, *et al.* Investigation of a mumps outbreak among university students with two measles-mumps-rubella (MMR) vaccinations, Virginia, September-December 2006. *J Med Virol* 2009; **81**(10): 1819–1825.
73. Strohle A, Bernasconi C, Germann D. A new mumps virus lineage found in the 1995 mumps outbreak in western Switzerland

identified by nucleotide sequence analysis of the SH gene. *Arch Virol* 1996; **141**(3–4): 733–741.
74. Lee JY, Na BK, Kim JH, Lee JS, Park JW, Shin GC, *et al.* Regional outbreak of mumps due to genotype H in Korea in 1999. *J Med Virol* 2004; **73**(1): 85–90.
75. Montes M, Cilla G, Artieda J, Vicente D, Basterretxea M. Mumps outbreak in vaccinated children in Gipuzkoa (Basque Country), Spain. *Epidemiol Infect* 2002; **129**(3): 551–556.
76. Agafonov AP, Neverov AA, Kameneva SN, Saraev DV, Ginko ZI, Blinov VM, *et al.* Isolation, genotyping and analysis of the complete nucleotide sequence of mumps virus Siberian strain. *Epidemiol Vakcinoprofil* 2007; **5**: 34–42.
77. Hindiyeh MY, Aboudy Y, Wohoush M, Shulman LM, Ram D, Levin T, *et al.* Characterization of large mumps outbreak among vaccinated Palestinian refugees. *J Clin Microbiol* 2009; **47**(3): 560–565.
78. Akcali A, Yilmaz N, Uyar Y, Ertek M, Buzgan T. Genotyping of mumps virus circulating in Turkey in the 2006-2007 winter season. *Arch Virol* 2009; **154**(11): 1807–1812.
79. Kidokoro M, Tuul R, Komase K, Nymadawa P. Characterization of mumps viruses circulating in Mongolia: identification of a novel cluster of genotype H. *J Clin Microbiol* 2011; **49**(5): 1917–1925.
80. Kashiwagi Y, Takami T, Mori T, Nakayama T. Sequence analysis of F, SH, and HN genes among mumps virus strains in Japan. *Arch Virol* 1999; **144**(3): 593–599.
81. Reid F, Hassan J, Irwin F, Waters A, Hall W, Connell J. Epidemiologic and diagnostic evaluation of a recent mumps outbreak using oral fluid samples. *J Clin Virol* 2008; **41**(2): 134–137.
82. Wu L, Bai Z, Li Y, Rima BK, Afzal MA. Wild type mumps viruses circulating in China establish a new genotype. *Vaccine* 1998; **16**(2-3): 281–285.
83. Shi H, Liu L, Ma S, Chen J, Che Y, Wang J, *et al.* Molecular epidemiology of mumps virus strains circulating in south-west China from 2007 to 2009. *J Med Microbiol* 2011; **60**(10): 1496–1501.
84. Saito H, Takahashi Y, Harata S, Tanaka K, Sano T, Suto T, *et al.* Isolation and characterization of mumps virus strains in a mumps outbreak with a high incidence of aseptic meningitis. *Microbiol Immunol* 1996; **40**(4): 271–275.
85. Lee JY, Kim YY, Shin GC, Na BK, Lee JS, Lee HD, *et al.* Molecular characterization of two genotypes of mumps virus circulated in Korea during 1998-2001. *Virus Res* 2003; **97**(2): 111–116.
86. Ivancic J, Gulija TK, Forcic D, Baricevic M, Jug R, Mesko-Prejac M, *et al.* Genetic characterization of L-Zagreb mumps vaccine strain. *Virus Res* 2005; **109**(1): 95–105.

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article:

Supplement 1. Diversity (min-max%) between 12 MuV genotypes based on 73 whole genomes, 120 SH, 94 HN and 98 F sequences (Neighbour-joining, P-distant, MEGA5.1-nt)
Supplement 2. The HN gene alignment of MuV strains and the Bat-MuV
Supplement 3. The F gene alignment of MuV strains and the Bat-MuV

DOI: 10.1002/rmv

CVI
Journals.ASM.org

Phase III Clinical Trials Comparing the Immunogenicity and Safety of the Vero Cell-Derived Japanese Encephalitis Vaccine Encevac with Those of Mouse Brain-Derived Vaccine by Using the Beijing-1 Strain

Chiaki Miyazaki,[a] Kenji Okada,[b] Takao Ozaki,[c] Mizuo Hirose,[d] Kaneshige Iribe,[e] Hiroyuki Yokote,[f] Yuji Ishikawa,[f] Takehiro Togashi,[g] Kohji Ueda[h]

Fukuoka-West Rehabilitation Center for Children, Fukuoka, Japan[a]; Department of Pediatrics, Fukuoka Dental College, Fukuoka, Japan[b]; Department of Pediatrics, Konan Kosei Hospital, Konan, Japan[c]; Hirose Pediatric Clinic, Saga, Japan[d]; Sakurajyuji Hospital, Kumamoto, Japan[e]; Chemo-Sero-Therapeutic Research Institute (KAKETSUKEN), Kumamoto, Japan[f]; School of Nursing, Sapporo City University, Sapporo, Japan[g]; Seinan Jo Gakuin University, Kitakyushu, Japan[h]

The immunogenicity and safety of an inactivated cell culture Japanese encephalitis vaccine (CC-JEV) were compared with those of an inactivated mouse brain-derived Japanese encephalitis vaccine (MB-JEV) in phase III clinical multicenter trials conducted in children. The vaccines contain the same Japanese encephalitis virus strain, the Beijing-1 strain. Two independent clinical trials (trials 1 and 2) were conducted. Trial 1 was conducted in 468 healthy children. Each subject was injected with 17 μg per dose of either CC-JEV or MB-JEV, and the immunogenicity and safety of the vaccines were investigated. Trial 1 showed that CC-JEV was more immunogenic and reactive than MB-JEV at the same dose. Therefore, to adjust the immunogenicity of CC-JEV to that of MB-JEV, a vaccine that has had a good track record regarding its efficacy for a long time, trial 2 was conducted in 484 healthy children. To improve the stability, CC-JEV was converted from a liquid type to a freeze-dried type of vaccine. Each subject was injected subcutaneously with either 4 μg per dose of CC-JEV, 8 μg per dose of CC-JEV, or 17 μg per dose of MB-JEV twice, at an interval of 2 to 4 weeks, followed by an additional booster immunization 1 to 15 months after the primary immunization. Based on the results of trial 2, 4 μg per dose of the freeze-dried CC-JEV (under the label Encevac) was selected as a substitute for the MB-JEV. Encevac was approved and launched in 2011 and has since been in use as a 2nd-generation Japanese encephalitis vaccine in Japan. (These studies have been registered at the JapicCTI under registration no. JapicCTI-132063 and JapicCTI-080586 for trials 1 and 2, respectively.)

Japanese encephalitis (JE) is an infectious disease caused by the JE virus (JEV), which is mediated by mosquitoes, such as *Culex tritaeniorhynchus* (1, 2). JE occurs not only in Japan but also in many other Asian countries, including Korea, Taiwan, China, Vietnam, Thailand, Malaysia, Myanmar, and India (3). The number of cases and fatalities due to JE are reported to be about 20,000 and 600 per year, respectively (1). To prevent this infectious disease, a JE vaccine derived from infected mouse brain tissue has been in use for a long time in Japan and other countries. Concurrently, a live-attenuated vaccine developed from a passaged culture of the JEV SA14 strain in primary hamster kidney cells and animals (mice and hamsters) with successive plaque purifications in primary chicken embryo cells, SA14-14-2, has been in use since 1989 in China and other countries (4). Moreover, an inactivated vaccine produced using the SA14-14-2 vaccine strain has been licensed in the United States, Europe, Canada, and Australia (5).

In Japan, mouse brain-derived JE vaccine (MB-JEV) was initially produced using mouse brains inoculated with JEV Nakayama-NIH as a vaccine virus strain. At that time, MB-JEV was produced by adding formalin to the centrifugal supernatant of a 5% emulsion of mouse brain to inactivate the JE virus (6). Later, the quality of MB-JEV was improved through purification processes. As for the virus strain used for vaccine production, the Nakayama-NIH strain was changed to the Beijing-1 strain in 1989. MB-JEV, using the Beijing-1 strain, showed neutralizing activities against a wide range of domestic and foreign JE viruses. Furthermore, this vaccine strain showed high productivity in vaccine manufacture and high antibody-positive rates and neutralizing antibody titers in vaccinees compared with the Nakayama-NIH strain.

From January 2005 to December 2007, acute disseminated encephalomyelitis (ADEM) occurred after vaccination with MB-JEV at a very low frequency of 0.8 per 100,000 children, according to a national investigation by pediatric departments in Japan (7). The Health, Labor, and Welfare Ministry of Japan admitted in 2005 that the ADEM cases occurring after vaccination with MB-JEV were health hazards, and accordingly, they issued a recommendation to withhold active recommendation of the MB-JEV. The following points were considered to be problems with MB-JEV: (i) a possible risk of it causing ADEM, (ii) difficulties with quality control, and (iii) the use of animals in vaccine production. To address this situation, two new freeze-dried inactivated cell culture JE vaccines (CC-JEV) produced using the Beijing-1 strain were approved as substitutes for the MB-JEV in Japan: JeBIK-V (Biken, The Research Foundation for Microbial Diseases of Osaka University, Kagawa, Japan), approved in 2009, and Encevac (Kaketsuken, the Chemo-Sero-Therapeutic Research Institute, Ku-

Received 29 June 2013 Returned for modification 23 July 2013
Accepted 8 December 2013
Published ahead of print 11 December 2013
Editor: M. F. Pasetti
Address correspondence to Hiroyuki Yokote, yokote@kaketsuken.or.jp.

doi:10.1128/CVI.00377-13

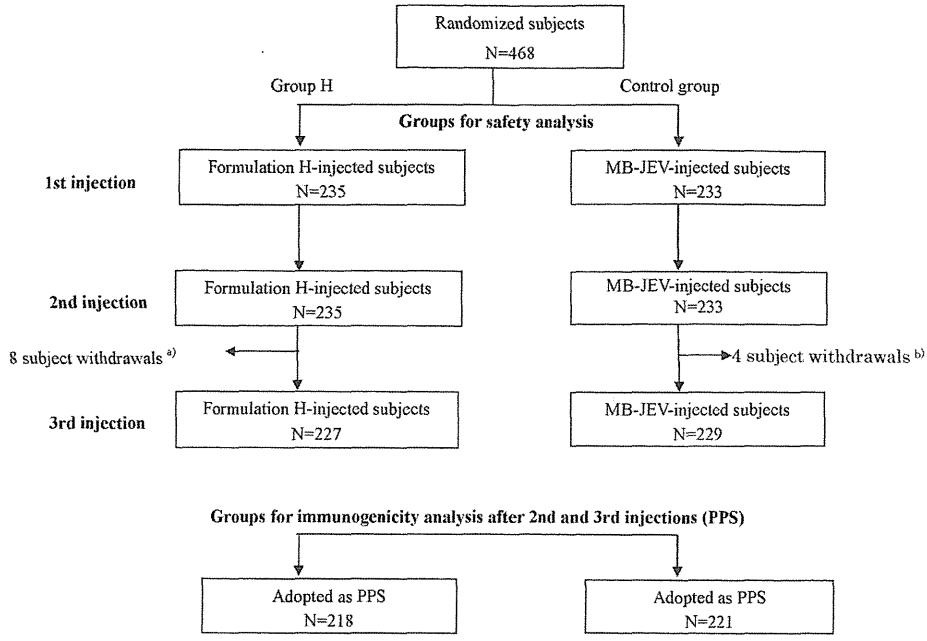

FIG 1 Disposition of the subjects in trial 1. [a], 8 subjects withdrew: 2 because of cancellations of the trial by parent or guardian of the subjects, 2 for the occurrence of adverse events, 1 because of change of residence, and 3 because of judgments by the primary investigator. [b], 4 subjects withdrew: 3 because of cancellations of the trial by parent or guardian of the subjects and 1 because of a positive antibody titer before injection.

mamoto, Japan), approved in 2011. In mice, JeBIK-V showed superior neutralizing antibody titers compared with those of MB-JEV (8). The safety and immunogenicity of JeBIK-V were also shown in children (9). However, as MB-JEV was not used as a comparator in the study, the comparisons of the immunogenicity and safety between the CC-JEV and MB-JEV were not performed simultaneously. The immunogenicity and safety of a CC-JEV vaccine, Ixiaro (Intercell Biomedical, Livingston, United Kingdom), assessed in clinical phase III trial, were also reported (10). This vaccine is a purified inactivated aluminum-adjuvanted JE vaccine

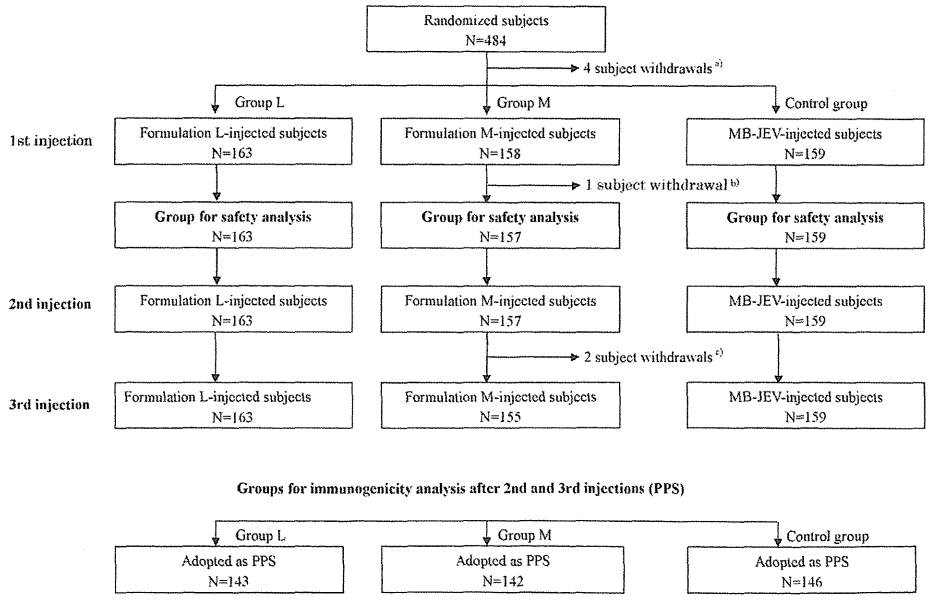

FIG 2 Disposition of the subjects in trial 2. [a], of the 484 randomized subjects, 4 subjects withdrew: 2 because of cancellations of the trial by parent or guardian of the subjects and 2 because of judgments by the investigator. [b], of the 158 formulation M-injected subjects, 1 subject withdrew because a drug with a nonallocated number was injected after the 2nd injection. [c], of the 157 formulation M-injected subjects, 2 withdrew: 1 because of cancellation of the trial by parent or guardian of the subject and 1 because of a positive antibody titer before injection.

TABLE 1 Background data of subjects in trial 1

Background factor	Group H (formulation H) (n = 218)[a] Value	95% CI (%)[b]	Control group (MB-JEV) (n = 221)[a] Value	95% CI (%)
Gender (n [%])				
Male	107 (49.1)	42.3–55.9	115 (52.0)	45.2–58.8
Female	111 (50.9)	44.1–57.7	106 (48.0)	41.2–54.8
Age (mean [SD]) (mo)	36.9 (13.4)	NC[c]	36.7 (15.5)	NC
Body wt (mean [SD]) (kg)	13. 9 (2.7)	NC	13.8 (3.3)	NC

[a] Injected vaccine.
[b] CI, confidence interval.
[c] NC, not calculated.

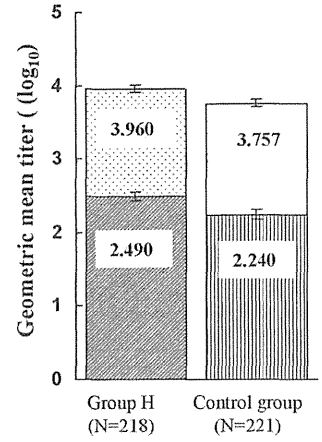

FIG 3 GMTs after the 2nd and 3rd injections in trial 1. Two groups of 235 and 233 subjects were injected subcutaneously with a series of two doses of 0.5 ml of formulation H or MB-JEV, respectively, at an interval of 1 to 4 weeks, and an additional injection was given 6 to 15 months after the 2nd injection. The GMTs after the 2nd and 3rd injections in trial 1 are shown after the 2nd (shaded area) and 3rd (dotted area) injections in group H and after the 2nd (striped area) and 3rd (white area) injections in the control group. The error bars represent 95% confidence intervals.

produced using the SA14-14-2 virus strain, which is used for persons ≥17 years of age in North America, Europe, and Australia (under the label Jespect) (11). In all these trials, however, a study on a direct comparison of the immunogenicity and safety between CC-JEV and MB-JEV produced using the Beijing-1 strain has not been reported so far. We report here the results of two series of phase III trials (trials 1 and 2) conducted in children simultaneously using CC-JEV (Encevac) and MB-JEV, produced using the same Beijing-1 strain (these studies have been registered at the JapicCTI under registration no. JapicCTI-132063 and JapicCTI-080586 for trials 1 and 2, respectively).

MATERIALS AND METHODS

Vaccines. The CC-JEV was manufactured according to the method described by Sugawara et al. (6). Briefly, Vero cells were passaged in a culture medium containing 2% fetal bovine serum and microcarriers. When cell numbers reached approximately 2×10^6 cells/ml, cells cultured by microcarriers were isolated and inoculated with the Beijing-1 strain. A serum-free medium was added after virus adsorption, and thereafter, the cells were cultured for 4 days at 37°C. The culture supernatant was then harvested and concentrated by ultrafiltration. After inactivation with formalin, the vaccine antigens were purified by sucrose density gradient centrifugation, in addition to the manufacturing method used for the production of the MB-JEV. For the production of the CC-JEV, additional affinity column chromatography using Cellufine sulfate (JNC Corporation, Tokyo, Japan) was added to achieve further purification by removing residual host cell-derived proteins and DNAs from the final bulk vaccine. Two series of phase III clinical trials, trials 1 and 2, were conducted using the CC-JEV as a test vaccine. In trial 1, formulation H, which was a liquid-type vaccine containing 17 μg/dose of CC-JEV in 0.5 ml, was used. In trial 2, two freeze-dried types of the CC-JEV vaccine instead of the liquid-type vaccine were used to increase the stability; these were formulations L and M, containing 4 μg/dose and 8 μg/dose of CC-JEV in 0.5 ml, respectively. The MB-JEV, used as a comparator vaccine in both trials, was manufactured by the Chemo-Sero-Therapeutic Research Institute (Kaketsuken), Kumamoto, Japan, as follows: the Beijing-1 strain was inoculated into the brains of 4-week-old ddy strain mice. The brains of the mice showing symptoms of encephalitis were collected to prepare a brain emulsion. After centrifugation, the supernatant of the emulsion was treated with protamine sulfate to remove the mouse brain-derived materials. The virus fluid was recovered from the supernatant, and the supernatant was subjected to the sucrose density gradient centrifugation. After centrifugation, the virus particle faction was collected and then treated with formalin for inactivation in order to prepare an inactivated purified virus preparation. Finally, the total amount of protein of the MB-JEV was adjusted to 34 μg/ml. In both trials, the liquid MB-JVE containing 17 μg/dose of vaccine antigen in 0.5 ml was used as a comparator vaccine. The vaccine was administered subcutaneously.

Vaccination design. Two series of phase III clinical trials, trials 1 and 2, were conducted as double-blinded randomized parallel-group studies in multiple facilities. The subjects were children 6 to 89 months of age without a history of JE vaccine or of JEV infection. The main exclusion criteria were: (i) subjects with acute serious diseases, (ii) subjects who clearly presented anaphylaxis to vaccine components or excipients, (iii) subjects who had received a blood transfusion or administration of gamma-globulin preparation within 3 months before the start of the trial or who had received a massive dose therapy of gamma-globulin preparation (≥200 mg/kg of body weight) within 6 months before the start of the trial, and (iv) subjects for whom enrollment in the trial was judged by the principal investigator or subinvestigators as not appropriate. Written informed consent was obtained from a parent or guardian of each subject before trial enrollment. Prior to the conduction of the trials, the trial-related forms, such as the trial protocols, were investigated by the institu-

TABLE 2 Seroconversion rates of neutralizing antibody after the 2nd and 3rd injections in trial 1

Time	Group H (formulation H) % (no. of positive conversions/no. of analyzed subjects)	95% CI[a]	Control group (MB-JEV) % (no. of positive conversions/no. of analyzed subjects)	95% CI	Noninferiority test
After 2nd injection	100.0 (218/218)	98.3–100.0	99.5 (220/221)	97.5–100.0	NC[b]
After 3rd injection	100.0 (218/218)	98.3–100.0	100.0 (221/221)	98.3–100.0	$P < 0.001$

[a] CI, confidence interval.
[b] NC, not calculated.

TABLE 3 Vaccine-related adverse events over three injections (≥5%) in trial 1

Reaction	Group H (formulation H) (n = 235)			Control group (MB-JEV) (n = 233)		
	n	%	95% CI[a]	n	%	95% CI
At injection site						
Erythema	22	9.4	6.0–13.8	13	5.6	3.0–9.4
Swelling	13	5.5	3.0–9.3	6	2.6	1.0–5.5
Systemic						
Fever	18	7.7	4.6–11.8	23	9.9	6.4–14.4

[a] CI, confidence interval.

tional review boards/independent ethics committees of the facilities conducting the trials or of the base hospital of the relevant network. The trials were conducted in compliance with the ethical doctrine based on the Helsinki Declaration, the good clinical practice regulations, and other relevant regulations.

Trial 1. Trial 1 was conducted from February 2003 to August 2004. A flowchart depicting the dispositions of the subjects in trial 1 is shown in Fig. 1. After obtaining informed consent, 468 qualified subjects were recruited for trial 1; among them, 235 and 233 subjects were assigned to two groups and were injected subcutaneously with a series of 2 doses of 0.5 ml of formulation H or the MB-JEV, respectively, at an interval of 1 to 4 weeks, and an additional injection was given 6 to 15 months after the 2nd injection. For the children <3 years of age, doses of 0.25 ml were given using the same vaccination schedule.

Trial 2. Trial 2 was conducted from June 2008 to May 2009. A flowchart depicting the dispositions of the subjects in trial 2 is shown in Fig. 2. After obtaining informed consent, 480 qualified subjects were recruited for trial 2; among them, 163, 158, and 159 subjects were assigned to three groups, and were injected subcutaneously with a dose of 0.5 ml of formulation M, formulation L, or the MB-JEV, respectively, at an interval of 2 to 4 weeks, and an additional injection was given 1 to 15 months after the 2nd injection. For the children <3 years of age, doses of 0.25 ml were given using the same vaccination schedule.

Randomization. In both trials, randomizations were performed according to a computer-generated algorithm. Trial 1 was performed with a permuted-block design. Trial 2 was performed with a stratified randomization as a stratified factor of the vaccine volumes. The eligible subjects were randomly assigned in a 1:1 ratio for trial 1 and a 1:1:1 ratio for trial 2. In both trials, the statistician generating the randomization algorithm was not involved in determining the eligibility, vaccination course, or determining outcomes of the subjects.

Blinding. In trial 1, the test vaccine and the comparator vaccine were liquid-type vaccines with an identical appearance. However, in trial 2, the test vaccine was a freeze-dried type, while the comparator vaccine was a liquid type. Therefore, to ensure blinding, an investigational drug coordinator who prepared the investigational drug for a doctor and cleared it off after the inoculation instead of the doctor was specially assigned at each hospital.

Safety analysis. In trial 1, a parent or guardian of each subject recorded the presence or absence of adverse events in a health diary every day for 7 days after each inoculation. In trial 2, a parent or guardian of each subject recorded the presence or absence of adverse events in a health diary every day for 13 days after each inoculation. Adverse events were recorded until 27 days after inoculation when they were recognized. Regarding local reactions, including erythema or swelling at the injection site, a reaction of ≥2 cm in diameter was recorded as an adverse event in trial 1, and any reaction regardless of its size was recorded as an adverse event in trial 2. A fever of ≥37.5°C was recorded as an adverse event in both trials. In trial 1, the severity of adverse events was judged by the principal investigator or a subinvestigator and was classified into three levels, mild, moderate, and severe, considered for their interference with normal daily activities. Similarly, in trial 2, the criteria were defined in terms of grades 1 to 4, according to severity. Adverse events for which a possible relationship with one of the test vaccines could not be denied were judged as vaccine-related adverse events.

Immunological analysis. In trial 1, blood serum samples for the measurement of antibody titers were obtained from each subject before the 1st injection and 2 to 6 weeks after the 2nd and 3rd injections. In trial 2, serum samples were obtained from each subject before the 1st injection and 4 to 6 weeks after the 2nd and 3rd injections. The sera were stored at −20°C until the time of measurement. Neutralizing antibodies against a JE virus strain, Beijing-1, were measured by a 50% plaque reduction method using Vero cells and calculated using the 3 points least-squares regression method (3LSRM) (12). Briefly, Vero cells grown in six-well plates (Costar six-well cell culture cluster, flat bottom, with lid, catalog no. 3506; Corning Incorporated, Corning, NY, USA) were used. Serial dilutions of the serum samples were carried out routinely to 1:10, 1:40, 1:160, 1:640, 1:2,560, and 1:10,240, and the challenge virus (Beijing-1 strain) was also diluted to give 100 plaques per well. One more dilution was added and the rest repeated if there was not a 50% plaque reduction. The same volume of diluted serum samples and virus were mixed and added to the cell-seeded wells in triplicate. The control virus was added to 12 wells. The plates were then incubated for 90 min at 37°C, and overlay medium was added. Following 5 days at 37°C incubation, 10% formalin was added, followed by methylene blue tetrahydrate to stain the virus plaques. If the average value of the number of plaques in ≥10 control wells was between 50 and 150, the assay was accepted. The neutralizing antibody titer was expressed as the reciprocal of the dilution of serum that caused a 50% reduction of plaque formation compared to the plaque number of the diluted challenge virus in the absence of antiserum. Antibody positive was defined as the neutralizing antibody titer being ≥1:10 (13).

Statistical analysis of antibody titers. In both trials, the primary endpoint was the seroconversion rate after the 3rd injection based on the

TABLE 4 Background data of subjects in trial 2

Background factor	Group (injected vaccine type) (n)					
	L (formulation L) (143)		M (formulation M) (142)		Control (MB-JEV) (146)	
	Value	95% CI[a]	Value	95% CI	Value	95% CI
Gender (n [%])						
Male	77 (53.8)	45.3–62.2	73 (51.4)	42.9–59.9	68 (46.6)	38.3–55.0
Female	66 (46.2)	37.8–54.7	69 (48.6)	40.1–57.1	78 (53.4)	45.0–61.7
Age (mean [SD]) (mo)	48.5 (18.2)	NC[b]	48.5 (16.1)	NC	47.7 (17.3)	NC
Body wt (mean [SD]) (kg)	15.7 (3.9)	NC	15.5 (3.1)	NC	15.4 (3.4)	NC

[a] CI, confidence interval.
[b] NC, not calculated.

TABLE 5 Seroconversion rates of neutralizing antibody after the 2nd and 3rd injections in trial 2

Time	Group L (formulation L)		Group M (formulation M)		Control group (MB-JEV)		Noninferiority test
	% (no. of positive conversions/no. of analyzed subjects)	95% CI[a]	% (no. of positive conversions/no. of analyzed subjects)	95% CI	% (no. of positive conversions/no. of analyzed subjects)	95% CI	
After 2nd injection	100.0 (143/143)	97.5–100.0	100.0 (141/141)	97.4–100.0	94.5 (138/146)	89.5–97.6	NC[b]
After 3rd injection	100.0 (143/143)	97.5–100.0	100.0 (140/140)	97.4–100.0	100.0 (146/146)	97.5–100.0	$P < 0.001$

[a] CI, confidence interval.
[b] NC, not calculated.

neutralizing antibody titer. The secondary endpoints were the seroconversion rate after the 2nd injection and the geometric mean antibody titers (GMTs) after the 2nd and 3rd injections. The per-protocol set (PPS) was used to represent the immunogenicity population. Statistical analyses were performed using SAS version 8.2 for trial 1 and SAS version 9.1 for trial 2.

Trial 1. In trial 1, the noninferiority in the seroconversion rate after the 3rd injection for the formulation H-injected group (group H) against the MB-JEV group (control group) was statistically analyzed by the method of Dunnett and Gent (14). A sample size of 100 subjects per group was calculated to verify the noninferiority in the seroconversion rate (assumed to have an expected seroconversion rate of 95%) in each group ($\alpha = 0.025$ [1-tailed test], $\beta = 0.10$, 90% power, and noninferiority limit $\delta = 10\%$). Assuming that the seropositive rate was 45% before the trial and the withdrawal rate was 10%, 204 subjects in each group, for a total of 408 subjects, were required.

Trial 2. In trial 2, the noninferiority in the seroconversion rates after the 3rd injection for the formulation L-injected group (group L) and the formulation M-injected group (group M) against the control group were statistically analyzed by the Farrington-Manning method (15). A sample size of 81 subjects for each group was calculated to verify noninferiority (assumed to have an expected seroconversion rate of 98% against JE virus after the 3rd injection in both groups ($\alpha = 0.025$ [1-tailed test], $\beta = 0.10$, 90% power, and noninferiority limit $\delta = 10\%$). Assuming that the seropositive rate was 10% before the trial and that the withdrawal rate was 10%, 100 subjects in each group, for a total 300 subjects, were required.

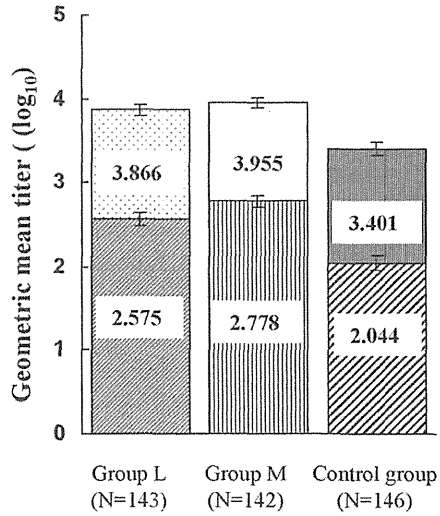

FIG 4 GMTs after the 2nd and 3rd injections in trial 2. Three groups of 158, 163, and 159 subjects were injected subcutaneously with a 0.5-ml dose of formulation L, formulation M, and MB-JEV, respectively, at an interval of 2 to 4 weeks, and an additional injection was given 1 to 15 months after the 2nd injection. The GMTs after the 2nd and 3rd injections in trial 2 are shown after the 2nd (shaded area) and 3rd (dotted area) injections in group L, after the 2nd (striped area) and 3rd (white area) injections in group M, and after the 2nd (shaded striped area) and 3rd (striped area) injections in the control group. The error bars represent 95% confidence intervals.

RESULTS

Trial 1. The main purpose of trial 1 was to verify the noninferiority in the seroconversion rates of group H to the control group after the 3rd injection. The subjects were injected with either formulation H or the MB-JEV. Among the 468 subjects enrolled in this trial, 235 subjects and 233 subjects were assigned randomly to either group H or the control group, respectively, and all of them were included in the safety population, while 439 subjects were included in the PPS immunogenicity population (group H, 218; control group, 221) (Fig. 1). The main protocol deviations were from specified inoculation and blood collection time points; subjects with deviations were excluded from the PPS. In trial 1, the seropositive rate was much lower than our *a priori* assumption of 45% based on the results of the National Epidemiological Surveillance of Vaccine-Preventable Diseases by the Ministry of Health, Labor, and Welfare. Although the number of subjects was greater than the planned sample size, the objective of the trial was achieved. The background data of the subjects are shown in Table 1. No statistically significant differences were observed in the baseline factors between the two groups.

Immunogenicity in trial 1. The seroconversion rates after the 3rd injection were 100% in both groups. The results analyzed by the Dunnett and Gent test verified that group H was not inferior to the control group in terms of the seroconversion rate, which met the primary endpoint. On the other hand, the seroconversion rate of group H after the 2nd injection was 100%, while that of the control group was 99.5% (Table 2). The GMTs of group H after the 2nd and 3rd injections were statistically higher than those of the control group (Fig. 3). The differences in the GMTs of subjects in group H and the control group were assessed using a *t* test ($P < 0.001$).

Safety in trial 1. In trial 1, vaccine-related adverse events with an incidence of ≥5% are listed in Table 3. The incidences of vaccine-related adverse events, such as injection site erythema and injection site swelling, were higher in group H than those of the control group; however, there were no statistically significant differences between the two groups. In group H, erythema exsudativum multiforme (a series of diseases in which erosion develops in the mucosa, such as the lips, oral cavity, and eyes, or multiple erythema or erosion lesions develop on the skin of the entire body) occurred in one subject 9 days after the 2nd injection with formulation H. The subject had recovered well 10 days after hospitaliza-

TABLE 6 GMTs after the 3rd injection in four patterns different in vaccine volume in trial 2

Vaccine volumes at 1st/2nd/3rd injections (ml)	Group (injected vaccine type) (*n*)[a]					
	L (formulation L) (143)		M (formulation M) (142)		Control (MB-JEV) (146)	
	No. receiving dose	GMT (log_{10}) (95% CI)	No. receiving dose	GMT (log_{10}) (95% CI)	No. receiving dose	GMT (log_{10}) (95% CI)
0.25/0.25/0.25	21	3.830 (3.612–4.047)	15	3.793 (3.518–4.069)	19	3.211 (2.944–3.478)
0.25/0.25/0.5	10	3.939 (3.744–4.134)	10	3.999 (3.801–4.197)	9	3.452 (3.109–3.796)
0.25/0.5/0.5	0	NA	2	4.535 (NA)	0	NA
0.5/0.5/0.5	112	3.866 (3.795–3.937)	113	3.962 (3.898–4.026)	118	3.428 (3.348–3.508)

[a] GMT, geometric mean titer; CI, confidence interval; NA, not applicable.

tion. The doctor who took care of this subject commented that a relationship between this case and formulation H could not be completely denied, although a viral infection was suspected because fever after pharyngeal erythema was observed. Based on this doctor's comment, this case was judged as a serious vaccine-related adverse event.

The immunogenicity of formulation H was greater than that of the MB-JEV. However, as the incidence of injection site vaccine-related adverse events for formulation H was higher than that of the MB-JEV and a serious vaccine-related adverse event occurred after the injection of formulation H, we conducted an additional trial, trial 2, to adjust the immunogenicity of the CC-JEV to match that of the MB-JEV.

Trial 2. The main purpose of trial 2 was to verify the noninferiority in the seroconversion rates of group L and group M to the control group after the 3rd injection. The subjects were injected with either formulation L, formulation M, or the MB-JEV. Among the 484 subjects enrolled in this trial, the subjects were assigned randomly into group L (n = 163), group M (n = 158), or the control group (n = 159), and group L (n = 163), group M (n = 157; one subject withdrew), or the control group (n = 159) were included in the safety test population. For the PPS immunogenicity test population, the numbers of included subjects were as follows: group L, 143; group M, 142; and the control group, 146 (Fig. 2). The main protocol deviations were the use of prohibited medications and deviations from the specified enrollment procedures; subjects with deviations were excluded from the PPS. In trial 2, the seropositive rate was much lower than our *a priori* assumption of 10%, which was made based on the results of trial 1. Although the number of subjects was greater than the planned sample size, the objective of the trial was achieved. The background data of the subjects are shown in Table 4. No statistically significant differences were observed in the baseline factors among the three groups.

Immunogenicity in trial 2. The seroconversion rates of all groups after the 3rd injection were 100%. The results analyzed by the Farrington-Manning test verified that groups L and M were not inferior to the control group in terms of the seroconversion rate, which met the primary endpoint. On the other hand, while the seroconversion rates of groups L and M after the 2nd injection were 100%, the seroconversion rate of the control group was 94.5% (Table 5). The GMTs of groups L and M after the 2nd and 3rd injections were statistically higher than those of the control group (Fig. 4). The differences in the GMTs of subjects in group L, group M, and the control group were assessed using a t test ($P <$ 0.001). These results showed that the neutralizing antibody titer

TABLE 7 Vaccine-related adverse events over three injections (≥5%) in trial 2

Reaction	Group (injected vaccine type) (*n*)								
	L (formulation L) (163)			M (formulation M) (157)			Control (MB-JEV) (159)		
	No. with adverse reaction	%	95% CI[a]	No. with adverse reaction	%	95% CI	No. with adverse reaction	%	95% CI
Local reactions									
Erythema	27	16.6	11.2–23.2	39	24.8	18.3–32.4	33	20.8	14.7–27.9
Swelling	11	6.7	3.4–11.8	13	8.3	4.5–13.7	13	8.2	4.4–13.6
Induration	3	1.8	0.4–5.3	8	5.1	2.2–9.8	4	2.5	0.7–6.3
Itching	1	0.6	0.0–3.4	2	1.3	0.2–4.5	13	8.2	4.4–13.6
Systemic reactions									
Fever	35	21.5	15.4–28.6	44	28.0	21.2–35.7	23	14.5	9.4–20.9
Grade ≥3 (≥39.0°C)	3	1.8	0.4–5.3	8	5.1	2.2–9.8	2	1.3	0.2–4.5
Coughing	13	8.0	4.3–13.3	9	5.7	2.7–10.6	11	6.9	3.5–12.0
Nasal drainage	11	6.7	3.4–11.8	11	7.0	3.5–12.2	8	5.0	2.2–9.7
Rash	9	5.5	2.6–10.2	4	2.5	0.7–6.4	4	2.5	0.7–6.3
Diarrhea	6	3.7	1.4–7.8	6	3.8	1.4–8.1	8	5.0	2.2–9.7
Grade ≥3[b]	0	0	0.0–2.2	0	0	0.0–2.3	1	0.6	0.0–3.5
Headache	4	2.5	0.7–6.2	4	2.5	0.7–6.4	8	5.0	2.2–9.7

[a] CI, confidence interval.
[b] Diarrhea grade of ≥3 defined as an increase in stool frequency of ≥9 times/day.

TABLE 8 Vaccine-related adverse event of fever over three injections in trial 2

Fever grade[a]	Group (injected vaccine type) (*n*)					
	L (formulation L) (163)		M (formulation M) (157)		Control (MB-JEV) (159)	
	No. with fever (%)	95% CI[b]	No. with fever (%)	95% CI	No. with fever (%)	95% CI
Total	35 (21.5)	15.4–28.6	44 (28.0)	21.2–35.7	23 (14.5)	9.4–20.9
1	22 (13.5)	8.7–19.7	20 (12.7)	8.0–19.0	14 (8.8)	4.9–14.3
2	10 (6.1)	3.0–11.0	16 (10.2)	5.9–16.0	7 (4.4)	1.8–8.9
3	2 (1.2)	0.1–4.4	8 (5.1)	2.2–9.8	1 (0.6)	0.0–3.5
4	1 (0.6)	0.0–3.4	0 (0.0)	0.0–2.3	1 (0.6)	0.0–3.5

[a] The most severe grade was counted when fever in the same subject occurred at different grades over the three injections. Grade 1, ≥37.5°C; grade 2, ≥38.0°C; grade 3, ≥39.0°C fever continued for less than a day; grade 4, ≥39.0°C fever continued for >2 days.
[b] CI, confidence interval.

against the Beijing-1 strain induced by formulations L or M was higher than that by the MB-JEV.

Table 6 shows the GMTs after the 3rd injection according to four patterns of injection volume (1st/2nd/3rd injection): 0.25/0.25/0.25 ml, 0.25/0.25/0.5 ml, 0.25/0.5/0.5 ml, and 0.5/0.5/0.5 ml. The number of subjects for whom the dose was changed from 0.25 ml to 0.5 ml was small. Comparing sets of two patterns, such as 0.25/0.25/0.25 ml and 0.5/0.5/0.5 ml of formulations L and M, respectively, there were no statistically significant differences between them. Based on these results, it is considered acceptable to inoculate children <3 years of age with 0.25 ml of any CC-JEV.

Safety in trial 2. In trial 2, vaccine-related adverse events with an incidence of ≥5% are listed in Table 7. In the local reactions, the most common vaccine-related adverse event was injection site erythema. There were no statistically significant differences in the incidences of injection site erythema among group L, group M, and the control group. In the systemic reactions, the most common vaccine-related adverse event was fever. The incidence of fever in group M was statistically higher than that in the control group (confidence interval for group M, 21.2 to 35.7; control group, 9.4 to 20.9). Most of the adverse events in both group L and group M were lower than grade 3 (<39.0°C) (Table 8). No serious vaccine-related adverse event was observed in trial 2. Based on these results, we selected formulation L containing 4 μg of CC-JEV per dose as the optimum dose.

Table 9 shows the vaccine-related adverse events after the 3rd injection according to four patterns of injection volume (1st/2nd/3rd injection): 0.25/0.25/0.25 ml, 0.25/0.25/0.5 ml, 0.25/0.5/0.5 ml, and 0.5/0.5/0.5 ml. The number of subjects for whom the dose was changed from 0.25 ml to 0.5 ml was small. Comparing sets of two patterns, such as 0.25/0.25/0.25 ml and 0.5/0.5/0.5 ml, of formulations L and M, respectively, there were no statistically significant differences between them. Based on these results, it is considered acceptable to inoculate children <3 years of age with 0.25 ml of formulation L.

DISCUSSION

We have developed a new type of JE vaccine, a freeze-dried CC-JEV vaccine derived from Vero cells instead of from mouse brain. The new type of JE vaccine contains a more highly purified antigen than in the previous vaccines, produced by adding an affinity column chromatography step to the manufacturing process used for the production of the MB-JEV. To evaluate the new type of JE vaccine, two series of phase III clinical trials, trials 1 and 2, were conducted as double-blinded randomized parallel-group studies. The trials showed that 4 μg per dose of formulation L has the same immunogenicity as 17 μg per dose of the MB-JEV, and formulation L was renamed Encevac. This is the first report on a direct comparison of immunogenicity and safety in children inoculated with the CC-JEV and MB-JEV derived from the Beijing-1 strain in phase III clinical trials.

After the launch of Encevac in 2011, an additional clinical study using Encevac was recently conducted to investigate its safety and immunogenicity, focusing on the 2nd stage of the Japanese public immunization program in children 9 to 12 years of age (K. Okada, personal communication). The GMT of 21 subjects who had received Encevac at the 1st stage was $10^{2.68}$ when measured prior to the booster injection at the 2nd stage, and the GMT had increased to $10^{3.84}$ by about 1 month after the booster injection of Encevac at the 2nd stage. On the other hand, the GMT of 34 subjects who had received MB-JEV at the 1st stage was $10^{2.37}$ when measured prior to the booster injection at the 2nd stage, and the GMT had increased to $10^{3.65}$ by about 1 month after the booster injection of Encevac at the 2nd stage. No serious vaccine-related adverse events were reported for any of the groups. Thus,

TABLE 9 Adverse vaccine reactions over three injections in four patterns different in vaccine volume in trial 2

Vaccine volumes at 1st/2nd/3rd injections (ml)	Group (injected vaccine type) (*n*)					
	L (formulation L) (163)		M (formulation M) (155)[a]		Control (MB-JEV) (159)	
	No. with adverse reaction/total no. receiving dose	% (95% CI)[b]	No. with adverse reaction/total no. receiving dose	% (95% CI)	No. with adverse reaction/total no. receiving dose	% (95% CI)
0.25/0.25/0.25	14/25	56.0 (34.9–75.6)	11/18	61.1 (35.7–82.7)	8/22	36.4 (17.2–59.3)
0.25/0.25/0.5	9/11	81.8 (48.2–97.7)	6/11	54.5 (23.4–83.3)	5/9	55.6 (21.2–86.3)
0.25/0.5/0.5	0	NA[c]	2/4	50.0 (6.8–93.2)	1/1	100.0 (2.5–100.0)
0.5/0.5/0.5	61/127	48.0 (39.1–57.1)	70/122	57.4 (48.1–66.3)	73/127	57.5 (48.4–66.2)

[a] Two subjects were excluded because they did not receive the 3rd injection.
[b] CI, confidence interval.
[c] NA, not applicable.

this clinical study targeting the booster injection at the 2nd stage has further confirmed the safety and immunogenicity of Encevac.

In the 1880s, incidents of ADEM related to a rabies vaccine that was prepared from rabbit spinal cord were reported, and protein contaminants deriving from the spinal cord were considered to be the cause of ADEM (16, 17). Incidents of ADEM after inoculation with a Japanese encephalitis vaccine were also suspected to be caused by protein contaminants derived from mouse brain. Based on these considerations, attempts have been undertaken to reduce the rate of ADEM by changing the materials of the vaccine from mouse brain to cell culture product. However, the World Health Organization (WHO) finally concluded (18) that no causal relationship exists between ADEM and inoculation with the Japanese encephalitis vaccine derived from mouse brain. Furthermore, the WHO Global Advisory Committee on Vaccine Safety concluded that no evidence exists regarding an increased risk of ADEM associated with administration of the inactivated JE vaccine. In fact, as the ADEM incidence rate of the newly approved CC-JEV in Japan was one case per approximately 1.3 million injections, while the ADEM incidence rate of the MB-JEV was one case per 0.7 to 2.0 million injections, the number of incidents of ADEM has not reduced after the introduction of the CC-JEV in Japan. However, the CC-JEV has the advantages of being able to reduce a possible risk caused by the contamination of unknown adventitious agents derived from mouse brain and to save animals necessary for manufacturing the JE vaccine.

Although the CC-JEVs used in Europe and the United States contain an aluminum hydroxide adjuvant (5), Encevac shows high immunogenicity even at a low vaccine antigen dose, without any adjuvant. Furthermore, Encevac has a 3-year shelf life under refrigerated conditions without containing any preservative, such as thimerosal. As JE is still the most common kind of viral encephalitis in Asia, especially in tropical and subtropical countries, the JE vaccine Encevac is expected to contribute to the prevention of JE.

ACKNOWLEDGMENTS

We thank the following pediatricians for their great contributions to the clinical trials described in this paper: Akio Furuse, Akira Akasawa, Atsuko Yamamoto, Eitaro Suzuki, Fuminari Ishibashi, Hideo Cho, Hiroshi Sakiyama, Hiroyuki Moriuchi, Hiroyuki Nunoi, Junichi Okada, Kaoru Oikawa, Katsutoshi Abe, Keiko Fukuhara, Keiko Kasai, Keinosuke Fujita, Kouichi Takeuchi, Kuniaki Iyoda, Kunihisa Shimomura, Kyoko Uramoto, Masakazu Inoue, Masakazu Kobayashi, Masami Sakaguchi, Masanobu Morimoto, Masanori Ikeda, Mitsunobu Miyazu, Naoki Hanada, Naoto Adachi, Ritsue Nii, Ryota Hosoya, Seiji Yamaguchi, Seishi Seguchi, Shigeru Ikezawa, Shigeto Sugino, Shinichi Tomoeda, Shinji Kido, Shiro Seto, Shoichi Chida, Shuhei Yajima, Susumu Hakamada, Susumu Kanzaki, Tadaomi Tokutake, Takanobu Ishihara, Takao Ashizawa, Takashi Nakano, Tatsuo Takeuchi, Tetsuro Kitamura, Toru Watanabe, Toshimi Yoshimoto, Tsuneyoshi Egami, Yasushi Shimada, Yoshiharu Eto, Yoshiaki Hirata, Yoshiko Hattori, Yoshiko Suginohara, Yoshitomo Okajima, Yoshizo Asano, Yougo Haraguchi, Yuichi Miura, and Yuji Mizumoto. We also thank Yasuo Ohashi, who was the trial statistician generating the randomization schedule. Finally, we thank the staff of Kaketuken for their great support in this work.

C.M., K.O., T.O., M.H., K.I., T.T., and K.U. have received consulting fees and payment for travel and lodging from Kaketsuken to attend investigators' meetings. H.Y. and Y.I. are employees of Kaketsuken.

REFERENCES

1. **Halstead SB, Jacobson J, Dubischar-Kastner K.** 2012. Japanese encephalitis vaccines, p 312–351. *In* Plotkin SA, Orenstein WA, Offit PA (ed), Vaccines, 6th ed. Elsevier, Amsterdam, the Netherlands.
2. **Sumiyoshi H, Mori C, Fuke I, Morita K, Kuhara S, Kondou J, Kikuchi Y, Nagamatu H, Igarashi A.** 1987. Complete nucleotide sequence of the Japanese encephalitis virus genome RNA. Virology **161**:497–510. http://dx.doi.org/10.1016/0042-6822(87)90144-9.
3. **Oya A, Kurane I.** 2007. Japanese encephalitis for a reference to international travelers. J. Travel Med. **14**:259–268. http://dx.doi.org/10.1111/j.1708-8305.2007.00134.x.
4. **Bista MB, Banerjee MK, Shin SH, Tandan JB, Kim MH, Sohn YM, Ohrr HC, Tang JL, Halstead SB.** 2001. Efficacy of single-dose SA 14-14-2 vaccine against Japanese encephalitis: a case control study. Lancet **358**:791–795. http://dx.doi.org/10.1016/S0140-6736(01)05967-0.
5. **Halstead SB, Thomas SJ.** 2011. New Japanese encephalitis vaccines: alternatives to production in mouse brain. Expert Rev. Vaccines **10**:355–364. http://dx.doi.org/10.1586/erv.11.7.
6. **Sugawara K, Nishiyama K, Ishikawa Y, Abe M, Sonoda K, Komatsu K, Horikawa Y, Takeda K, Honda T, Kuzuhara S, Kino Y, Mizokami H, Mizuno K, Oka T, Honda K.** 2002. Development of Vero cell-derived inactivated Japanese encephalitis vaccine. Biologicals **30**:303–314. http://dx.doi.org/10.1006/biol.2002.0345.
7. **Yamaguchi Y, Kira R, Hara T.** 2010. Acute disseminated encephalomyelitis and pediatric multiple sclerosis: current states in Japan. No To Hattatsu **42**:227–229. (In Japanese.)
8. **Kikukawa A, Gomi Y, Akechi M, Onishi T, Manabe S, Namazue J, Fuke I, Ishikawa T, Okuno Y, Ueda S.** 2012. Superior immunogenicity of a freeze-dried, cell culture-derived Japanese encephalitis vaccine (inactivated). Vaccine **30**:2329–2335. http://dx.doi.org/10.1016/j.vaccine.2012.01.054.
9. **Okada K, Iwasa T, Namazue J, Akechi M, Ueda S, JE Vaccine Clinical Study Group.** 2012. Safety and immunogenicity of a freeze-dried, cell culture-derived Japanese encephalitis vaccine (Inactivated) (JeBIK-V) in children. Vaccine **30**:5967–5972. http://dx.doi.org/10.1016/j.vaccine.2012.07.034.
10. **Tauber E, Kollaritsch H, Korinek M, Rendi-Wagner P, Jilma B, Firbas C, Schranz S, Jong E, Klingler A, Dewasthaly S, Klade CS.** 2007. Safety and immunogenicity of a Vero-cell-derived, inactivated Japanese encephalitis vaccine: a non-inferiority, phase III, randomised controlled trial. Lancet **370**:1847–1853. http://dx.doi.org/10.1016/S0140-6736(07)61780-2.
11. **Schuller E, Klingler A, Dubischar-Kastner K, Dewasthaly S, Müller Z.** 2011. Safety profile of the Vero cell-derived Japanese encephalitis virus (JEV) vaccine Ixiaro. Vaccine **29**:8669–8676. http://dx.doi.org/10.1016/j.vaccine.2011.08.117.
12. **Abe M, Kuzuhara S, Kino Y.** 2003. Establishment of an analyzing method for a Japanese encephalitis virus neutralization test in Vero cells. Vaccine **21**:1989–1994. http://dx.doi.org/10.1016/S0264-410X(02)00772-7.
13. **Oya A.** 1988. Japanese encephalitis vaccine. Acta Paediatr. Jpn. **30**:175–184.
14. **Dunnett CW, Gent M.** 1977. Significance testing to establish equivalence between treatments, with special reference to data in the form of 2×2 tables. Biometrics **33**:593–602. http://dx.doi.org/10.2307/2529457.
15. **Farrington CP, Manning G.** 1990. Test statistics and sample size formulae for comparative binomial trials with null hypothesis of non-zero risk difference or non-unity relative risk. Stat. Med. **9**:1447–1454. http://dx.doi.org/10.1002/sim.4780091208.
16. **Bennetto L, Scolding N.** 2004. Inflammatory/post-infectious encephalomyelitis. J. Neurol. Neurosurg. Psychiatry **75**(Suppl 1):i22–i28. http://dx.doi.org/10.1136/jnnp.2003.034256.
17. **Menge T, Hemmer B, Nessler S, Wiendl H, Neuhaus O, Hartung HP, Kieseier BC, Stüve O.** 2005. Acute disseminated encephalomyelitis: an update. Arch. Neurol. **62**:1673–1680. http://dx.doi.org/10.1001/archneur.62.11.1673.
18. **WHO.** 2010. The immunological basis for immunization series: module 13: Japanese encephalitis. World Health Organization, Geneva, Switzerland. http://whqlibdoc.who.int/publications/2010/9789241599719_eng.pdf.

研　究

特別支援学校に在籍する小児の予防接種実施状況に関する調査（第2報）

—予防接種に関する養育者の要望—

古藤　雄大[1)]，岡部　里佳[2)]，宮崎　千明[3)]，稲垣　二郎[4)]
山岸　義晃[5)]，田辺　卓也[6)]，永井利三郎[7)]

〔論文要旨〕

本研究では，特別支援学校に在籍する小児の養育者3,799名を対象として質問紙調査を行い，1,752名（回収率46.1%）から回答を得た。対象児の背景や予防接種状況などの点に関しては第1報で報告した。今回は，質問紙の自由記載の予防接種に関する養育者の要望を内容分析の手法を用いて分析を行ったので報告する。

要望として特徴的であったものとして，【接種場所に関して考慮してほしい】，【接種時期に関して考慮してほしい】，【情報がほしい】，【費用に関して考慮してほしい】，【発達と運動機能に課題のある子どもへの配慮がほしい】などが挙げられた。一般児に比べ，体調のコントロールなどが難しい対象児においては，環境の整備や十分な情報提供を行って予防接種を受けやすくする支援の必要性が示された。

Key words：予防接種，特別支援学校，内容分析，養育者

Ⅰ．諸　　言

基礎疾患をもつ特別支援学校在籍児は，感染症の罹患によって基礎疾患の症状が悪化することがある。また，予防接種を打つことで起きうる副反応の発熱は，健常児には大きな問題とならない場合でも，てんかんのような基礎疾患をもつ小児にとっては発作を誘発する因子となることがある。

基礎疾患をもつ小児の予防接種状況に関する調査としては，乳児重症ミオクロニーてんかん（Severe myoclonic epilepsy in infancy：SMEI）症例のワクチン接種状況調査[1)]や重症心身障害児の予防接種状況[2)]が行われているが，養育者の予防接種についての考えや未接種の理由，医療者に対する要望等を検討したものは少ない。そこで，本研究では，大阪府立の特別支援学校に在籍する小児を対象に，小児の疾患と予防接種状況との関連や，予防接種を未接種の理由，養育者の予防接種に対する要望などを調査した。第1報[3)]では，対象児の疾患と予防接種率との関係について検討し，てんかんや重症心身障害をもつ小児の定期予防接種率が低い一方で，任意予防接種率は一般児と比して高い傾向にあり，養育者の感染予防への意識が高かったことを報告した。

Present Situation and Parents' Needs in Vaccination for Children of Special Education School（2nd Report）
Yuta Koto，Rika Okabe，Chiaki Miyazaki，Jirou Inagaki，
Yoshiaki Yamagishi，Takuya Tanabe，Toshisaburou Nagai
1）大阪府立母子保健総合医療センター（看護師）
2）大阪大学大学院医学系研究科保健学専攻（看護師）
3）福岡市立心身障がい福祉センター（医師／小児科）
4）独立行政法人国立病院機構九州がんセンター（医師／小児科）
5）独立行政法人医薬品医療機器総合機構（医師／小児科）
6）田辺こもどクリニック（医師／小児科）
7）大阪大学大学院医学系研究科保健学専攻（医師／小児科）
別刷請求先：古藤雄大　大阪大学大学院医学系研究科保健学専攻　〒565-0871 大阪府吹田市山田丘1－7
Tel/Fax：06-6879-2531

〔2601〕
受付 14．1．6
採用 14．6．8

Ⅱ. 研究目的

質問紙の自由記載の分析を行い，養育者の視点から考えた，予防接種を受けやすい環境作りと接種勧奨のあり方を検討する。

Ⅲ. 方　法

1. 対象と方法

調査対象は，大阪府立の特別支援学校全24校のうち，調査協力を得られた19校の特別支援学校に在籍する小児の養育者3,799名を対象として，無記名自記式質問紙調査を行った。

2. 期　間

調査期間は，2009年12月～2010年1月の2か月間であった。

3. 調査内容

調査内容は，回答者の背景，対象児の背景，予防接種対象疾患への罹患歴，ワクチン接種の有無，接種した場合の副反応の有無，未接種ワクチン名とその理由，小児が接種を受ける際にひきつけを起こさないように，養育者が何らかの配慮を行っているか，そして養育者の予防接種に対する要望であった。

4. 分析方法

今回は，調査内容のうち，養育者の要望について分析を行った。分析には，Berelson Bの内容分析[4]の手法を参考にし，特別支援学校在籍児の養育者の予防接種に対する要望を明らかにした。分析は，質問紙の各自由記載欄に書かれた要望を一つの記録単位として類似の内容からコード化を行い，コードをカテゴリーに分類した。分析の信頼性確保のために，分析中は研究者4名による討議を行い，分析結果の研究者間での単純一致率およびスコットの一致率πを算出した。

5. 倫理的配慮

本研究では，質問紙の配布・回収は各学校から行った。質問紙は記入後，封をして，回収時に養育者が強制力を感じることのないように設けられた回収ボックスに入れて，回収後大学へ郵送してもらった。また回収ボックスの設置場所は，各クラスに設置するのではなく，学校全体で一つのものとし，回答者が特定されることのない場所に設置するよう，各学校に配慮を求めた。また，質問紙と共に，研究の目的および倫理的配慮について説明した文書を配布し，回答を以て同意とみなした。なお，本研究は大阪大学医学部保健学倫理委員会の承認を得て実施した。

Ⅳ. 結　果

1. 回収結果

回答が得られた養育者は，1,752名（回収率46.1％）であり，有効回答数は1,694名（有効回答率96.7％）であった。

表1　定期予防接種についての要望〈記録単位数〉

（n＝261，π＝0.807）

コード	カテゴリー
学校で接種してほしい（集団接種にしてほしい）〈100〉	接種場所に関して考慮してほしい〈122〉
指定病院以外でも接種してほしい〈8〉	
主治医に打ってほしい〈7〉	
個別接種してほしい（医療機関で打ちたい）〈7〉	
接種時期等の情報がほしい〈61〉	情報がほしい〈66〉
母子手帳などに予防接種を一覧にしてほしい〈5〉	
時期を過ぎても行ってほしい〈23〉	接種時期に関して考慮してほしい〈39〉
接種できる日にち・時間を拡大してほしい〈11〉	
接種回数を減らしてほしい（まとめられるものはまとめてほしい）〈3〉	
ワクチンの接種可能年齢を引き下げてほしい〈2〉	
発達と運動機能に課題のある子どもが受けやすい環境を作ってほしい〈22〉	発達と運動機能に課題のある子どもへの配慮がほしい〈34〉
子どもの身体的影響への対策がほしい（副反応の情報がほしい）〈8〉	
てんかん発作があっても予防接種をしてほしい〈4〉	

2．対象児の背景

対象児の性別は，男児990名（58.4％），女児535名（31.6％）であった。年齢は，最小値3歳10か月，最大値19歳6か月，中央値14歳2か月であった。小児の基礎疾患の種類を見ると，発達障害806名（47.6％）が一番多く，次いでてんかん441名（26.0％）や染色体異常213名（12.6％）などであった（第1報[3]参照）。

3．養育者の要望

各要望の単純一致率およびスコットの一致率πは，定期予防接種への要望85.0％（π＝0.807），任意予防接種への要望87.1％（π＝0.808），周知方法への要望83.9％（π＝0.819），予防接種全般への要望81.8％（π＝0.787）であった。本研究では，カテゴリーを【　】，コードを『　』で示した。

1）定期予防接種について（表1）

定期予防接種についての要望は261記録単位が示され，それらは内容の類似性から13コード，4カテゴリー【接種場所に関して考慮してほしい】，【情報がほしい】，【接種時期に関して考慮してほしい】，【発達と運動機能に課題のある子どもへの配慮がほしい】に分類された。

2）任意予防接種について（表2）

任意予防接種についての要望は411記録単位が示され，それらは内容の類似性から9コード，5カテゴリー【費用に関して考慮してほしい】，【接種場所に関して考慮してほしい】，【発達と運動機能に課題のある子どもへの配慮がほしい】，【情報がほしい】，【ワクチンに関して考慮してほしい】に分類された。

3）周知方法について（表3）

周知方法についての要望は174記録単位が示され，それらは内容の類似性から12コード，4カテゴリー【情報の内容に関して考慮してほしい】，【情報の発信源に関して考慮してほしい】，【発達と運動機能に課題のある子どもへの配慮がほしい】，【接種場所に関して考慮してほしい】に分類された。

表2　任意予防接種についての要望〈記録単位数〉

（n＝411，π＝0.808）

コード	カテゴリー
費用が安くなるように見直してほしい〈217〉	費用に関して考慮してほしい〈326〉
定期接種に（無料化）してほしい〈70〉	
価格を一律にしてほしい〈39〉	
集団接種にしてほしい〈29〉	接種場所に関して考慮してほしい〈34〉
主治医に打ってほしい〈5〉	
発達と運動機能に課題のある子どもへの補助・優先枠がほしい〈25〉	発達と運動機能に課題のある子どもへの配慮がほしい〈25〉
情報提供してほしい〈21〉	情報がほしい〈21〉
ワクチンの数量を十分に用意してほしい〈3〉	ワクチンに関して考慮してほしい〈5〉
インフルエンザも1回接種で済ませてほしい〈2〉	

表3　周知方法についての要望〈記録単位数〉

（n＝174，π＝0.819）

コード	カテゴリー
（具体的に内容や情報源についての記載はないが）情報がほしい〈25〉	情報の内容に関して考慮してほしい〈80〉
接種時期等の情報がほしい〈21〉	
予防接種そのものや副反応についての情報がほしい〈20〉	
予防接種の一覧表がほしい〈14〉	
市町村から情報がほしい〈26〉	情報の発信源に関して考慮してほしい〈61〉
学校から情報がほしい〈24〉	
病院などの医療機関から情報がほしい〈7〉	
今ある方法以外の方法で知らせてほしい〈4〉	
発達と運動機能に課題のある子どもが受けやすい環境を作ってほしい〈12〉	発達と運動機能に課題のある子どもへの配慮がほしい〈20〉
子どもが納得して接種できるための方法が知りたい〈5〉	
発達と運動機能に課題のある子ども向けの情報がほしい〈3〉	
集団接種にしてほしい〈13〉	接種場所に関して考慮してほしい〈13〉

表4 予防接種全般への要望〈記録単位数〉

(n=295, π=0.787)

コード	カテゴリー
集団接種にしてほしい〈96〉	接種場所に関して考慮してほしい〈111〉
主治医に打ってほしい〈8〉	
市外でも接種してほしい〈4〉	
安全ならば，医療機関以外でも接種してほしい〈3〉	
発達と運動機能に課題のある子どもが予防接種を受けやすい環境を作ってほしい〈32〉	発達と運動機能に課題のある子どもへの配慮がほしい〈90〉
医療者側が発達と運動機能に課題のある子どもに対してもっと理解をしてほしい〈21〉	
身体への影響が心配（副反応についての情報がほしい）〈15〉	
注射以外の方法があってほしい〈11〉	
パンデミックインフルエンザのワクチン接種優先対象者にしてほしい〈9〉	
介護者もパンデミックインフルエンザの接種対象者にしてほしい〈2〉	
情報がほしい〈30〉	情報がほしい〈30〉
ワクチンの種類を減らしてほしい（まとめることができるものはまとめてほしい）〈7〉	ワクチン・抗体検査に関して考慮してほしい〈30〉
安心して受けることができるワクチンを作ってほしい〈7〉	
接種できるワクチンの量を増やしてほしい〈6〉	
流行時には行政に迅速に対応してほしい〈5〉	
接種できるワクチンの種類を増やしてほしい〈3〉	
抗体検査を簡単にできるようにしてほしい〈2〉	
接種の期限をなくしてほしい〈8〉	接種時期に関して考慮してほしい〈18〉
予防接種の機会と回数を増やしてほしい〈5〉	
予約を取りやすくしてほしい〈5〉	
任意をもっと安くしてほしい〈9〉	費用に関して考慮してほしい〈16〉
病院ごとの対応・料金を一律にしてほしい〈4〉	
任意を定期にしてほしい〈3〉	

4) 予防接種全般について（表4）

予防接種全般についての要望は295記録単位が示され，それらは内容の類似性から23コード，6カテゴリー【接種場所に関して考慮してほしい】，【発達と運動機能に課題のある子どもへの配慮がほしい】，【情報がほしい】，【ワクチン・抗体検査に関して考慮してほしい】，【接種時期に関して考慮してほしい】，【費用に関して考慮してほしい】に分類された。

V. 考　　察

1. 定期予防接種について

養育者の定期予防接種に関する要望から，接種場所および時期の考慮や定期予防接種に関する情報，基礎疾患をもつ小児に対する配慮が求められていることが示された。

接種場所について，本調査では，『学校で接種してほしい（集団接種にしてほしい）』といった学校での集団接種の要望が結果として示された。個別接種のワクチンを受けるために病院へ行くことや，待合室で長時間待つことに難しさを感じている意見が多かった。今回の調査当時，対象地である大阪府では，BCGとポリオは保健センター等での集団接種，それ以外のものは市内の病院での個別接種であった。学校における集団接種は2003年のBCGワクチンの接種中止以降行われていない[5]。体調面や病院への移動に考慮が必要である小児においては，特別支援学校での接種が望まれており，校医や自治体との連携をもとに，学校での接種を検討していく必要がある。

また接種費用について，現在，定期予防接種の公費負担は居住市内での病院に限られている。本調査の対象児では市外の大学病院などをかかりつけ医にしている小児も多い。田辺ら[1]の報告において，保護者は主治医と居住地が異なる場合の費用負担に困難を感じていることが示されている。居住地外での接種の公費負担は，居住地の市町村長から接種先の市町村長へ予防接種実施の依頼を行うことにより可能であり[6]，特別

支援学校在籍児が定期予防接種を受ける機会が減ることのないよう，居住市外における費用負担制度を簡易に利用できるような配慮が必要であると考える。

接種時期に関しては，『時期を過ぎても行ってほしい』といった，公費負担対象期間中に接種できなかった場合でも接種費用の負担を行うことが挙がった。本調査実施後，2013年には「予防接種法施行令の一部を改正する政令」が施行され，対象疾病にかかったこと等により定期接種機会を逸した者に定期接種の機会が確保された[6]。また，『接種できる日にち・時間を拡大してほしい』といった，土日や夜間での接種を望む養育者がいることが示された。接種時間の拡張は，一般児においても土日や夜間での接種が希望されている[7]。本調査の対象のように基礎疾患をもつ小児は体調のコントロールが難しく，接種時間の拡大を検討する必要が示唆された。

2．任意予防接種について

養育者からの任意予防接種に関する要望としては，費用に関する要望が多く，『費用が安くなるように見直してほしい』や『定期接種に（無料化）してほしい』などの費用負担の軽減や無料化のほかに，『価格を一律にしてほしい』といった医療機関の料金の統一が求められていた。先行研究でも，費用負担を軽減してほしいという要望が挙げられている[7]。任意予防接種の公費補助制度を実施している自治体における調査では，補助を出すことで社会的視点における便益が高くなることを示唆している[8]。また，任意予防接種の無料化，定期接種化と合わせて，諸外国で使用されている５種混合ワクチンなどの導入により接種回数が減ることでの養育者の負担軽減が期待される。

今回の調査では，2009年に流行したパンデミックインフルエンザ（A/H１N１ pdm2009）の影響もあり，『ワクチンの数量を十分に用意してほしい』，『インフルエンザも１回接種で済ませてほしい』といった要望が挙げられた。1994年の予防接種法改正により，インフルエンザワクチンは任意接種のワクチンとなり，学童集団接種は行われなくなった[9]。その結果，インフルエンザワクチンの接種率は大きく低下した[10]。わが国ではインフルエンザの集団予防効果について懐疑的な意見が大勢を占めるが，児童生徒に対するインフルエンザワクチンの有効率は70～90％と言われており[11]，接種することによるインフルエンザの軽症化効果も示唆されている[10]。養育者からは，定期接種と同様に体調面や病院への移動の難しさを理由に，『集団接種にしてほしい』と希望しており，障害をもった小児が受けやすい環境を検討すべきである。

3．周知方法について

予防接種の周知方法に関する要望として，情報の内容や情報源についての考慮などが挙げられた。情報の内容としては，接種スケジュールに関することやワクチンの副作用についての情報を求めていることがわかった。また，障害をもっている小児に対応できる病院などに関する情報を養育者が求めていることもわかった。

たびたびの予防接種実施に関する法改正[12]により，養育者は混乱を来していると推測される。加えて『予防接種そのものや副反応についての情報がほしい』といった予防接種の副反応に関する具体的な情報を求めていることがわかった。予防接種の副反応としては，1993年のMMRワクチンによる無菌性髄膜炎の問題[13]や2005年の日本脳炎ワクチンによる急性散在性脳脊髄炎（Acute disseminated encephalomyelitis：ADEM）の問題があった[14]。しかし，予防接種後の副反応と報告されているものには，ワクチン接種との因果関係が明らかではないものも含まれている。ワクチンの接種に際しては，100％安全なワクチンは困難であり，ワクチンの副反応の頻度は自然感染したときの合併症の頻度と比較してみるとワクチンの有用性は明らかであるという見方を養育者に周知していくことが大切である[15]。養育者が副反応に関して不安を感じることは当然であるが，そういった不安に対して医療者側が適切なデータに基づく情報を提供することで，不安を解消し，接種を受けやすい環境ができると考えられる。

また，発達障害児の養育者は，待ち時間が長い場合に小児がおとなしく待っていることができず，医師や他の患者の視線が辛いと感じており[16]，「しつけ」の問題だと勘違いされ，周囲の厳しい評価に追い込まれている[17]。今回の調査でも，『発達と運動機能に課題のある子どもが受けやすい環境を作ってほしい』という要望が挙がった。予防接種と同様に侵襲を伴う歯科での調査では，保護者が他の患者にも気を使っていることが示唆されており，保護者が「障害について理解がある」，「無理をしない」ことを要望していることが明らかとなっている[18]。養育者と小児が安心して予防

接種を受けることができるために，医療者側の発達障害やその対応に関する認知を高めていく必要がある。

情報源に関しては，学校，市町村の広報，病院の三者からのアプローチを養育者が求めていることが明らかとなった。市役所からの広報や病院からの情報提供の重要性は従来から提唱されており[19,20]，学校の校医や看護師と連携し養育者に対し情報提供を行っていくことで接種率向上につながると推測される。特に，小児の居住地と特別支援学校の所在地が異なる場合，小児を通した養育者同士の情報共有が促進されにくいことも挙げられており，複数の自治体に所轄する特別支援学校においては，自治体ごとの情報を提供するなどのサポートが望まれる。

4．予防接種全般について

予防接種全般への要望では，定期予防接種や任意予防接種，周知方法で述べた要望のほかに，ワクチンに関するものや基礎疾患，発達障害をもつ小児への配慮が挙げられた。

2009年に流行したパンデミックインフルエンザの影響から，『流行時には行政に迅速に対応してほしい』という要望が挙げられた。パンデミックインフルエンザの出現によりパニックが起これば，抗インフルエンザ薬やワクチンの備蓄があっても多くの患者が診療を受けられない事態も想定され[21]，一般の人はもとより基礎疾患を有し，罹患によるリスクの高い特別支援学校在籍児への対応の確立が求められる。また，小児だけでなく，介護者への優先接種を求める養育者もおり，家族や学校関係者まで含んだ対策が必要である。

その他には，『注射以外の方法があってほしい』と望む養育者もいた。感覚過敏などをもつ発達障害児においては痛みの少ない方法による予防が望まれており，経鼻噴霧による生ワクチン[22]など新たなワクチンの導入によって予防接種を受けやすい環境づくりを目指すことも必要である。

今回の分析では質問紙の回答者すべてを分析対象としており，疾患やADLによる養育者の要望の違いは検討できておらず，今後分析を行っていく必要がある。

Ⅵ．結　　論

特別支援学校に在籍する小児の養育者に対して行った予防接種に関する質問紙調査から，養育者の要望が明らかとなった。

要望として，【接種場所に関して考慮してほしい】，【接種時期に関して考慮してほしい】，【情報がほしい】，【費用に関して考慮してほしい】，【ワクチンに関して考慮してほしい】，【発達と運動機能に課題のある子どもへの配慮がほしい】などが挙げられた。感染症罹患を防ぐため基礎疾患をもつ小児が予防接種を受けることができるよう，環境整備や十分な情報提供を行う必要が示された。

本研究の一部は第57回小児保健学会で発表した。なお，本研究は大阪大学大学院医学系研究科修士論文の一部を加筆・修正したものである。

本研究にご協力いただいた前田由美先生，伊予田邦昭先生に感謝申し上げます。

また，本研究は利益相反に関する開示事項はない。

文　　献

1) 田辺卓也，粟屋　豊，松石豊次郎，他．乳児重症ミオクロニーてんかん（SMEI）症例のワクチン接種状況調査．脳と発達　2004；36：318-323.
2) 皆川公夫．道立小児総合保健センター神経外来における重症心身障害児の予防接種状況．小児科診療　2007；70：509-512.
3) 古藤雄大，宮崎千明，前田由美，他．支援学校に在籍する小児の予防接種実施状況に関する調査（第1報）．小児保健研究　2013；72：282-288.
4) Berelson B．稲葉三千男，他訳．内容分析．第1版．東京：みすず書房，1957.
5) 岡田賢司．小・中学校におけるツベルクリン反応・BCG接種廃止と小児結核予防対策．小児保健研究　2003；62：402-404.
6) 予防接種ガイドライン等検討委員会監修．予防接種ガイドライン2013年度版．第1版．東京：予防接種リサーチセンター，2013.
7) 小野靖彦．予防接種に対する保護者の意識．日本小児科医師会会報　2007；33：145-150.
8) 大日康史，菅原民枝，三野正博，他．水痘予防接種に対する公費補助制度の政策評価．感染症学雑誌　2010；84：159-164.
9) 中山哲夫，木村慶子．保育園，幼稚園，小・中学校でのインフルエンザ対策．インフルエンザ　2001；2：137-142.
10) 木村慶子．インフルエンザワクチン集団接種の有効