ORIGINAL ARTICLE

Laboratory diagnosis of vaccine-associated measles in Zhejiang Province, China

Chang-Ping Xu, Min-Hong Li, Han-Qing He, Yi-Yu Lu, Yan Feng*

Key Laboratory of Emergency Detection for Public Health of Zhejiang Province, Zhejiang Provincial Center for Disease Control and Prevention, Hangzhou, Zhejiang Province, China

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KEYWORDS
allelic discrimination; measles virus; real-time reverse transcriptase polymerase chain reaction; vaccine-associated measles

Abstract
Background/Purpose: Along with the improving vaccine coverage, suspected vaccine-associated measles has been reported in Zhejiang Province, China. In order to maintain the accuracy of the measles surveillance system, it is critical to discriminate between measles vaccine and wild-type virus.

Methods: Eight suspected cases of vaccine-associated measles were reported in Zhejiang Province during 2011 and 2014. Sera collected within 4 days and throat swabs collected within 6 days after rash onset were tested with immunoglobulin M and measles virus (MeV) RNA to confirm MeV infection. In order to further identify the vaccine-associated cases, throat swabs with positive MeV RNA were tested using an allelic discrimination real-time reverse transcriptase polymerase chain reaction (rRT-PCR) assay developed in this study, RT-PCR-restriction fragment length polymorphism (RFLP) recommended by the National Measles Laboratory, and RT-PCR followed by sequencing and genotyping.

Results: Combining anti-measles immunoglobulin M and RNA testing, eight cases were confirmed as MeV infection. Of the eight, two were identified as vaccine-associated cases by the allelic discrimination rRT-PCR assay, and one was identified by RT-PCR-RFLP. Subsequent sequencing and genotyping confirmed that the sequences of the two cases were identical to that of the Chinese vaccine strain. The developed allelic discrimination rRT-PCR was 10 times more sensitive than the RT-PCR-RFLP assay when RNA standards generated from three genotypes of MeV were tested.

* Corresponding author. Key Laboratory of Emergency Detection for Public Health of Zhejiang Province, Zhejiang Provincial Center for Disease Control and Prevention, 3399 Binsheng Road, Hangzhou, Zhejiang Province, 310051, China.
E-mail address: fengyanzjh@aliyun.com (Y. Feng).

These two authors contributed equally to this article.

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Introduction

Measles is a highly contagious disease, which resulted in millions of deaths in the pre-vaccine era. The morbidity and mortality of measles have declined considerably since the 1960s, when the attenuated or killed measles vaccine was developed and used successfully throughout the world.\(^1\)\(^,\)\(^2\) After decades of improved immunization coverage and decreasing incidence, the elimination of measles became the goal of the World Health Organization (WHO) in its four regions by 2015.\(^3\) To achieve this goal in China, WHO has recommended a measles mass immunization campaign (MMIC) in addition to two doses of routine vaccination.

Measles-containing vaccines, the most effective means to control and interrupt measles infection at present,\(^4\) have been used globally for ~50 years. Although the vaccines have been proved to be safe and effective, previous studies have found that the live attenuated vaccine may cause measles-like symptoms, such as moderate fever, rash, and conjunctivitis, in 5–15% of recipients.\(^5\)\(^,\)\(^6\) With the apparent drop in the incidence of wild-type measles, the epidemiology and clinical pattern of the disease have changed, and vaccine-associated measles has been reported in many countries.\(^7\)\(^–\)\(^10\) In order to ensure the accuracy of the measles surveillance system during the period of measles elimination, vaccine-associated measles should be distinguished from wild-type measles virus (MeV) infection when cases are reported.

Differentiation of vaccine-associated measles based on clinical symptoms or laboratory methods routinely used in MeV surveillance, such as immunoglobulin M (IgM) testing, virus isolation, and RNA amplification, is unworkable or unreliable. Sequencing combined with genotyping has always been the most accessible way to identify vaccine-associated cases\(^11\); however, this method is time consuming, and not every clinical specimen contains sufficient amount of viral RNA to perform sequencing analysis.\(^12\) A reverse transcriptase polymerase chain reaction-restriction fragment length polymorphism (RT-PCR-RFLP) assay has been developed and recommended for discriminating between measles vaccine and wild-type strains by the Chinese National Measles Laboratory.\(^13\) The process of the RT-PCR-RFLP is divided into RT-PCR plus enzyme digestion and gel electrophoresis, which are not only labor intensive but can easily cause crossover contamination as well. A loop-mediated isothermal amplification assay has also been developed for differentiating vaccine from wild-type MeVs in Japan.\(^12\) This assay is simpler than RT-PCR-RFLP, but its sensitivity for detecting wild-type MeV strains is ~10 times lower than it is for detecting vaccine-type strains.\(^12\)

Conclusion: Vaccine-associated measles has been identified in Zhejiang. The developed allelic discrimination real-time RT-PCR (rRT-PCR) assay and applied it to discriminate between measles vaccine and wild-type strains. Our research provides data for the laboratory diagnosis of vaccine-associated measles case and contributes to the accuracy of the measles case surveillance system.

Methods

Suspected vaccine-associated measles

Eight suspected vaccine-associated measles were reported in Zhejiang Province, China during 2011 and 2014. All the suspected cases were vaccinated with live attenuated measles and rubella combined vaccine 4–11 days prior to the onset of rash. Patients presented with symptoms of fever and rash, which are consistent with measles clinically (Table 1). Cases 1 and 7 were reported to present with typical Koplik’s spots. Cases 2–4 and 6 presented with symptoms of cough, conjunctivitis, and coryza (3C). Case 1 was also reported to have enlarged lymph nodes. Cases 1, 2, and 6 had been hospitalized prior to onset of illness (Table 1).

Specimens

Clinical specimens of eight suspected vaccine-associated cases were used in this study. Serum samples collected within 4 days after rash onset were tested by enzyme-linked immunosorbent assay kit (Virion\textregistered Serion, Würzburg, Germany). Throat swab specimens collected within 6 days after rash onset were analyzed by a multiplex rRT-PCR for MeV and rubella virus (RV) routinely used in Zhejiang Provincial Center for Disease Control and Prevention (CDC),\(^14\) as well as used for virus isolation.\(^15\) Afterwards, throat swabs with positive MeV RNA were tested using the allelic discrimination rRT-PCR developed in this study, RT-PCR-RFLP recommended by the National Measles Laboratory,\(^13\) and RT-PCR followed by sequencing and genotyping.\(^16\)

RNA extraction

Total RNA was extracted from clinical specimens or cell culture supernatants using the High Pure Viral Nucleic Acid Kit (Roche, Welwyn Garden City, UK). Briefly, 200 μL of clinical specimen or cell culture supernatant was added to a lysis buffer containing poly (A) and proteinase K and
incubated at 72°C for 10 minutes. The sample was run through the High Pure Spin Filter column and the RNA was bound to the glass fiber fleece. The contaminants, including salts, protein, and cellular contaminants, were removed through several wash and spin steps. The purified RNA was eluted into 50 μL Elution Buffer and then stored at −80°C.

Allelic discrimination rRT-PCR assay

Whole-genome sequences of 16 MeVs, including seven vaccine strains (genotype A) and nine wild-type strains (genotypes H1a, H1b, B3, D3, D5, D6, D7, D8, and G3), were downloaded from GenBank. Multiple sequence alignments were performed using BioEdit software (Figure 1). The allelic discrimination site was searched through the whole genome. Primer pair and TaqMan-minor groove binder (MGB) probes containing the allelic discrimination site were designed using PrimerExpress 3.0 software (Applied Biosystems, Foster City, CA, USA). Sequences and positions of the primer pair and TaqMan-MGB probes are shown in Table 2. The probe for detecting wild-type MeV (wild-type probe: 5′-FAM-CTTCCAGCGCCAGG-NFQ-MGB) was labeled with FAM (6-carboxy-fluorescein) at the 5′ end, while the probe for detecting measles-vaccine strain (Vaccine probe: 5′-VIC-CATGCTTCCAGCGCCAGG-NFQ-MGB) was labeled with VIC fluorescence. MGB was labeled at the 3′ end of the two probes.

The allelic discrimination rRT-PCR was performed using One Step PrimeScript RT-PCR (Perfect Real-time) kit (TaKaRa Biotechnology Co., Kusatsu, Japan). Each 25-μL reaction mixture contained 12.5 μL 2× One Step RT-PCR Buffer, 0.5 μL TaKaRa Ex Hot start (HS) Taq, 0.5 μL PrimeScript RT Enzyme Mix, 0.5 μL each primer, 0.25 μL each probe, 5 μL deionized distilled water, and 5 μL RNA template. The final concentration of each primer and probe was 0.4 μM and 0.2 μM, respectively. The optimal amplification conditions are shown in Table 2. FAM and VIC fluorescence signals were acquired at the end of each annealing step.

Preparation of MeV RNA standards

Three MeV strains were used to prepare RNA standards. Measles vaccine was produced by Shanghai Institute of Biological Products, Shanghai, China, of which genotype A

### Table 1. Clinical information for eight suspected vaccine-associated measles

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Date of birth</th>
<th>Date of vaccination</th>
<th>Date of fever</th>
<th>Date of rash onset</th>
<th>Clinical symptoms</th>
<th>Ever been hospitalized</th>
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<td>28 Jul 2010</td>
<td>18 Apr 2011</td>
<td>27 Apr 2011</td>
<td>28 Apr 2011</td>
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<td>15 Mar 2013</td>
<td>22 Mar 2013</td>
<td>25 Mar 2013</td>
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<td>No</td>
</tr>
<tr>
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<td>24 Jul 2012</td>
<td>26 Mar 2013</td>
<td>2 Apr 2013</td>
<td>4 Apr 2013</td>
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<td>No</td>
</tr>
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<td>11 May 2013</td>
<td>12 May 2013</td>
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<td>No</td>
</tr>
<tr>
<td>6</td>
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<td>18 Mar 2013</td>
<td>19 Mar 2013</td>
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<tr>
<td>7</td>
<td>18 Jun 2013</td>
<td>20 Feb 2014</td>
<td>28 Feb 2014</td>
<td>2 Mar 2014</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>8</td>
<td>13 Jun 2013</td>
<td>13 Feb 2014</td>
<td>22 Feb 2014</td>
<td>24 Feb 2014</td>
<td>No</td>
<td>No</td>
</tr>
</tbody>
</table>

Figure 1. Primer pair and probes designed for the allelic discrimination real-time reverse transcriptase polymerase chain reaction assay. The upper part shows sequence alignments between seven vaccine strains and nine wild-type strains. The lower part shows sequences of the primer pair, vaccine probe and wild-type probe. The highlighted location indicates the nucleotide that is different between vaccine and wild-type strains. The vaccine probe and wild-type probe is labeled with VIC and FAM (6-carboxy-fluorescein) fluorescence, respectively, at the 5′ end.
strain Shanghai-191 (GenBank accession No. FJ416067) was incorporated. Genotype H1 strain MVi/Zhejiang.CHN/12.09/1 was isolated from an acute measles case in Zhejiang CDC. Genotype D4 strain was donated by the National Measles Laboratory.

Three genotypes of RNA standard were generated as previously described.\(^17\) H gene fragment [nucleotides (nt) 7178\(^e\)–9224\(^e\)] was amplified using RNA template of three MeV strains with primers containing T7 promoter sequence and were in vitro transcribed with T7 RNA polymerase (TaKaRa Biotechnology Co.). The sequence of the forward primer was 5\(^{\prime}\)-ACTCGTTAATACGACTCACTATAGGGAGCCAGCATCAATCCCACCTGAA-3\(^{\prime}\) (T7 promoter sequence were highlighted in bold) (nt 7178\(^e\)–7198) and the reverse primer was 5\(^{\prime}\)-CCACTTGGACCCTACGTT-3\(^{\prime}\) (nt 9224\(^e\)–9207).

The synthetic RNA transcripts were purified, qualified and then 10-fold diluted, ranging from 10\(^6\) copies/\(\mu\)L to 100 copies/\(\mu\)L.

Specificity and sensitivity of the allelic discrimination rRT-PCR

Specificity of the allelic discrimination rRT-PCR assay was determined using RNA extracted from cell culture supernatant of three genotypes of MeV, RV, mumps virus, and influenza virus subtype A, as well as the clinical specimens from patients infected with enterovirus 71 and coxsackievirus A16. Sensitivity was assessed through performing the allelic discrimination rRT-PCR in parallel with the RT-PCR-RFLP on serial diluted (10\(^6\)–10\(^0\) copies/\(\mu\)L) RNA standards generated from three genotypes of MeV. Throat swabs from eight suspected vaccine-associated cases were also used to validate the developed assay.

RT-PCR-RFLP

We used the RT-PCR-RFLP assay recommended for discriminating between MeV vaccine and wild-type strains in China.\(^13\) A fragment located within nt 7516–7953 on the MeV H gene was amplified by RT-PCR. The 438 nt RT-PCR product contained a restriction enzyme cutting site at nt 7666–7671. Measles vaccine strain (genotype A) can be cut into two fragments (287 nt and 151 nt length bands) after enzyme digestion; however, none of the wild-type strains can be cut by enzyme, and only one band can be observed in gel electrophoresis.

RT-PCR followed by sequencing and genotyping

The 450 nt sequence at the C terminus of the N gene was amplified by RT-PCR using PrimeScript One Step RT-PCR Kit (TaKaRa Biotechnology Co.) and primer pair MeV214 and MeV216 that were routinely used to perform genotyping of MeV.\(^16\) Five microliters of RNA was used as a template for RT-PCR. The first round of nested RT-PCR was generated by primer pair MeV214 and MeV216.\(^16\) The primer pair for the second round was originated from MeV214 and MeV216 with slight modifications. The sequence of the forward primer was 5\(^{\prime}\)-GGAGCTATGCCATGGGAGTAG-3\(^{\prime}\) (nt 1105–1125) and the reverse primer was 5\(^{\prime}\)-AACAATGATGGAGGGTAGGCG-3\(^{\prime}\) (nt 1736–1716). RT-PCR products were purified using the MinElute PCR Purification Kit (Qiagen, Hilden, Germany) and sequenced by Sangon Biotech (Shanghai, China). The phylogenetic tree was drawn based on the 450 nt sequences of 23 reference strains,\(^18\) and six sequences generated in this study (Table 3), as well as Chinese vaccine strain Shanghai-191 (GenBank accession No. FJ416067), using the neighboring-joining and the Kimura two-parameter model of MEGA version 5.0 software (http://www.megasoftware.net).\(^19\)

Results

Confirmation of MeV infection

For case confirmation, anti-measles IgM testing, MeV and RV RNA amplification, and virus isolation were performed. Of the eight cases, six were positive for anti-measles IgM, but
Cases 4 and 8 whose serum specimens were collected within 2 days after rash onset gave negative results (Table 3). All eight cases were positive for MeV RNA but negative for RV RNA (Table 3). Combining the results of anti-measles IgM and MeV RNA, the eight cases were all confirmed as MeV infection. In addition, four MeV strains were isolated from Cases 2–4 and 6 (Table 3).

**Evaluation of the allelic discrimination rRT-PCR assay**

An allelic discrimination rRT-PCR assay was developed for differentiating between MeV vaccine and wild-type strains. Based on the result of multiple sequence alignments on the MeV genome (Figure 1), site nt 8419 located within the H gene was selected as an allelic discrimination site. MeV vaccine strains contain nucleotide A, while wild-type strains contain nucleotide G (Figure 1) on the site. Two TaqMan-MGB probes used in the assay were designed to hold the allelic discrimination site (nt 8419) and were labeled with different fluorescence (VIC and FAM). MeV vaccine and wild-type strains can be differentiated based on fluorescence signals acquired after amplification.

Specificity evaluation of the allelic discrimination rRT-PCR showed that the assay has no cross-reactivity against other respiratory viruses, except for three genotypes of MeV. Moreover, positive result on the VIC channel was only generated by genotype A MeV, while positive results on the FAM channel were detected from genotype H1 or D4 MeVs.

Sensitivity of the allelic discrimination rRT-PCR was assessed and compared with the RT-PCR-RFLP assay. When RNA standards were tested, detection limits of the allelic discrimination rRT-PCR assay were 10 copies/μL. Through enzyme digestion, two specific bands with 287 nt and 151 nt in length were observed in the gel picture of genotype A RNA standard (Figure 2B), but only one band of 438 nt in length was observed in the picture of genotype H1 or D4 RNA standards, respectively (Figure 2D and F). In addition, the allelic discrimination rRT-PCR was faster than RT-PCR-RFLP, as the whole process only needed ~1.5 hours.

**Identification and confirmation of vaccine-associated cases**

In order to discriminate whether or not the eight cases were caused by measles vaccine, throat swabs were tested using the allelic discrimination rRT-PCR in parallel with RT-PCR-RFLP assay. All the cases tested positive for MeV RNA using allelic discrimination rRT-PCR, two of which (Cases 1 and 8) acquired positive results from channel VIC and were identified as vaccine-associated measles, while the other six were tested as positive in channel FAM and identified as wild-type MeV infection (Table 3). Five (Cases 1–4 and 6) of the eight cases were detected as positive by the RT-PCR-RFLP assay, among which only Case 1 was identified as a vaccine-associated case, as there were two bands observed in the electrophoretogram (Table 3). RT-PCR followed by sequencing and genotyping was used to confirm the results of allelic discrimination rRT-PCR. Six of the eight tested positive by RT-PCR, of which, two (MVs/Zhejiang.CHN/17.11 and MVs/Zhejiang.CHN/12.14) were directly generated from clinical specimens of Cases 1 and 8, and another four were generated from cell culture supernatants. The sequence of Case 8 was generated by nested RT-PCR, since there was a weak band after routine RT-PCR amplification.
The phylogenetic tree (Figure 3) showed that MVs/Zhejiang.CHN/17.11 and MVs/Zhejiang.CHN/12.14 were located in the same branch with Edmonston-wt. USA/54, the reference strain of genotype A MeV, and were identical to the Chinese vaccine strain Shanghai-191 (GenBank accession No. FJ416067). Hence, these two were confirmed as vaccine-associated measles. The other four sequences generated from Cases 2–4 and 6 had the nearest distances to reference strain Hunan.CHN/93/7, and were identified as genotype H1 strains. Three of these four sequences (Cases 3, 4, and, 6) were identical and had seven nucleotides different from the MVi/Zhejiang.CHN/13.13/12 strain, which was generated from Case 2.

Discussion

With the acceleration of measles control and elimination in recent years, atypical and vaccine-associated measles have been monitored worldwide.7–10 Many cases suspected to correlate with vaccine administration have been reported in Zhejiang Province, China, since a nationwide MMIC carried out in 2010 when >2 million children aged from 8 months to 4 years were immunized in Zhejiang Province with measles-containing vaccine.20

In this study, we focused on eight suspected cases of vaccine-associated measles that occurred in Shaoxing City of Zhejiang Province during 2011 and 2014. On arrival at the hospital, all of the patients presented with symptoms of fever and red maculopapules. They were clinically diagnosed as measles and reported to the local CDC. It was subsequently discovered that they had been immunized with measles and rubella combined vaccine 4–11 days prior to rash onset. Based on the first definition of vaccine-associated measles by the WHO,21–23 hospital staff doubted whether the cases were caused by the measles vaccine. In order to determine if there was a connection, serum and throat swab specimens were collected and used to perform laboratory diagnosis.
negative results in the first 3 days after rash onset. However, Case 8 was tested as negative by RT-PCR-RFLP, which suggests that the allelic discrimination rRT-PCR assay would be in detection of the other 19 genotypes of MeV. Another limitation of the study was that only three genotypes were available in our laboratory. Even though the multiple sequence alignment assures the accuracy of the assay theoretically, we cannot be completely certain what the performance of the allelic discrimination rRT-PCR assay would be in detection of the other 19 genotypes of MeV, based on VIC fluorescent signals. Of the eight cases, two were identified as vaccine-associated measles by the new rRT-PCR method. One of these cases (Case 1) was also identified by the recommended RT-PCR-RFLP assay. However, Case 8 was tested as negative by RT-PCR-RFLP, which suggests that the allelic discrimination rRT-PCR assay would be in detection of the other 19 genotypes of MeV. Another limitation of the study was that only a small number of specimens (8 suspected cases) were detected using the allelic discrimination rRT-PCR, which may have been insufficient to precisely evaluate the sensitivity and specificity of the assay. Therefore, the assay should be further validated with different genotypes of MeV as well as a large number of clinical samples.
In conclusion, the process described in this study for the identification and confirmation of vaccine-associated measles cases could improve the accuracy of the measles case surveillance system. Moreover, the allelic discrimination rRT-PCR assay provides a good alternative for differentiating between vaccine and wild-type MeV.

Conflicts of interest

All contributing authors declare no conflicts of interest.

Acknowledgments

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