

Molecular epidemiology study of measles viruses in Kunming area of China

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Received June 17, 2016; Accepted April 21, 2017

DOI: 10.3892/etm.2017.5033

Abstract. The present study assessed the variation of measles viruses (MV) and its association with clinical manifestations in patients with MV. A total of 38 pediatric patients with MV at the acute infection stage were selected and 2 ml venous blood was collected from each of them. Serum immunoglobulin M antibodies were determined by ELISA. Urine specimens were collected from 30 of the 38 patients and associated genetic structures were detected by reverse-transcription polymerase chain reaction mapping. At the same time, clinical epidemiological manifestations were collected to perform an epidemiological analysis. The MV-positive rate within the cohort determined in serum was 100%. Seven MV strains were isolated from urine specimens of 30 patients and the positive rate was 23.33%. Four MV strains were randomly selected from the 7 strains and the results revealed that they were all of the H_{1a} genotype. In addition, there was no significant correlation between clinical manifestation of pediatric patients with measles and the genotype of the MV. In conclusion, the preponderant genotype of MV in Kunming was H_{1a} and there was obvious nucleotide or amino acid mutation. The clinical manifestation of MV infection in pediatric patients was not associated with the MV genotype.

Introduction

Measles is a highly infectious and potentially dangerous disease, and a major killer of children (1). Subsequent to contact with MV, almost all children without immunity are infected. The clinical characteristics of measles include fever, upper respiratory tract infection, conjunctivitis, oral cavity mucous membrane measles, maculopapule on the whole body and pigmentation after back rash (2). MV, a paramyxovirus of the *Morbillivirus* genus of the paramyxovirus family, is an enveloped virus containing a single-stranded, minus (-) sense 50S RNA genome (3). MV infection causes profound immunosuppression, which makes measles patients susceptible to secondary infections accounting for high morbidity (4). It is responsible for an acute childhood illness that infects >40 million individuals and leads to >1 million mortalities per annum (5). It is generally thought that patients with measles have infectivity 5 days prior to and after its outbreak and the infective period may be prolonged to 10 days when complications are present.

Genetic heterogeneity exists in the wild-type MV, but there is only one serotype (6). Since the 1980s, MV has had a genetic drift in all countries, as indicated by the antigenic variation of MV and nucleotide sequence analysis. The MV P gene encodes eight proteins, including the P protein and the two non-structural proteins C and V (7). The proteins C and V are formed by selective translation of viral RNA encoding phosphoprotein. For the other 6 structural proteins, P protein, giant protein (L protein) and nucleocapsid protein (N protein) constitute a capsid wrapping viral RNA, while hemagglutinin protein (H protein), fusion protein (F protein) and matrix protein (M protein) combine with lipids from the membrane of host cells to form the envelope of virus (8,9). The N protein of MV consists of an N-terminal moiety, N CORE, which is resistant to proteolysis and a C-terminal moiety, N TAIL, which is hypersensitive to proteolysis and not visible as a distinct domain by electron microscopy (10). At present, MV is thought to have 23 genotypes, including A, B₁-B₃, C₁ and C₂, D₁-D₁₀, E, F, G₁-G₃, as well as H₁ and H₂, 5 of which (B₁, D₁, E, F and G₁) have no activity since their genotypes have not

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Key words: measles virus, genotype, molecular epidemiology, Kunming

been found in the past 15 years (11). Gene sequence analysis of MV isolated from Chinese provinces including Shandong, Hebei, Hunan, Beijing, Anhui, Hainan and Shanxi showed that the H₁ genotype is predominant in China and that intra-type variation will continue (12,13).

Various MV genotypes have a differential geographical distribution and various regions have different local epidemic strains (11,13-15). In addition, time has a certain relevance in the global epidemic of measles, providing a theoretical basis for monitoring the global molecular epidemiology of measles. Yunnan is a Chinese province with a high incidence of measles due to its unique geographical location, bordering with certain Southeast Asian countries with a high incidence of measles (15). At present, the clinical manifestations of measles in pediatric patients are diverse with heterogeneity in symptoms and occurrence of complications. In addition, to the best of our knowledge, no previous study has assessed the molecular epidemiology of MV in Kunming (China).

The present study assessed the molecular epidemiology of MV in 38 pediatric patients with measles in Kunming from 2008 by reverse-transcription quantitative polymerase chain reaction (RT-qPCR) analysis to determine the major genotype of wild-type MV in Kunming. The degree of genotype variation and the possible association with differential clinical manifestations between patients were also assessed.

Materials and methods

Sample collection and preparation. The study was approved by Ethics Committee of the Second Affiliated Hospital of Kunming Medical University (Kunming, China). The parents or guardians of all patients provided written informed consent prior to enrollment in the study.

Pediatric patients (age, <12 years) were recruited at the Second Affiliated Hospital of Kunming Medical University (Kunming, China) and the Children's Hospital of Kunming City (Kunming, China) between January 1, 2008 and March 31, 2008, and 38 cases (21 from the Second Affiliated Hospital of Kunming Medical University and 17 from the Children's Hospital of Kunming City) were selected. Venous blood (2 ml) was collected from each patient and urine was collected from 30 of the 38 cases. The inclusion criteria conformed to the diagnostic criteria for measles included in the seventh edition 'pediatrics' (16) and the disease being in its infectious stage (3 days before or 6 days after rash; this duration was chosen by researchers to collect positive samples).

The samples were stored at -80°C. Blood samples were centrifuged at 4°C, 5,478.2 x g for 10 min to obtain serum samples, which were stored at -20°C until analysis.

RNA isolation and RT-PCR. For RNA isolation, total MV RNA was extracted from urine specimens using the mirVana miRNA isolation kit (Ambion; Thermo Fisher Scientific, Inc., Waltham, MA, USA) after the sample collection. After isolation, the RNA concentration was assessed using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Inc.) and the RNA solution was stored at -80°C for further use.

RT-PCR was performed in 50- μ l reaction volumes. The amplification reaction mixture contained 0.2 mM of each primer, 1X Ex-Taq buffer, 0.25 mM of each deoxynucleotide

phosphate, 0.25 U Ex-Taq DNA polymerase (Takara Bio Inc., Otsu, Japan) and 2.5 μ l of the extracted template DNA in DNase-free water. The upstream primer of measles virus was 5'-GCTATGCCATGGGAGTAGGAGTGG-3', and the downstream primer was 5'-CCTCGGCCTCTCGCACCTAGT-3'. Sequence data were deposited in GenBank under accession number (EU090820). The reaction conditions were as follows: Initially denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 30 sec, annealing at 52°C for 30 sec and elongation at 72°C for 1 min, and a final elongation step at 72°C for 7 min. Distilled water was used as a negative control here.

The amplified products (50 μ l) were visualized by staining with ethidium bromide and separation by electrophoresis on 1.0% agarose gels (Beijing Borunlaite Science & Technology Co., Ltd., Beijing, China). The PCR products were purified with a BioSpinGel Extraction kit (Bioer Technology Co., Ltd., Beijing, China). The PCR products were purified and cloned into the pEASY-T1 vector (Beijing TransGen Biotech Co., Ltd., Beijing, China) (17). The vectors were then transduced into Trans1-T1 Phage Resistant Chemically Competent Cells (Beijing TransGen Biotech Co., Ltd.). The positive clones were selected and sequenced by Invitrogen Shanghai (Thermo Fisher Scientific, Inc.). The sequencing results were edited using DNASTar software 7.0 (DNASTar, Inc., Madison, WI, USA).

Detection of antibodies of MV. A total of 38 serum samples were evaluated for antibodies using ELISA. The procedures for detection were also performed according to the manufacturer's instructions.

Sequence analysis. The nucleotide sequences designated in the present study were compared with the 23 genotypes of MV by using the Basic Local Alignment Search Tool (Blast; <https://blast.ncbi.nlm.nih.gov/Blast.cgi>). Relevant sequences were also downloaded from GenBank (<https://www.ncbi.nlm.nih.gov/genbank/>). The sequences were assembled and aligned using DNASTar (<http://www.dnastar.com/>). To analyze the association between the variation of MV and clinical manifestation of measles in infected patients, a tree was generated based on the sequences of the complete H_{1/2} gene. Phylogenetic analysis was performed using the MP method in the software MEGA 5.0 (<http://www.megasoftware.net/>) and the European Bioinformatics Institute Tool ClustalW2 (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>), and the confidence level of the branch was assessed using bootstrap analysis with 1,000 replicates. The tree was rooted, and human parvovirus B19 and MV were used as out groups.

Statistical analysis. Differences between two proportions were measured by using Pearson's Chi-squared test or Fisher's exact test. P<0.05 was considered to indicate a statistically significant difference. Statistical analyses were performed by using SPSS 13.0.0 statistical software for Windows (SPSS, Inc., Chicago, IL, USA).

Results

MV determination. Urine specimens were collected from 30 of the 38 pediatric patients and 7 strains were isolated with a

positive rate of 23.33%. The results demonstrated that it was possible to isolate MV from urine specimens of patients up to 6 days after presenting with a rash. The positive rate for the 21 patients whose specimens were taken 1-3 days after rash was 19.05% and that of the 9 patients whose samples were taken at 4-6 days after the rash was 33.33%. Statistical analysis of the positive rate for the two time-periods using Fisher's exact test revealed no statistically significant difference ($P=0.640$). Venous blood was collected from 38 patients with measles and serum antibody IgM was determined. The results showed that the positive rate of IgM antibody was 100%. Statistical analysis of the positive rate of MV in urine specimens compared with that of serum IgM antibody revealed a statistically significant difference ($\chi^2=44.024$; $P<0.001$).

RT-PCR of N gene. Seven strains of MV isolated from urine specimens of 30 subjects were amplified and purified. After identification by 1.5% agarose gel electrophoresis, an obvious positive band was observed at 516 bp (Fig. 1). Four strains were selected from the 7 strains of MV after purification. Sequence determination analysis was automatically completed on an ABI 3730 sequencer by Invitrogen Shanghai (Thermo Fisher Scientific, Inc.) and representative sequencing data are presented in Fig. 2.

Sequence analysis

Analysis of genetic kinship. A total of 543 nucleotide sequences at the carboxyl terminal of the N gene in 4 strains of wild-type MV were compared with the 23 genotypes of MV from GenBank by using the Basic Local Alignment Search Tool (Blast; <https://blast.ncbi.nlm.nih.gov/Blast.cgi>). The results demonstrated that all of the 4 strains of MV were of the H genotype, which was consistent with the predominant genotype in China. MEGA 5.0 software and the European Bioinformatics Institute Tool ClustalW2 (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>) were then used to perform a genetic kinship analysis between the 543 nucleotide sequences at the carboxyl terminal of the N gene in the 4 strains of wild-type MV and the representative strain at WHO for China between 1993 and 1994 and the corresponding sequences of referential strains (China93-7, China93-2, China94-7 and China94-1) for the H₁ and H₂ genotypes (13). The genetic kinship trees of nucleotide and amino acid sequences are displayed in Fig. 3A and B, respectively. The 4 strains of MV were all of the H₁ genotype and belonged to the same branch as China93-7. The kinship between isolated MV and referential strain China93-2 (H_{1a} subtype) was closest. It was therefore indicated that the genotype of the 4 MV strains was the H_{1a} subtype of the H₁ genotype.

Variation analysis of nucleotides and amino acids. Genetic distance analysis within and between gene sequences of 4 MV strains and WHO referential strains China93-7, China93-2, China94-7 and China94-1 were performed. The results presented in Table I demonstrated that the genetic distance between nucleotide sequences of the 4 MV strains from the H₁ and H₂ genotype referential strains China93-7 and China94-1 was 0.031-0.047 and 0.332-0.398, respectively. The genetic distance for the H_{1a} genotype (standard strain China93-2) was 0.010-0.033, and that between the 4 strains was 0.005-0.040.

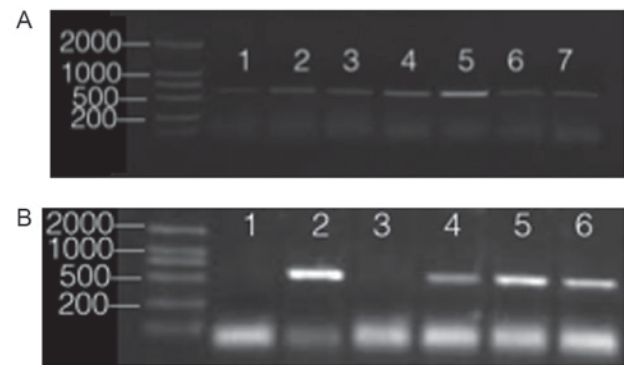


Figure 1. Results of agarose gel electrophoresis of MV. (A) Nucleotide fragment amplification of N gene in MV. Lane 1, Kunming08-6 strain; lane 2, Kunming08-1 strain; lane 3, Kunming08-5 strain; lane 4, Kunming08-2 strain; lane 5, Kunming08-3 strain; lane 6, Kunming08-7 strain; lane 7, Kunming08-4 strain. (B) Electrophoresis chart of the 4 MV strains after purification. MV, measles virus. Lane 1, ddH₂O negative control; lane 2, Kunming08-3 strain; lane 3, ddH₂O negative control; lane 4, Kunming08-1 strain; lane 4, Kunming08-1 strain; lane 5, Kunming08-2 strain; lane 6, Kunming08-3 strain.

The genetic distance between amino acid sequences of the 4 MV strains and those of the H₁ and H₂ genotype referential strains China93-7 and China94-1 was 0.088-0.149 and 0.205-0.282, respectively. The distance for the H_{1a} genotype was 0.003-0.066, and that between the 4 strains was 0.010-0.073.

Homology analysis of nucleotides and amino acids. The homology of 543 nucleotides at the carboxyl terminal of the N gene in the 4 strains of wild-type MV was 96-98.9%. The nucleotides differed in 6-12 bases and the homology of amino acids was 95.6-99.6%. The nucleotide homology of the 4 strains of wild-type MV with the H₁ standard strain China93-7 was 95.9-99.7%, while the amino acid homology was 89.9-94.8%. The nucleotide homology with the H_{1a} standard strain China93-2 was 96.9-99.8% and the amino acid homology was 94.8-99.7% (Table II).

Epidemiology analysis

Morbidity situation. The number of pediatric patients who presented with measles at the Children's Hospital of Kunming City and the Second Affiliated Hospital of Kunming Medical University between January 2008 and March 2008 was 51, while that in January, February and March 2006 was 122, 113 and 129, respectively. The numbers in January, February and March of 2007 were 124, 55 and 35, respectively. The morbidity decreased with increasing time, which may be associated with the strength of immunization to MV in 2006 due to a pandemic of measles.

Age distribution. The age of the 38 pediatric patients with measles enrolled in the present study ranged from 51 days to 11 years. The number of children below the age of 1 year was 21 and accounted for 55.26% of the total patients selected. The number of children aged from 1 to 2 years old was 9, accounting for 23.68%. The number of patients aged between 2-3, 7-8 and 8-9 years old was 2 in each age range, respectively, accounting for 5.26% of all the recruited children. In total, the number of patients below the age of 2 years was 30 and

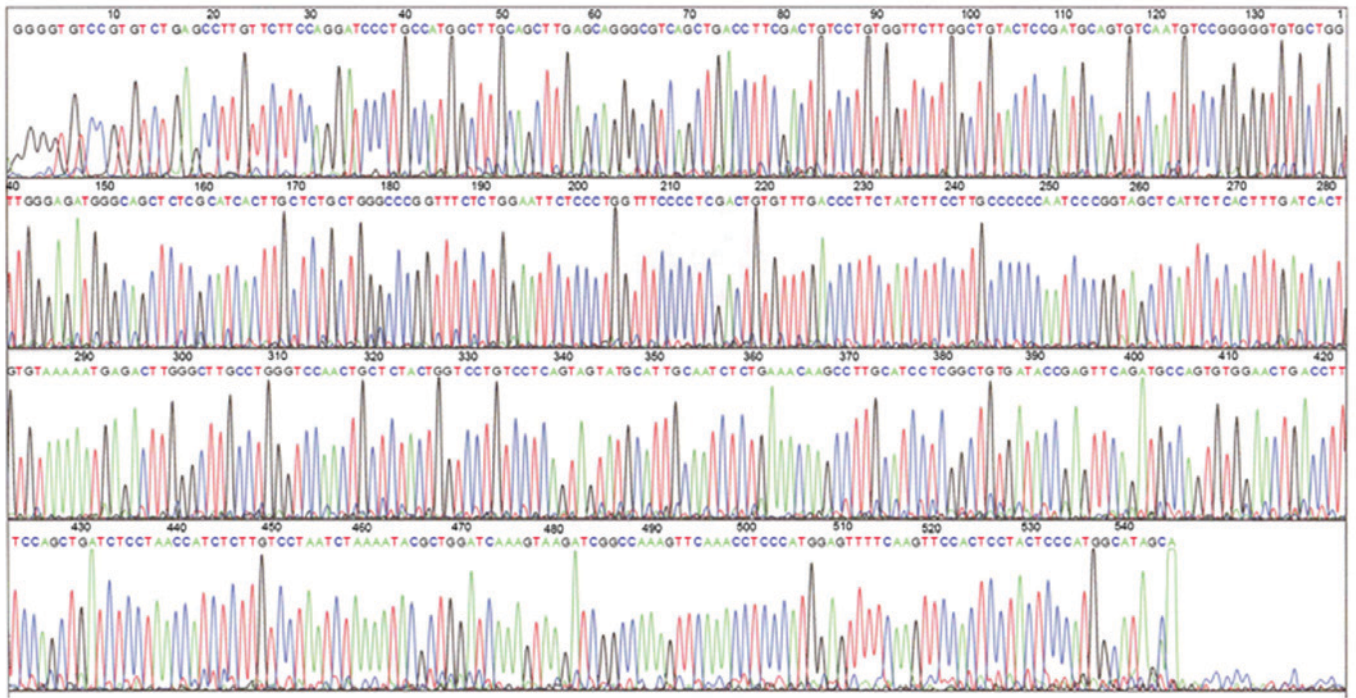


Figure 2. Original figure of sequencing results on Kunming08-3 measles virus strains produced by a sequencing instrument. A total of 543 nucleotide sequences at the carboxyl terminal of the N gene in Kunming08-3 strain was compared with the 23 genotypes of MV from GenBank. The homology of 543 nucleotides at the carboxyl terminal of the N gene was 96-98.9% with China93-2 strain, which indicated that the genotype was the H_{1a} subtype of the H₁ genotype.

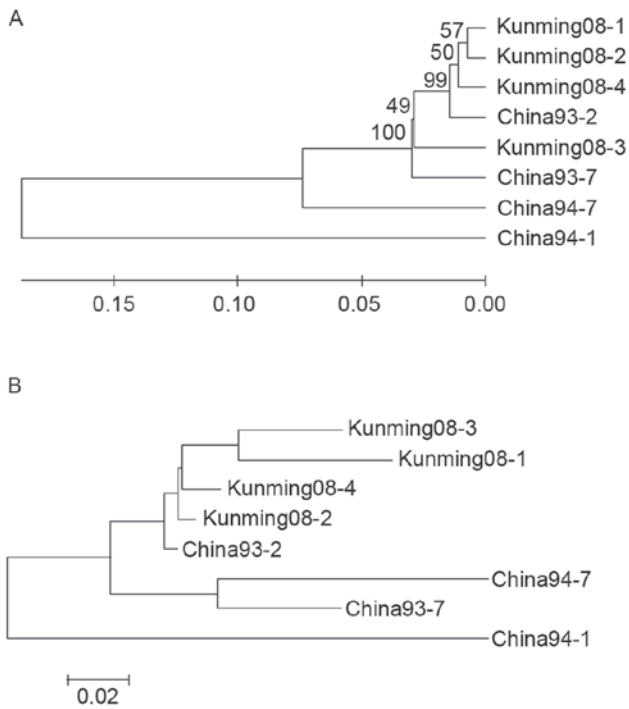


Figure 3. Kinship analysis on nucleotide and amino acid sequences of virus strains. (A) Kin relationship tree of nucleotide sequences in 4 wild-type measles virus strains and referential strains popular in China. The scales indicate homology from the Genbank, which demonstrated the kinship between isolated measles viruses and referential strains. (B) Kin relationship tree of amino acid sequences in 4 wild-type measles virus strains and referential strains popular in China.

accounted for 78.95% of the 38 patients. A high incidence of measles was therefore encountered in children aged <2 years.

Within the cohort of 38 pediatric patients, there were 23 male and 15 females with a proportion of 60.53 and 39.47%, respectively, indicating that the amount of male children was much higher than that of female children.

Regional distribution. Within the cohort of the present study (n=38), the regional distribution within Kunming was as follows: Wuhua, 7; Panlong, 4; Guandu, 5; Xishan, 7; Dongchuan, 1; Chenggong, 1; Jinning, 2; Fumin, 2; Chongming, 1; Luquan, 7; and Anning, 1. This indicated that the number of patients with measles in Wuhua and Xishan was highest in Kunming city. The cases of measles in the present study were sporadic cases and not affected by any pandemic. The differences in the number of infected patients in different regions may be associated with the imbalance of immunization work. Within the cohort comprising 38 cases, 7 had been vaccinated against measles, 30 had not been vaccinated and the vaccination status of the remaining case was unknown. Cases with no immunity and ominous immune history accounted for 81.58% of the total patient population. Among the 7 patients vaccinated against measles, 5 had primary immunization failure and the other 2, who were aged >7 years, had received no second vaccination.

Association between clinical manifestation and genotype. Four strains were randomly selected from the 7 strains of MV isolated from urine specimens of measles patients. Sequence determination and analysis showed that they were all of the H_{1a} genotype, but there was a great difference the clinical manifestation of measles in the 4 patients (Table II). This indicated that the clinical manifestation of measles in different patients was not obviously associated with the genotype of MV.

Table I. Genetic distance analysis of the nucleotides and amino acids of the 4 wild-type measles virus strains isolated from Kunming area and referential strains.

A, Nucleotides								
Strain	Kunming08-1	Kunming08-2	Kunming08-3	Kunming08-4	China93-7	China93-2	China94-7	China94-1
Kunming08-1								
Kunming08-2	0.005							
Kunming08-3	0.029	0.039						
Kunming08-4	0.016	0.008	0.040					
China93-7	0.031	0.035	0.047	0.047				
China93-2	0.010	0.018	0.033	0.029	0.039			
China94-7	0.114	0.107	0.167	0.089	0.160	0.132		
China94-1	0.353	0.348	0.398	0.332	0.399	0.380	0.271	

B, Amino acids								
Strain	Kunming08-1	Kunming08-2	Kunming08-3	Kunming08-4	China93-7	China93-2	China94-7	China94-1
Kunming08-1								
Kunming08-2	0.010							
Kunming08-3	0.059	0.042						
Kunming08-4	0.003	0.010	0.052					
China93-7	0.066	0.073	0.073	0.059				
China93-2	0.156	0.156	0.156	0.149	0.197			
China94-7	0.188	0.213	0.273	0.205	0.282	0.282		
China94-1	0.088	0.095	0.118	0.088	0.149	0.118	0.273	

Kunming08, wild-type strains identified in the present study; China93/4, referential strains.

Discussion

Measles is an acute viral infectious disease caused by MV and is characterized by fever, upper respiratory tract infection and maculopapules over the entire body surface (18). According to an estimation by the world health organization, ~45 million measles cases occur and 1 million children die from measles and its complications each year (19). Monitoring of the genotype distribution of MV in different areas is of important significance for identifying scientific approaches for blocking the spread of MV and developing a regional measles elimination plan (20).

MV is a RNA virus of the genus *Morbillivirus* within the family Paramyxoviridae, and each virus has antigen cross-reactivity within the *Morbillivirus* genera (21). MV only has one serotype, but it has multiple genotypes. In the present study, 38 blood samples and 30 urine specimens were collected from patients with measles. Previous studies showed that at the prodromal stage of measles and on the first day that a rash appears on the body, MV is easy to be separated from the patient's blood and respiratory secretions (22). In addition, studies reported that multinuclear giant cells in the nasopharynx of measles patients were rapidly reduced after the rash, but multinuclear giant cells were still found in the urine sediment a few days later (23). Therefore, the time window for virus collection from the urine may be longer than that from other fluids. The results of the present study showed that MV may be isolated from urine specimens within 6 days

after rash. The virus separation rate within 4-6 days after rash (33.33%) was higher than that within 3 days (19.05%), while there was no statistical difference between them. In addition, the determination of measles IgM antibody from the serum of the 38 cases of measles at the acute stage provided a positive rate of 100%. This indicated that the detection rate of measles IgM antibody from serum was evidently higher than the separation rate of MV from urine.

The H and N genes have the greatest variation among the structural genes of MV (24). The N gene encodes 525 amino acids, the H gene encodes 617 amino acids and they have 7% nucleotide variation in their sequences (15). Particularly the 450 nucleotides at the carbon end of the N gene is the largest zone for MV gene variations and the degree of genetic variation of MV is up to 12%. The 1,851 nucleotides contained in the H gene, 1,575 in the N gene and 450 in the N gene carbon end were the main subjects of a previous epidemiological study on MV (25). Their sequence analysis results have been widely used to describe the genotypes of MV. In the present study, 4 MV strains were randomly selected from the 7 isolated MV strains and a molecular epidemiological analysis was performed. Blast comparison and genetic affinity analysis of the 543 nucleotides at the carboxyl terminal of the N gene in 4 strains of wild-type MV and 23 genotypes of MV from GenBank as well as a vaccine strain was performed. A total of 4/23 genotypes were Chinese representatives, including China93-7, China93-2, China94-7 and China94-1. By comparison with these 4 strains which were the most

Table II. Homology analysis of nucleotides and amino acids of the 4 wild-type measles virus strains isolated from Kunming area and referential strains.

A, Nucleotides								
Strain	Kunming08-1	Kunming08-2	Kunming08-3	Kunming08-4	China93-7	China93-2	China94-7	China94-1
Kunming08-1								
Kunming08-2	0.969							
Kunming08-3	0.960	0.969						
Kunming08-4	0.978	0.989	0.978					
China93-7	0.959	0.988	0.959	0.997				
China93-2	0.969	0.998	0.969	0.988	0.989			
China94-7	0.869	0.886	0.868	0.897	0.915	0.906		
China94-1	0.778	0.787	0.778	0.808	0.819	0.809	0.859	

B, Amino acids								
Strain	Kunming08-1	Kunming08-2	Kunming08-3	Kunming08-4	China93-7	China93-2	China94-7	China94-1
Kunming08-1								
Kunming08-2	0.959							
Kunming08-3	0.956	0.968						
Kunming08-4	0.956	0.996	0.969					
China93-7	0.899	0.927	0.909	0.948				
China93-2	0.948	0.996	0.959	0.997	0.929			
China94-7	0.839	0.877	0.868	0.868	0.906	0.868		
China94-1	0.789	0.858	0.788	0.827	0.789	0.855	0.788	

Kunming08, wild-type strains identified in the present study; China93/4, referential strains.

Table III. Clinical manifestation of measles in pediatric patients subjected to sequence determination.

Genotype code of measles virus	Clinical manifestation of measles				
	Body temperature	Rash	Respiratory symptoms	Koplik spot	Conjunctivitis
Kunming08-1	Ardent fever	Yes	Severe, pneumonia	Yes	No
Kunming08-2	Cardiothoracic fever	Yes	Mild, cough	Yes	Yes
Kunming08-3	Cardiothoracic fever	Yes	Severe, pneumonia	Yes	No
Kunming08-4	Ardent fever	Yes	Severe, pneumonia	No	No

common genotypes in China, genetic drift and variation would be confirmed and this would help to build the surveillance of MV and to evaluate the efficacy of vaccination. The results showed that the sequences from all of the 4 strains of MV were of the H genome and of the H_{1a} subtype. The genotype of the 4 wild-type MV strains from Kunming area was consistent with the genotype of the major epidemic strain in China. The genetic distance, nucleotide homology and amino acid homology between the corresponding sequences of the 543 nucleotides at the carboxyl terminal of the N gene of the 4 wild-type MV strains and referential strains were assessed. The results revealed only a minor difference between the 4 MV strains. The genotype of the wild-type MV strains encountered in Kunming area from January 2008 to March 2008 was consistent with that reported in Yunnan province in 2005 (26).

Yunnan province has planned immunization-projects since the early 1980s and measles has been effectively controlled due to the enhancement of the measles vaccine inoculation rate (27). Kunming is the capital of Yunnan province and the epidemiological characteristics of measles in Kunming area have a certain representativeness. The present study assessed the age at onset and gender distribution, epidemiology of measles as well as genetic variations of MV and their association with clinical manifestation of measles in patients. The results demonstrated that the clinical manifestation of measles had no obvious correlation with the genotype of wild-type MV.

In conclusion, the preponderant genotype of MV in Kunming area was H_{1a}, which was consistent with the genotype of the most common strain in Yunnan province. While the age range of measles patients was large, the disease mainly

occurred in patients aged <2 years and the majority of patients had not been vaccinated against measles.

Acknowledgements

The present study was supported by the National Natural Science Foundation of China (grant nos. 81100091 and 81200067), the Shanghai Municipal Commission of Health and Family Planning, China (grant nos. 20134Y33 and 2010Y145), Young Medical Talents Training Program of Pudong Health Bureau of Shanghai, China (grant no. PWRq2011-02) and the Research Fund of Science and Technology Commission of Shanghai Municipality (grant no. 14140903600).

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