



The Brazilian Journal of INFECTIOUS DISEASES

www.elsevier.com/locate/bjid



Original article

Molecular epidemiology of measles virus infection in Shanghai in 2000–2012: the first appearance of genotype D8

Shuhua Li^a, Xiaohua Qian^a, Zhengnan Yuan^b, Xiaodong Sun^b, Chongshan Li^b,
Xian Tang^a, Yanji Yang^a, Xiangzhen Gong^{a,*}, Guangwen Cao^{c,*}

^a Hongkou Center for Diseases Control and Prevention, Shanghai, China

^b Shanghai Center for Diseases Control and Prevention, Shanghai, China

^c Department of Epidemiology, Second Military Medical University, Shanghai, China

ARTICLE INFO

Article history:

Received 6 March 2014

Accepted 17 May 2014

Available online 1 October 2014

Keywords:

D8 genotype

Importation

Measles virus

Molecular epidemiology

ABSTRACT

Purpose: The purpose of this study was to identify measles virus in Shanghai in 2012 and study the genotype trend of measles virus epidemic strains during 2000–2012.

Methods: Nose and throat swab specimens were collected from 34 suspected measles cases in Shanghai. Measles virus was isolated using Vero-SLAM cells (African green monkey kidney cells/lymphoid signal activating factor-transfected African green monkey kidney cells). The 450 bp of C terminus of the N gene and the entire hemagglutinin gene sequence was amplified using RT-PCR. Phylogenetic analysis was performed by comparing the seven measles strains in Shanghai with the reference strains for H1a, H1b and D8 genotypes, as well as the Chinese measles virus vaccine strain.

Results: Seven measles viruses strains were isolated from the 34 throat swap specimens. Six strains were genotype H1a, which is the predominant strain in China and one strain was genotype D8, which is the first imported strain since 2000. All these seven strains maintained most of the glycosylation sites except subtype H1a, which lost one glycosylation site.

Conclusion: Since 2000, measles virus strains in Shanghai are consistent with measles virus from other provinces in China with H1a being the predominant genotype. This study is also the first report of genotype D8 strain in Shanghai. All strains maintained their glycosylation sites except H1a that lost one glycosylation site. These strains could still be neutralized by the Chinese measles vaccine. We suggest that Shanghai Center for Disease Control laboratories should strengthen their approaches to monitor measles cases to prevent further spread of imported strains.

© 2014 Elsevier Editora Ltda. Este é um artigo Open Access sob a licença de [CC BY-NC-ND](http://creativecommons.org/licenses/by-nc-nd/4.0/)

* Corresponding authors.

E-mail addresses: xzongsh@hotmail.com (X. Gong), gcao@smmu.edu.cn (G. Cao).

<http://dx.doi.org/10.1016/j.bjid.2014.05.018>

1413-8670/© 2014 Elsevier Editora Ltda. Este é um artigo Open Access sob a licença de [CC BY-NC-ND](http://creativecommons.org/licenses/by-nc-nd/4.0/)

Introduction

Measles is a leading cause of childhood mortality in developing countries. Although an effective measles vaccine has been available for more than 40 years and greatly reduced measles outbreak globally, risk for illness and death from measles still exist in countries with poor routine vaccination, and measles outbreaks can still be a threat in these countries.^{1,2} Measles is caused by infection of measles virus (MVs), which is an enveloped virus of the *Morbillivirus* genus in the *Paramyxoviridae* family. Since Shanghai carried out the measles surveillance in 2000, 10,684 measles cases were reported and 488 strains of MVs were monitored until 2012. In 2012, 592 measles cases were reported and 248 strains of MVs were monitored in Shanghai. Although MVs is serologically monotypic, genetic characterization of this virus has identified eight classes (A–H), which can be subdivided into 24 genotypes.^{3,4}

The distribution of each genotype is chronically and geographically patterned. For example, genotype A has circulated in the United States, United Kingdom, Russia, China, and Argentina during the last 20 years.⁵ Group B and C viruses were mostly isolated in Japan, Philippines, Micronesia⁶ and South Africa.⁷ Groups D and E viruses appear to be circulating widely in many countries in Western Europe, particularly Germany, Spain, and United Kingdom⁸; Group F viruses have been isolated in the African countries.⁹ Group G viruses were first isolated in Montreal, Canada, in 1988 and have been predominantly identified in Indonesia and Malaysia recently.¹⁰ Group H viruses form a highly distinct group isolated in four provinces within China during the early 1990s¹¹ and had then been detected in Korea and Japan.^{12–14} MVs surveillance in China since 1993 revealed that the H1 genotype is the endemic dominant strain, which has H1a, H1b and H1c subtypes, with H1a being the dominant subtype circulating in China.^{11,15–19}

To assist the goal of globally eliminating measles, it is important to carry out molecular epidemiology investigation besides classic epidemiology studies. Molecular epidemiology can support classical epidemiology by identifying transmission pathways and confirming whether the isolated virus genotype is consistent with the predominant genotype circulating in the country or whether it is an imported strain, and region from which the case was imported.^{20,21} The present study performed detailed genetic characterization on seven measles strains isolated in Shanghai, tempting to trace the source of an imported strain and eventually give suggestions on how to perform genetic surveillance on MVs in China.

Materials and methods

Sample preparation

Nose and throat swab specimens were collected from 34 suspected measles cases in Hongkou District of Shanghai between March 1, 2012 and November 1, 2012. Specimens were kept in 2 mL MEM media and stored at 4 °C to maintain viral activity. All specimens were sent to the Shanghai Municipal Center for Disease Control and Prevention within

24 h for molecular analysis. The specimens were treated with antibiotics (penicillin and streptomycin) overnight and centrifuged at 2000 rpm for 5 min at 4 °C to remove precipitation. The supernatant was preserved at –80 °C for further experiments.

Culture of Vero cells

The Vero and Vero-SLMA cells were allowed to quickly thaw into T25 flasks. Cells were cultured in a humidified air incubator at 37 °C with 5% CO₂, changing media every 2 days. Once cells reached confluency, cells were digested with 1× trypsin–EDTA at 37 °C for 5 min. Then, cells were seeded at a density of 5 × 10⁵ cells/mL into 24 well plates and incubated at 37 °C in a humidified 5% CO₂ incubator until forming a confluent monolayer.

Isolation of MVs

Vero and Vero-SLMA cells in 24 well plates were infected with 0.2–0.3 mL/well of the viral supernatant. Each viral sample was inoculated into three wells and each plate contained three control wells, which were inoculated with 0.2–0.3 mL MEM media. After 1 h of incubation at 37 °C, 1 mL of fresh MEM media were added into each well to replace virus-containing media. The cells were then cultured for 7–10 days to induce cytopathogenic effect (CPE). China measles vaccine strain S191 was used as positive control strain. Cells were observed 24 h, 48 h and 72 h after inoculation. CPE+ cells were grown for five passages. CPE– strains were discarded after three passages if they remain CPE–.

Pathological diagnosis of MVs

The MVs were identified using indirect fluorescence assay. Once the adherent cells on 24 well plates showed CPE++ – +++, the cells were scraped and plated onto 12 well slides. Two replicates were examined for each sample including control cells. Samples were allowed to air dry and then fixed with cold acetone–methanol (1:1, v/v) solution. Assays were performed according to the manufacturer's instructions of immunofluorescence antibody (IFA) assay kit and peripheral fluorescent signal was detected by fluorescence microscopy for IFA+.

Viral RNA extraction and RT-PCR

The second passages of CPE+ cells were collected and RNA was extracted using TIANamp virus RNA Kit (TianGen biotech, DP315-R) according to the manufacturer's instruction. The primers were designed according to the entire hemagglutinin (HA) gene and the 450 bp C terminus region of the nucleocapsid (N) gene using primer 5.0 software. The primers were synthesized by Shanghai Biological Engineering Technology Co., Ltd. and these sequences of the primers are listed in Table 1.

RT-PCR was performed using one step RT-PCR kit (TianGen Biotech). The one step RT-PCR was carried out in a 50 µL reaction volume containing 2 µL template RNA, 5 µL 10× reaction buffer, 2 µL dNTP Mix (10 mM each), 10 µL 5× RT-PCR enhancer, 0.5 µL RNasin (40 U/µL), 1 µL HotmasterTaq

Table 1 – Primers to amplify the C terminus 450bp of the measles virus N gene.

Gene	Primer	Position	Sequence (5'–3')	Fragment (bp)
N	N-P1	1198–1219	TAGGGCAAGAGATGGTAAGGAG	568
	N-P2	1745–1765	TGTGTGGACTGGTTCCTAAG	
HA	H1-P1	7244–7265	AAAACCTTAGGGTGCAAGATCAT	845
	H1-P2	8070–8089	TGTCATATGGAACACCGGAG	
	H2-P1	7904–7925	CGAGGTTACAATGTGTCATCTA	677
	H2-P2	8560–8580	TGTGTGATCAATGGCCCGAAT	
	H3-P1	8488–8507	GATTCCCTTCATACGGGGTCT	730
	H3-P2	9195–9217	GACCCTACGTTTTTCTTAATTCT	

polymerase (2.5 U/ μ L), 0.5 μ L Quant RTase (for one step), 3 μ L upstream primer (10 μ M), 3 μ L downstream primer (10 μ M), and 23 μ L nuclease free water. The reaction mixture was amplified at the following conditions: reverse transcription at 50 °C for 30 min, initial denaturation at 94 °C for 2 min, followed by 40 cycles at 94 °C for 0.5 min, 56–61 °C for 1 min, 65 °C for 2 min, and a final step at 65 °C for 10 min.

DNA sequencing and genetic characterization

Amplification products were separated by electrophoresis using 1.5% agarose gel containing 0.5 μ g/mL ethidium bromide, and imaged on Gel Doc™ XR System (Bio-Rad, Hercules, CA). PCR products were purified and sequenced on an ABI 3730XL automated sequencer in Maipu biotech, Shanghai. Sequencing results were analyzed by Chromas software.

The isolated virus strains were characterized according to the World Health Organization (WHO) recommended “MV's genotype identification standard method”.²² Sequences of 23 MVs representative strains and the sequence of Chinese measles vaccine strain, S191 were downloaded from GenBank (Table S1). Target strain sequence and existing sequences in 2000–2012 in Shanghai were analyzed using the Molecular Evolutionary Genetics Analyses (MEGA) software version 4.0.²³ The reliability of the analyses was estimated using 1000 bootstrap replicates.

Supplementary Table S1 related to this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.bjid.2014.05.018>.

Results

Virus isolation and pathological analysis

Thirty-four suspected measles cases were reported in 2012, and 27 cases were confirmed in the research lab and one case was confirmed by clinical diagnosis. The other six cases (rubella or other cases) were excluded for this study. Throat swab specimens were taken from 34 patients and seven of them showed CPE+ after infecting Vero-SLMA cells (Fig. 1A and B). The CPE+ cells showed positive peripheral fluorescent signal by IFA, confirming that the isolated virus were MVs (Fig. 1C and D). The epidemiological information of the seven patients is listed in Table 2. According to the WHO systematic nomenclature for MVs, the seven isolated viruses were named and their short names were SHHK2012-01, SHHK2012-02, SHHK2012-03, SHHK2012-04, SHHK2012-05, SHHK2012-06,

SHHK2012-07 in this study. RT-PCR showed 568 bp for N gene, and 845 bp, 677 bp, and 730 bp for HA gene (Fig. 2), which are all the characteristic bands for MVs.

Genetic analysis for measles strains

NCBI search revealed 23 MVs reference strains²⁴ and 14 measles subtype reference strains¹¹ in China between 1993 and 1994. Phylogenetic trees were constructed by comparing the C terminal 450bp of N gene (Fig. 3A) and entire HA gene (Fig. 3B) of these reference strains with those of the seven strains isolated in Shanghai in 2012. Among the 14 strains isolated in 1993–1994, 12 belong to the H1 gene family, one (China93-5) belongs to the A gene family, and one (China94-1) belongs to the H2 gene family. Among the 12 H1 strains, China93-2 and China93-4 were H1a subtype; China94-7 is H1b subtype; China93-1, China93-3, China93-6, China93-7, China94-2, China94-3, China 94-4, China 94-5 and China94-6 are H1c subtypes. The China93-7 (AF045212) and China94-1 (AF045217) are standard reference strains for H1 and H2 genotypes, respectively.

Analysis on the N gene sequence indicated that the seven strains isolated in 2012 are not identical. The gene sequences of three strains including SHHK2012-01, SHHK2012-03 and SHHK2012-04 in the same group are identical to that of MVs/HongKong.CHN/49.12 (KC417295.1). The gene sequences of other three strains, including SHHK2012-02, SHHK2012-05 and SHHK2012-06 in another group, were identical. Only one base pair is different between the gene sequences of the two groups (499/450). The difference between the N gene sequences of these six strains and the H1 reference strain Hunan.CHN/93/7 (china93-7, AF045212) is 2.22–2.45% (440/450–439/450, < 2.5%). The difference is 6.67% (420/450) when compared with the H2 reference strain china94-1 (AF045217) and 7.11–11.33% (418/450–399/450) when compared with the other 21 representative strains (Table S1). Therefore, according to the WHO reference sequence for each genotype, these six strains belong to the H1 genotype.²⁴

The sequence of the seventh strain SHHK2012-07 varies from the other six strains by 8.89–9.11% (410/450–409/450), suggesting that the seven strains do not belong to the same gene family. However, SHHK2012-07 is identical to strains MVs/New-York.USA/37.12 [D8] (gb|KC492072.1), MVs/HongKong.CHN/46.12 [D8] (gb|KC288107.1), and MVs/Taunton.GBR/27.12/[D8] (gb|JX984461.1) (450/450), which were isolated in 2012 from USA, Hong Kong and England, respectively. The difference between SHHK2012-07 and the D8 genotype reference strain Manchester.UNK/30.94

Table 2 – Information of the patient infected by the seven measles strains.

Number	Gender	Age	Occupation	Residence	Onset of disease	Date of diagnosis	Type of diagnosis	Length of residence	Vaccine history	Source of vaccine history	Nomenclature	NCBI accession number	Abbreviations	Genotype
1	M	26	Government staff	Local resident	2012/8/6	2012/8/10	Laboratory diagnosis	>3 months	Unavailable	Parent memory	MVi/shanghai .CHN/30.12/03	Unregistered	SHHK2012-01	H1
2	M	26	Business	Other province	2012/7/26	2012/7/29	Laboratory diagnosis	>3 months	Unavailable	Parent memory	MVi/shanghai .CHN/30.12/04	Unregistered	SHHK2012-02	H1
3	M	36	Food industry	Local resident	2012/8/16	2012/8/19	Laboratory diagnosis	>3 months	Unavailable	Parent memory	MVi/shanghai .CHN/32.12/05	Unregistered	SHHK2012-03	H1
4	F	36	Housekeeping or unemployed	Local resident	2012/8/8	2012/8/12	Laboratory diagnosis	>3 months	Unavailable	Parent memory	MVi/shanghai .CHN/32.12/06	Unregistered	SHHK2012-04	H1
5	F	36	Government staff	Local resident	2012/7/22	2012/7/29	Laboratory diagnosis	>3 months	Unavailable	Parent memory	MVi/shanghai .CHN/33.12/06	Unregistered	SHHK2012-05	H1
6	F	42	Housekeeping or unemployed	Other province	2012/8/17	2012/8/20	Laboratory diagnosis	>3 months	Unavailable	Parent memory	MVi/shanghai .CHN/33.12/07	Unregistered	SHHK2012-06	H1
7	F	1	Children	Local resident	2012/5/23	2012/5/29	Laboratory diagnosis	>3 months	Unvaccinated	Vaccine record	MVi/shanghai .CHN/40.12/01	Unregistered	SHHK2012-07	D8

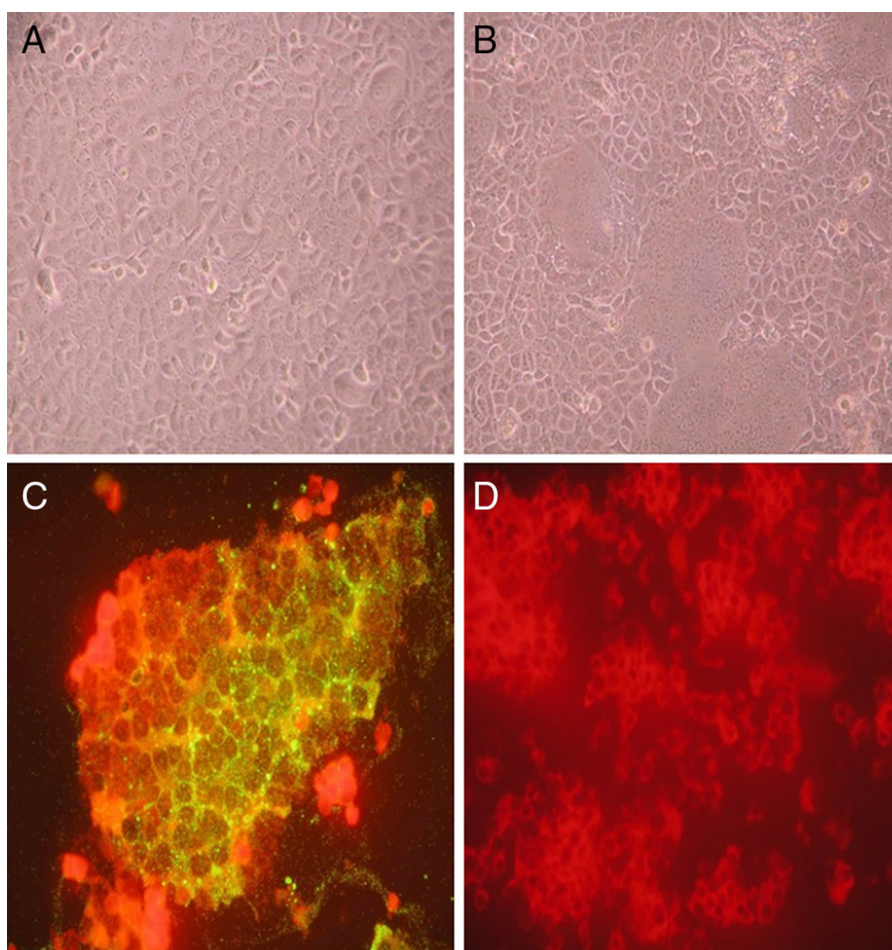


Fig. 1 – Isolation and pathological diagnosis of measles virus by cytopathic effect (CPE) and indirect fluorescence assay. Vero-SLAM cells were infected with control cell culture media (A, D) and virus collected from throat swabs (B, C) for 72 h. Cells infected with virus collected from throat swabs showed obvious CPE (B) while cells infected with control media were normal (A). Indirect fluorescence assay was performed on cells infected with control virus and throat swab material. Viral infected cells showed peripheral green fluorescence staining (C) which was absent in control cells (D).

(AF280803) is only 2.22% (440/450), and the difference is 4.89% (428/450)–10.0% (405/450) when compared to reference strains from other genotypes. Therefore, we conclude that the SHHK2012-07 belongs to the D8 gene family.

The six strains that belong to the H1 gene family were also compared with reference strains of H1 subtypes. The difference between the six strains with H1a reference strains (China93-2, China93-4) is 1.1–1.3% (445/450–444/450),

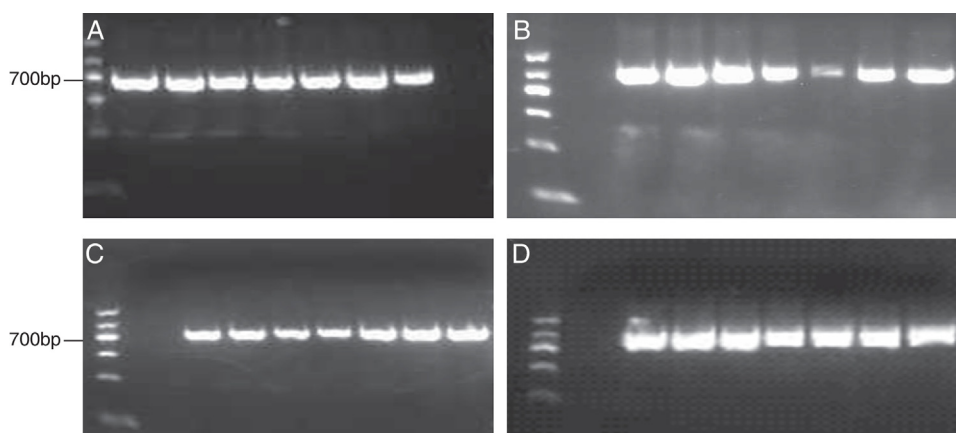


Fig. 2 – RT-PCR amplification products were separated by electrophoresis on 1.5% agarose gel. All seven strains have the characteristic bands, which are 568 bp for N gene (A), 845 bp for H1 (B), 677 bp for H2 (C), and 730 bp for H3 (D).

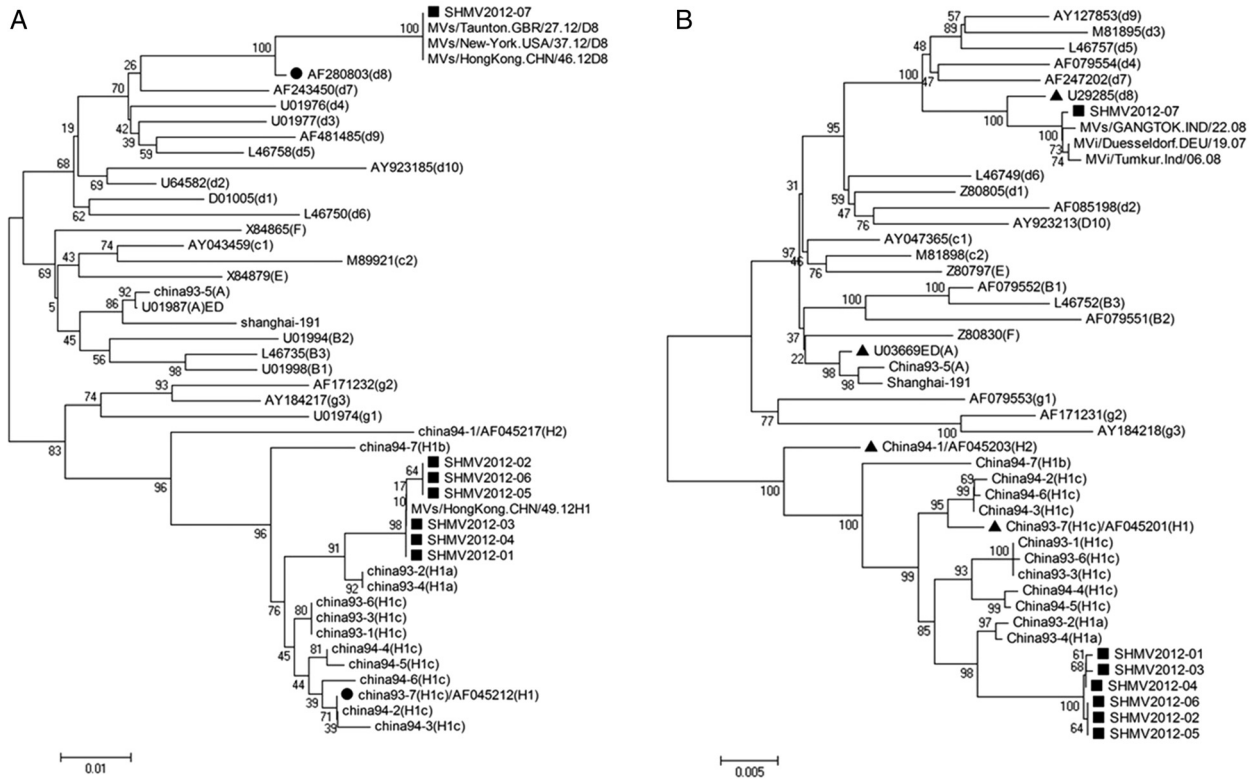


Fig. 3 – Phylogenetic analysis of MVs genotype reference strains and the seven measles strains in Shanghai in 2012 using the C terminal 450 bp of N gene (A) and sequence the entire HA gene (B). The dendrogram was constructed using MEGA 4 software. Bootstrap confidence limits were based on 1000 replicates. Numbers at the nodes indicate bootstrapping values. Reference strains of each genotype and subtype are indicated by black triangles. Strains isolated in Shanghai in 2012 are indicated with black squares. D8 and H1c reference strains are indicated by black circles.

3.33–3.11% (435/450–436/450) when compared with H1b subtype (China94-7), and 2.22–2.89% (440/450–437/450) when compared with H1c subtype (China93-7). Because the sequence difference between subtypes was less than 2%, we conclude that these six strains belong to the H1a subtype.

In addition to the nucleotide sequence comparison with the N gene, we also evaluated the differences in entire HA gene sequences of the seven new isolated strains and of the reference strains. Analysis on HA gene sequences demonstrated that there was only 0.1% (1852/1854) variation among the six H1 genotype strains. The difference is 2.10% (1815/1854) when compared with China93-7 (Hunan.CHN/93/7, AF045201), 3.13% (1796/1854) when compared to H2 reference strain China94-1 (Beijing.CHN/94/1, AF045203), and 5.0–7.0% when compared with the other 21 strains (Table S1).

SHHK2012-07 differs with the other six strains by 6.58–6.63% (1732/1854–1731/1854), by 0.86% (1838/1854) when compared to D8 reference strain Manchester.UNK/30.94 (U29285), and by 2.21% (1813/1854)–5.88% (1745/1854) when compared to other 22 D8 strains.

The six H1 strains belong to the same branch with the 12 H1 subtypes from 1993 to 1994. Phylogenetic analysis indicated that these six strains vary by 1.24–1.18% (1831/1854–1832/1854) when compared with H1a reference strains (China93-2, China93-4), by 2.75% (1803/1854) when

compared to H1b subtype (China94-7) and by 2.26–1.89% (1812/1854–1819/1854) when compared to H1c strain (China93-7). Therefore, these six strains belong to H1a subtype (Table S1).

Phylogenetic analysis on MVs strain in Shanghai in 2000–2012

NCBI search resulted in 104 measles strains during the 2000–2012 in Shanghai. Phylogenetic analysis was performed for these 104 strains and the 14 strains isolated between 1993 and 1994, as well as the Chinese measles vaccine strain S191 (Fig. 4A). Phylogenetic analysis demonstrated that 103 of the 104 strains are genotype H1, while one strain was genotype D8. Among the 103 H1 strains, 97 belong to the H1a subtype. These 97 strains belong to the same cluster with China93-2 and China93-4 with the internal variation of 0–2.22% (440/450). Another six strains together with China94-7 belong to the H1b subtype with an internal variation of 2.45–0.67% (439/450–447/450). Although there are nine H1c subtypes isolated in 1993–1994 with the internal variation of 0–1.33% (444/450–450/450), there was no H1c strain in 2000–2012, suggesting that these strains might have been eliminated in Shanghai. The 97 H1a strains can be further separated into two clusters: 62 strains belong to cluster1 and 35 strains belong to cluster2.

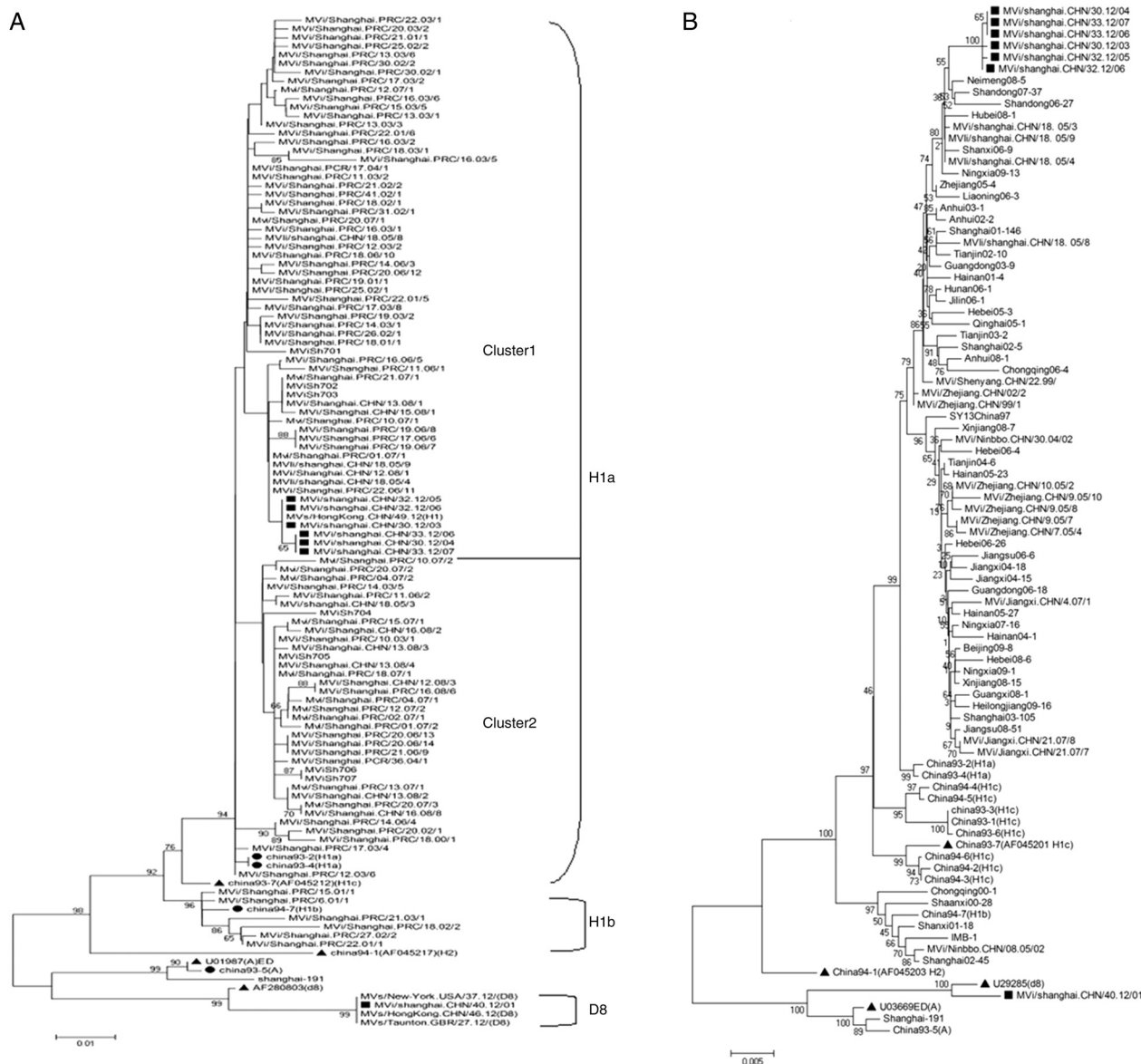


Fig. 4 – Phylogenetic analysis of the C terminal 450 bp of N gene sequence (A) and the entire HA gene sequence (B) of 104 measles strains isolated in Shanghai during 2000–2012, the 14 strains between 1993 and 1994, and the Chinese measles vaccine strain S191. The dendrogram was constructed using MEGA 4 software. Bootstrap confidence limits were based on 1000 replicates. Numbers at the nodes indicate bootstrapping values. Reference strains of each genotype and subtype are indicated by black triangles. Strains isolated in Shanghai in 2012 are indicated with black squares. H1 reference strains are indicated by black circles.

Comparison between the epidemic strain and the Chinese measles vaccine strain

We obtained the HA gene sequences of 65 MVs in China. Using these sequences, we constructed phylogenetic trees for the seven strains in this study, 14 strains in 1993–1994 and the Chinese measles vaccine strain S191 (Fig. 4B).

All 71 H1 genotypes were from 25 provinces in China among which 65 strains were in the same group of China93-4 and China93-2 as H1a subtype with internal variation of 0–2.05% (1816/1854–1854/1854). Another six strains were in the same group of H1b reference strain China94-7 with an

internal variation of 0.38–0.92% (1937/1854–1847/1854). The nine strains from 1993 to 1994 were the only cases of H1c subtype with internal variation of 0.05–1.56% (1825/1854–1853/1854), consistent with the conclusion that the H1c subtype might have been eliminated.

The only D8 virus differs from the H1 genotype by 6.63–5.99% (1731/1854–1743/1854) and differs with the Chinese measles vaccine strain S191 by 4.53–5.61% (1770/1854–1750/1854), confirming that this strain does not belong to H1 strain, but rather forms a separate cluster by itself.

In order to determine whether the current MVs vaccine is able to protect against the newly imported D8 strain, we

	*** ** ** ** **
Shanghai-191	ELMNALVNSTLLETRITNQLAVSKGNCSGPTTIRGQFSNMSSLDDLYLGRGYNVSSIVTMTSQGMYGGTYLVEKPNLSSKRSELSQ
China93-2 (H1a)	ELMNALVNSTLLETRITNQLAVSKGNCSGPTTIRGQFSNMSSLDDLYLGRGYNVSSIVTMTSQGMYGGTYLVEKPNLSSKRSELSQ
China93-4 (H1a)	ELMNALVNSTLLETRITNQLAVSKGNCSGPTTIRGQFSNMSSLDDLYLGRGYNVSSIVTMTSQGMYGGTYLVEKPNLSSKRSELSQ
Shanghai01-146	ELMNALVNSTLLETRITNQLAVSKGNCSGPTTIRGQFSNMSSLDDLYLGRGYNVSSIVTMTSQGMYGGTYLVEKPNLSSKRSELSQ
Shanghai02-5	ELMNALVNSTLLETRITNQLAVSKGNCSGPTTIRGQFSNMSSLDDLYLGRGYNVSSIVTMTSQGMYGGTYLVEKPNLSSKRSELSQ
Shanghai03-105	ELMNALVNSTLLETRITNQLAVSKGNCSGPTTIRGQFSNMSSLDDLYLGRGYNVSSIVTMTSQGMYGGTYLVEKPNLSSKRSELSQ
MVi/shanghai.CHN/18.05/4	ELMNALVNSTLLETRITNQLAVSKGNCSGPTTIRGQFSNMSSLDDLYLGRGYNVSSIVTMTSQGMYGGTYLVEKPNLSSKRSELSQ
MVi/shanghai.CHN/30.12/03	ELMNALVNSTLLETRITNQLAVSKGNCSGPTTIRGQFSNMSSLDDLYLGRGYNVSSIVTMTSQGMYGGTYLVEKPNLSSKRSELSQ
MVi/shanghai.CHN/33.12/07	ELMNALVNSTLLETRITNQLAVSKGNCSGPTTIRGQFSNMSSLDDLYLGRGYNVSSIVTMTSQGMYGGTYLVEKPNLSSKRSELSQ
China94-7 (H1b)	ELMNALVNSTLLETRITNQLAVSKGNCSGPTTIRGQFSNMSSLDDLYLGRGYNVSSIVTMTSQGMYGGTYLVEKPNLSSKGLGLSQ
Shanxi01-18	ELMNALVNSTLLETRITNQLAVSKGNCSGPTTIRGQFSNMSSLDDLYLGRGYNVSSIVTMTSQGMYGGTYLVEKPNLSSKGLGLSQ
Shanghai02-45	ELMNALVNSTLLETRITNQLAVSKGNCSGPTTIRGQFSNMSSLDDLYLGRGYNVSSIVTMTSQGMYGGTYLVEKPNLSSKGLGLSQ
Chongqing00-1	ELMNALVNSTLLETRITNQLAVSKGNCSGPTTIRGQFSNMSSLDDLYLGRGYNVSSIVTMTSQGMYGGTYLVEKPNLSSKGLGLSQ
China93-1 (H1c)	ELMNALVNSTLLETRITNQLAVSKGNCSGPTTIRGQFSNMSSLDDLYLGRGYNVSSIVTMTSQGMYGGTYLVEKPNLSSKGLGLSQ
China94-3 (H1c)	ELMNALVNSTLLETRITNQLAVSKGNCSGPTTIRGQFSNMSSLDDLYLGRGYNVSSIVTMTSQGMYGGTYLVEKPNLSSKGLGLSQ
China94-6 (H1c)	ELMNALVNSTLLETRITNQLAVSKGNCSGPTTIRGQFSNMSSLDDLYLGRGYNVSSIVTMTSQGMYGGTYLVEKPNLSSKGLGLSQ
AF045201 (H1.China93-7)	ELMNTALVNSTLLETRITNQLAVSKGNCSGPTTIRGQFSNMSSLDDLYLGRGYNVSSIVTMTSQGMYGGTYLVEKPNLSSKRSELSQ
AF045203 (H2.China94-1)	ELMNALVNSTLLETRITNQLAVSKGNCSGPTTIRGQFSNMSSLDDLYLGRGYNVSSIVTMTSQGMYGGTYLVEKPNLSSKRSELSQ
AF079552 (B1)	ELMNALVNSTLLETRITNQLAVSKGNCSGPTTIRGQFSNMSSLDDLYLGRGYNVSSIVTMTSQGMYGGTYLVEKPNLSSKRSELSQ
AF079551 (B2)	ELMNALVNSTLLETRITNQLAVSKGNCSGPTTIRGQFSNMSSLDDLYLGRGYNVSSIVTMTSQGMYGGTYLVEKPNLSSKRSELSQ
L46752 (B3)	ELMNALVNSTLLETRITNQLAVSKGNCSGPTTIRGQFSNMSSLDDLYLGRGYNVSSIVTMTSQGMYGGTYLVEKPNLSSKRSELSQ
AJ239133 (B3)	ELMNALVNSTLLETRITNQLAVSKGNCSGPTTIRGQFSNMSSLDDLYLGRGYNVSSIVTMTSQGMYGGTYLVEKPNLSSKRSELSQ
AY047365 (C1)	ELMNALVNSTLLETRITNQLAVSKGNCSGPTTIRGQFSNMSSLDDLYLGRGYNVSSIVTMTSQGMYGGTYLVEKPNLSSKRSELSQ
M81898 (C2)	ELMNALVNSTLLETRITNQLAVSKGNCSGPTTIRGQFSNMSSLDDLYLGRGYNVSSIVTMTSQGMYGGTYLVEKPNLSSKRSELSQ
Z80808 (C2)	ELMNALVNSTLLETRITNQLAVSKGNCSGPTTIRGQFSNMSSLDDLYLGRGYNVSSIVTMTSQGMYGGTYLVEKPNLSSKRSELSQ
Z80805 (D1)	ELMNALVNSTLLETRITNQLAVSKGNCSGPTTIRGQFSNMSSLDDLYLGRGYNVSSIVTMTSQGMYGGTYLVEKPNLSSKRSELSQ
AF085198 (D2)	ELMNALVNSTLLETRITNQLAVSKGNCSGPTTIRGQFSNMSSLDDLYLGRGYNVSSIVTMTSQGMYGGTYLVEKPNLSSKRSELSQ
M81895 (D3)	ELMNALVNSTLLETRITNQLAVSKGNCSGPTTIRGQFSNMSSLDDLYLGRGYNVSSIVTMTSQGMYGGTYLVEKPNLSSKRSELSQ
AF079554 (D4)	ELMNALVNSTLLETRITNQLAVSKGNCSGPTTIRGQFSNMSSLDDLYLGRGYNVSSIVTMTSQGMYGGTYLVEKPNLSSKRSELSQ
AF009575 (D5)	ELMNALVNSTLLETRITNQLAVSKGNCSGPTTIRGQFSNMSSLDDLYLGRGYNVSSIVTMTSQGMYGGTYLVEKPNLSSKRSELSQ
L46757 (D5)	ELMNALVNSTLLETRITNQLAVSKGNCSGPTTIRGQFSNMSSLDDLYLGRGYNVSSIVTMTSQGMYGGTYLVEKPNLSSKRSELSQ
L46749 (D6)	ELMNALVNSTLLETRITNQLAVSKGNCSGPTTIRGQFSNMSSLDDLYLGRGYNVSSIVTMTSQGMYGGTYLVEKPNLSSKRSELSQ
AF247202 (D7)	ELMNALVNSTLLETRITNQLAVSKGNCSGPTTIRGQFSNMSSLDDLYLGRGYNVSSIVTMTSQGMYGGTYLVEKPNLSSKRSELSQ
AY043461 (D7)	ELMNALVNSTLLETRITNQLAVSKGNCSGPTTIRGQFSNMSSLDDLYLGRGYNVSSIVTMTSQGMYGGTYLVEKPNLSSKRSELSQ
U29285 (D8)	ELMNALVNSTLLETRITNQLAVSKGNCSGPTTIRGQFSNMSSLDDLYLGRGYNVSSIVTMTSQGMYGGTYLVEKPNLSSKRSELSQ
MVi/shanghai.CHN/40.12/01 (D8)	ELMNALVNSTLLETRITNQLAVSKGNCSGPTTIRGQFSNMSSLDDLYLGRGYNVSSIVTMTSQGMYGGTYLVEKPNLSSKRSELSQ
AY127853 (C9)	ELMNALVNSTLLETRITNQLAVSKGNCSGPTTIRGQFSNMSSLDDLYLGRGYNVSSIVTMTSQGMYGGTYLVEKPNLSSKRSELSQ
AF079553 (G1)	ELMNALVNSTLLETRITNQLAVSKGNCSGPTTIRGQFSNMSSLDDLYLGRGYNVSSIVTMTSQGMYGGTYLVEKPNLSSKRSELSQ
AF171231 (G2)	ELMNALVNSTLLETRITNQLAVSKGNCSGPTTIRGQFSNMSSLDDLYLGRGYNVSSIVTMTSQGMYGGTYLVEKPNLSSKRSELSQ
AY184218 (G3)	ELMNALVNSTLLETRITNQLAVSKGNCSGPTTIRGQFSNMSSLDDLYLGRGYNVSSIVTMTSQGMYGGTYLVEKPNLSSKRSELSQ
U03669ED (A)	ELMNALVNSTLLETRITNQLAVSKGNCSGPTTIRGQFSNMSSLDDLYLGRGYNVSSIVTMTSQGMYGGTYLVEKPNLSSKRSELSQ
Z80797 (E)	ELMNALVNSTLLETRITNQLAVSKGNCSGPTTIRGQFSNMSSLDDLYLGRGYNVSSIVTMTSQGMYGGTYLVEKPNLSSKRSELSQ
Z80830 (F)	ELMNALVNSTLLETRITNQLAVSKGNCSGPTTIRGQFSNMSSLDDLYLGRGYNVSSIVTMTSQGMYGGTYLVEKPNLSSKRSELSQ

Fig. 5 – The location and mutations of the glycosylation site in measles strains isolated in Shanghai compared to the H1c subtype reference strains and the S191 vaccine strain. The H1 strains isolated in Shanghai lost glycosylation site at 238–240 (N-L-S) whereas the D8 strain maintained this glycosylation site.

compared the amino acid sequences besides the nucleotide analyses. There was 0–3.73% (594/617) difference within the 71 H1 strains. The variation between these strains with D8 was 6.32–4.53% (578/617-589/617) and 5.51–3.89% (583/618-593/618) when compared with the S191 vaccine strain.

Glycosylation of the HA gene is important for neutralization of the antigenic sites,²⁵ proper folding of the proteins, and has big influence on the antigenicity and immunogenicity of the HA glycoprotein.^{26,27} In order to understand whether the current Chinese measles vaccine can induce protection against the seven isolated MVs strains, we compared the locations and mutations of the glycosylation sites. The results indicated that the serine (S) 240 of the H1a subtype was mutated to asparagine (N) by a single point mutation of adenine 719 to guanine, which destroyed one glycosylation site, while this site was maintained in H1b, H1c subtype and in genotype D8 (Fig. 5). When we examine the reference strain for the other 21 genotypes, we found that all strains maintained five N glycosylation sites except for genotype D5, B3 and G3. Genotype D5 lost the glycosylation site at 169, while B3 and G3

lost a glycosylation site at 238. Based on the analysis on the glycosylation site, we predict that the Chinese measles vaccine is still able to induce protection against the predominant strains as well as against the 22 imported strains.

Description of the D8 genotype appearance

Epidemiological surveys show that the D8 strain infected patient was a female child born in Shanghai on January 26, 2012. The patient started to have fever in the morning on September 28, 2012, who was later diagnosed of “suspected measles” at Fudan University Children’s Hospital. This case was reported through the Internet on October 2nd at 2:34 pm. Throat swab and serum specimens were sent to the laboratory for virus testing. On October 9th, collected serum turned out positive for measles IgM antibody. Shanghai Center for Disease Control (CDC) reported positivity for MVs on November 6th and further examination at Chinese Center for Disease Control confirmed that the MVs was of genotype D8 on December 24th.

The father of the child started presenting with fever in the morning on September 18th, 2012. He was also diagnosed as "suspected measles" by Shanghai Public Health Clinical Center at 14:00 on September 23 and the patient was immediately isolated for treatment. Serological testing turned out positive for measles IgM antibodies on September 24th. The patient was hospitalized until September 28th to collect throat swab specimens. Before the onset of fever, the child patient had never traveled overseas. Her father, however, did two business trips between Shanghai and South Korea 7–21 days before onset of his disease. He could have potentially got in contact with the virus on public transportations (such as airplane or airport shuttles) where he came across many international tourists. The child might have been infected by MVs from her father who had imported the virus on his business trips.

Discussion

Molecular epidemiological surveillance of MVs is an important part of global measles control and elimination, which can be achieved by monitoring the distribution of genotypes in different countries and different regions of the same country. The present study performed detailed molecular characterization on seven measles strains identified in Shanghai during 2000–2012. Through the detailed analysis of the HA and C terminus 450bp of the N gene, we concluded that six of the seven measles strains belong to the H1 genotype and H1a subtype. The remaining strain was of genotype D8. There was no H1c strain in 2000–2012, suggesting that this strain might have been eliminated in Shanghai. This trend is consistent throughout the country.^{15–19} China started surveillance for MVs in 1993.¹⁷ According to recent studies on circulation of MVs strains in China, H1c might have been eliminated because the last cases of H1c subtype occurred in 1993–1994 and there has been no further report of H1c strain. However, H1a and H1b were co-circulating between 1993 and 2005. The circulation of H1b already started to decrease following vaccination in 2005, and only H1a has remained as the predominant strain in China.¹⁷ The virus strain isolated in Shanghai is consistent with the geographic and genotypic distributions in other provinces of China, suggesting that the current measles vaccine should be sufficient to protect people in Shanghai and district specific immunization is not necessary at the current stage.

This study is the first report for the occurrence of a D8 strain in Shanghai. D8 MVs is predominantly circulating in Bangladesh, India and Nepal.^{28,29} There have also been D8 import reports in the USA, UK, Australia, and Central Europe since 2000. Because of the wide distribution of the D8 virus, it is difficult to determine the original source of the D8 strain. In China, there have only been four genotypes of imported MVs cases previously. One H2 genotype was detected in Beijing in 1994, one D9 genotype in Sichuang in 2009, one D4 case from Shanxi, and one D11 case in Yunnan. Following detection of the last case in Yunan a severe measles outbreak has ensued in the subsequent 2 years.^{30–33} Although there have been several reports on MVs analysis in Shanghai in the past few years,^{34,35} this is the first time that the MVs epidemic analysis went through a 12-year span and provided important information on MVs control.

Glycosylation of the HA gene, which is important for neutralization of the antigenic sites²⁵ and proper folding of the proteins, has big influence on the antigenicity and immunogenicity of the HA glycoprotein.²⁶ For example, the Edmonston MVs vaccine strain in 1960 contains five N linked glycosylation sites.²⁷ These sites are located at the N terminus 168–170 (NST), 187–189 (NCS), 200–202 (NMS), 215–217 (NVS) and 238–240 (NLS).^{27,36} Mutations at the glycosylation sites will cause instability and structural change on HA protein and influence its immunogenicity. Analysis on amino acid sequences revealed that most of the glycosylation sites are maintained except for H1a subtype containing one amino acid mutation. Although mutations at the glycosylation sites have big impact on antigenicity,^{25,27} H1a subtypes and D8 strain both maintained most of the glycosylation sites, suggesting that the current vaccine can still induce protection against the D8 imported strain.

However, the importance of detecting an imported strain cannot be neglected because imported strains caused severe measles outbreak during the past 20 years.^{37–40} China has committed to eliminate endemic MVs transmission by 2012.⁴¹ It is critical to investigate the genotype of circulating MVs in order to control the spread of imported strains.

In conclusion, the present study performed detailed molecular characterization on seven MVs strains in Shanghai in 2012. Six strains belonged to the H1a subtype, which is consistent with the predominant strain in other provinces in China. One strain was genotype D8 and was detected for the first time in China. These strains contain most of the N linked glycosylation sites. Therefore, the current measles vaccines used in China can induce protection against these strains.

Shanghai as an international metropolis serves as one of the major trading ports and gateways for all over the world. The fast economic developments make Shanghai not only a diversified international culture exchange center, but also make the city vulnerable for imported virus strains and make it challenging for diseases control at the same time. With the development of molecular biology techniques, it is entirely feasible to carry out gene sequencing and molecular characterization of MVs surveillance in regional laboratories. This can help strengthen the measles laboratory monitoring network and improve monitoring efficiency.

Funding

This work was supported by grants from: 1. the Excellent Academic Leaders of Shanghai public Health field (No.GWDTR201225). 2. the Shanghai Municipal Health & Family planning Commission (No.20114067).

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgements

The authors thank the work team of Department of Immunization programs (District Center for Diseases Control and Prevention of Hongkou, Shanghai, China) for collecting

measles swabs samples and epidemiological investigations of cases.

REFERENCES

1. Featherstone DA, Rota PA, Icenogle J, et al. Expansion of the global measles and rubella laboratory network 2005–09. *J Infect Dis.* 2011;204 Suppl. 1:S491–8.
2. World Health Organization. Global measles and rubella laboratory network – update. *Wkly Epidemiol Rec.* 2005;80:384–8.
3. Kremer JR, Brown KE, Jin L, et al. High genetic diversity of measles virus. World Health Organization European Region, 2005–2006. *Emerg Infect Dis.* 2008;14:107–14.
4. Update of the nomenclature for describing the genetic characteristics of wild-type measles viruses: new genotypes and reference, strains. *Relev Epidemiol Hebd/Sect d'hygiene Secret Societe Nations.* 2003;78:229–32.
5. Bellini WJ, Rota PA. Genetic diversity of wild-type measles viruses: implications for global measles elimination programs. *Emerg Infect Dis.* 1998;4:29–35.
6. Guris D, Auerbach SB, Vitek C, et al. Measles outbreaks in Micronesia, 1991 to 1994. *Pediatr Infect Dis J.* 1998;17:33–9.
7. Smit SB, Hardie D, Tiemessen CT. Measles virus genotype B2 is not inactive: evidence of continued circulation in Africa. *J Med Virol.* 2005;77:550–7.
8. Rima BK, Earle JA, Yeo RP, et al. Temporal and geographical distribution of measles virus genotypes. *J Gen Virol.* 1995;76 Pt 5:1173–80.
9. Christensen LS, Scholler S, Schierup MH, Vestergaard BF, Mordhorst CH. Sequence analysis of measles virus strains collected during the pre- and early-vaccination era in Denmark reveals a considerable diversity of ancient strains. *APMIS.* 2002;110:113–22.
10. Rota PA, Liffick S, Rosenthal S, Heriyanto B, Chua KB. Measles genotype G2 in Indonesia and Malaysia. *Lancet.* 2000;355:1557–8.
11. Xu W, Tamin A, Rota JS, Zhang L, Bellini WJ, Rota PA. New genetic group of measles virus isolated in the People's Republic of China. *Virus Res.* 1998;54:147–56.
12. Okafuji T, Okafuji T, Fujino M, Nakayama T. Current status of measles in Japan: molecular and seroepidemiological studies. *J Infect Chemother.* 2006;12:343–8.
13. Na BK, Lee JS, Shin GC, et al. Sequence analysis of hemagglutinin and nucleoprotein genes of measles viruses isolated in Korea during the 2000 epidemic. *Virus Res.* 2001;81:143–9.
14. Riddell MA, Rota JS, Rota PA. Review of the temporal geographical distribution of measles virus genotypes in the prevaccine and postvaccine eras. *Virol J.* 2005;2:87.
15. Zhang Y, Ji Y, Jiang X, et al. Genetic characterization of measles viruses in China, 2004. *Virol J.* 2008;5:120.
16. Zhang Y, Xu S, Wang H, et al. Single endemic genotype of measles virus continuously circulating in China for at least 16 years. *PLOS ONE.* 2012;7:e34401.
17. Zhang Y, Zhu Z, Rota PA, et al. Molecular epidemiology of measles viruses in China, 1995–2003. *Virol J.* 2007;4:14.
18. Ji Y, Zhang Y, Xu S, et al. Measles resurgence associated with continued circulation of genotype H1 viruses in China, 2005. *Virol J.* 2009;6:135.
19. Ji Y, Xu S, Zhang Y, et al. Genetic characterization of wild-type measles viruses isolated in China, 2006–2007. *Virol J.* 2010;7:105.
20. Rota JS, Heath JL, Rota PA, et al. Molecular epidemiology of measles virus: identification of pathways of transmission and implications for measles elimination. *J Infect Dis.* 1996;173:32–7.
21. Rota PA, Liffick SL, Rota JS, et al. Molecular epidemiology of measles viruses in the United States, 1997–2001. *Emerg Infect Dis.* 2002;8:902–8.
22. World Health Organization. Update of the nomenclature for describing the genetic characteristics of wild-type measles viruses: new genotypes and reference, strains. *Wkly Epidemiol Rec.* 2003;78:229–32.
23. Tamura K, Dudley J, Nei M, Kumar S. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Mol Biol Evol.* 2007;24:1596–9.
24. Update of the nomenclature for describing the genetic characteristics of wild-type measles viruses: new genotypes and reference, strains. *Wkly Epidemiol Rec.* 2003;78:229–32.
25. Hu A, Cattaneo R, Schwartz S, Norrby E. Role of N-linked oligosaccharide chains in the processing and antigenicity of measles virus haemagglutinin protein. *J Gen Virol.* 1994;75 Pt 5:1043–52.
26. Panda A, Elankumaran S, Krishnamurthy S, Huang Z, Samal SK. Loss of N-linked glycosylation from the hemagglutinin-neuraminidase protein alters virulence of Newcastle disease virus. *J Virol.* 2004;78:4965–75.
27. Parks CL, Lerch RA, Walpita P, Wang HP, Sidhu MS, Udem SA. Comparison of predicted amino acid sequences of measles virus strains in the Edmonston vaccine lineage. *J Virol.* 2001;75:910–20.
28. Shakya AK, Shukla V, Maan HS, Dhole TN. Identification of different lineages of measles virus strains circulating in Uttar Pradesh, North India. *Virol J.* 2012;9:237.
29. Truong AT, Mulders MN, Gautam DC, et al. Genetic analysis of Asian measles virus strains – new endemic genotype in Nepal. *Virus Res.* 2001;76:71–8.
30. Zhang Y, He J-L, Sun L. D Measles virus was first isolated from an imported measles in Sichuan Province of China. *Chin J Vaccine Immun.* 2009;15.
31. Wang HL, Zheng L, Wang JT, et al. The first imported measles case associated with genotype D4 measles virus in China. *Bing Du Xue Bao.* 2010;26:103–8.
32. Zhang Y, Ding Z, Wang H, et al. New measles virus genotype associated with outbreak, China. *Emerg Infect Dis.* 2010;16:943–7.
33. Li L-Q, Pang Y-K, Wang H-L, et al. Epidemic distribution characteristics of wild-type measles viruses genotypes in Yunnan Province. *Chin J Vaccines Immun.* 2012;18:105–9.
34. Wang J-g, Tang W, Li C-s. Genetic characterization of measles virus isolated in Shanghai during 2004–2006. *Int J Biol.* 2008;31:148–52.
35. Li S-h, Ni Z, Ju L-w, et al. Sequence analysis on measles viruses isolated in Shanghai in 2005. *Chin J Epidemiol.* 2007;28:165–8.
36. Bankamp B, Takeda M, Zhang Y, Xu W, Rota PA. Genetic characterization of measles vaccine strains. *J Infect Dis.* 2011;204 Suppl. 1:S533–48.
37. Cheng WY, Yang CF, Hou YT, et al. Imported measles and implications for its elimination in Taiwan. *Emerg Infect Dis.* 2011;17:1523–6.
38. Cheng WY, Tung HP, Wang HC, Lee LL, Wu HS, Liu MT. Molecular epidemiology of measles virus in Taiwan in 2010–2011: the common genotype changed from H1 to D9 and the first appearance of D4. *J Med Virol.* 2013;85:1095–9.
39. Salimi V, Mokhtari-Azad T, Abbasi S, et al. Molecular epidemiology of measles virus in Iran 2009–2010: first detection of measles genotype H1. *J Med Virol.* 2011;83:2200–7.
40. Cheng WY, Lee L, Rota PA, Yang DC. Molecular evolution of measles viruses circulated in Taiwan 1992–2008. *Virol J.* 2009;6:219.
41. Utikal J, Gratchev A, Muller-Molinat I, et al. The expression of metastasis suppressor MIM/MTSS1 is regulated by DNA methylation. *Int J Cancer.* 2006;119:2287–93.