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Original Article

Reevaluation of Laboratory Methods for Diagnosis of Measles

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SUMMARY: The purpose of this study is to reevaluate the sensitivities of different methods used in the diagnosis of measles including virus isolation, RT-PCR, and measurement of IgM. Sixty-three throat swabs, 84 peripheral blood mononuclear cell (PBMC) samples, and 85 plasma samples were collected from 85 cases of suspected measles. The sensitivity of virus isolation using throat swabs and PBMC in comparison with RT-PCR was 58.1 and 93.5%, respectively. We defined laboratory-confirmed cases as those in which at least one of the methods was positive. The percentage of positive results by the different methods was compared among 49 laboratory-confirmed cases. The percentage of positive results from PBMC by RT-PCR and virus isolation was 100 and 91.7%, respectively. The percentage of positive results from throat swabs by RT-PCR and virus isolation was 91.2 and 52.8%, respectively. The percentage of IgM positive (79.6%) was significantly lower than that of PBMC by RT-PCR. Ten of 27 plasma samples collected within 5 days of the onset of fever were IgM negative. In contrast, all of the 21 plasma samples collected 6 days after the onset of fever were IgM positive. In conclusion, the detection of measles virus RNA in PBMC by RT-PCR was the most effective method for diagnosis of measles.

INTRODUCTION

Measles is a highly infectious respiratory virus infection, with typical symptoms that include maculopapular rash, fever, cough, coryza, and conjunctivitis (1). The introduction of the live attenuated measles virus (MV) vaccine has decreased the frequency of measles outbreaks. As the prevalence of measles has declined, laboratory confirmation has become increasingly important (2,3). The detection of MV-specific IgM antibodies is recommended as the standard method for laboratory diagnosis by the World Health Organization (WHO) (4) and is used in countries throughout the world. However, the level of IgM may be low or absent in patients sampled in the early stage of infection (5). Virus isolation is widely used as a valuable diagnostic method. The detection of MV RNA by reverse transcription-polymerase chain reaction (RT-PCR) has been shown to be very effective for the diagnosis of measles (6,7). Throat swabs and peripheral blood mononuclear cells (PBMC) are used for virus isolation and RT-PCR (8). The detection of MV depends on the time of sample collection after the onset of symptoms. Therefore, to determine the sensitivity of different methods requires the comparison of the number of days that have elapsed since the onset of fever. The purpose of this study is to compare the percentage of MV-positive results by different methods including virus isolation, RT-PCR, and the measurement of IgM for diagnosis of measles.

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MATERIALS AND METHODS

Clinical samples: From May 2007 to August 2008, a total of 85 cases were reported to the Kobe Institute of Health as measles based on the typical symptoms of measles. The median age of patients was 15 years and patients' ages ranged from 0 to 44 years. Throat swabs and peripheral blood were collected from 0 to 14 days after the onset of fever. Informed consent was obtained from patients or their guardians. Sixty-three throat swabs, 84 PBMC samples, and 85 plasma samples were collected from 85 cases. Both virus isolation and RT-PCR were performed on 58 throat swabs and 54 PBMC samples. The samples were kept at 4°C until inoculation for virus isolation. The throat swabs were eluted into 2 ml of virus transport medium (bovine serum albumin, penicillin/streptomycin, amphotericin B in Dulbecco's modified Eagle medium) and centrifuged at 3,000 rpm for 10 min and filtered through 0.45 μm filter membrane. The throat swabs for RNA extraction were stored at −80°C until use. Two milliliters of peripheral blood was treated with EDTA, and plasma was collected after centrifugation at 3,000 rpm for 10 min. PBMC were separated by Ficoll-Hypaque graduation centrifugation. PBMC used for RNA extraction were resuspended into 1 ml of cellbanker +1 (Zenoaq, Fukushima, Japan) and stored at −80°C until use.

Virus isolation: Throat swabs and PBMC were inoculated with B95a cells and cultured at 37°C. When multinucleated giant cells were observed, MV isolation was confirmed by an indirect immunofluorescent test using an antibody specific to MV (9).

RNA extraction: RNA was extracted from 140 μl of throat swabs using the QIAamp Viral RNA Mini Kit (Qiagen, Tokyo, Japan) and from PBMC with the

QIAamp RNA Blood Mini Kit (Qiagen). RNA was resuspended in RNAase free H_2O or AVE buffer and stored at −80°C.

Nested RT-PCR: The primer sets for the nested RT-PCR were recommended by the National Institute of Infectious Diseases of Japan (10,11). Each procedure including RNA extraction, first and nested RT-PCR, and agarose gel electrophoresis was performed in separate laboratories to avoid laboratory contamination. The MV RNA was first converted to cDNA using PrimerScript™ RT Reagent Kit (Takara, Otsu, Japan). Reverse transcription was performed at 37°C for 15 min. The first and nested PCR were performed using PerfectShot™ Ex Taq (Takara). The 50 μl-reaction mixture used for the first PCR contained 20 pmol of each primer. The first PCR was performed with primers specific for the H gene (forward, AACGGATGAT CCAGTGATAG and reverse, TTGAATCTCGGTAT CCACTC) and involved 30 cycles of 98°C for 10 s, 53°C for 30 s, and 72°C for 1 min. The nested PCR was performed with the following primer set: forward, TACCTCTCATCTCACAGAGG and reverse, CAC CTAAGGCTAGGTTCTTC. The 50 μl-reaction mixture used for the nested PCR contained 20 pmol of each primer. Five microliters of the first round of PCR products was amplified. Amplification was performed via 30 cycles of 98°C for 10 s, 55°C for 30 s, and 72°C for 1 min.

Anti-MV IgM antibodies: MV-specific IgM antibodies in plasma were assayed using Measles IgM-EIA (Denka Seiken, Tokyo, Japan).

Statistical analysis: The percentage of IgM-positive results was compared with virus isolation and RT-PCR using the chi-square test. P values of <0.05 were considered to be statistically significant.

RESULTS

Table 1 shows the comparison of RT-PCR with virus isolation. Of the 58 throat swabs, 18 samples were virus isolation positive and 40 samples were virus isolation negative. Of the 40 virus isolation-negative throat swabs, 13 throat swabs were found to be positive by RT-PCR, indicating that the sensitivity of virus isolation using throat swabs was 58.1%. Of the 54 PBMC samples, 29 samples were virus isolation positive and 25 samples were virus isolation negative. Of the 25 virus isolation-negative throat swabs, 2 throat swabs were found to be positive by RT-PCR, indicating that the sensitivity of virus isolation using PBMC was 93.5%. All virus isolation-positive samples from throat swabs and PBMC were found to be positive by RT-PCR, indicating that the specificity of virus isolation was 100% when compared with RT-PCR. Forty-nine cases were diagnosed as measles on the basis that at least one of the methods showed a positive result.

The onset day was not known in 1 of 49 cases. Table 2 shows the detection of MV in throat swabs and PBMC in accordance with the sampling time in days after the onset of fever among 48 laboratory-confirmed measles cases. All throat swabs and PBMC collected from 4 to 5 days after the onset of fever were found to be MV positive by either virus isolation or RT-PCR. The MV-positive ratio by virus isolation was lower in throat swabs

Table 1. Comparison of RT-PCR with virus isolation

Throat swab

RT-PCR	Virus isolation		Total
	Positive	Negative	
Positive	18	13	31
Negative	0	27	27
Total	18	40	58

PBMC

RT-PCR	Virus isolation		Total
	Positive	Negative	
Positive	29	2	31
Negative	0	23	23
Total	29	25	54

Table 2. Detection of measles virus by days after fever onset

Throat swab

Days after fever onset	Virus isolation			RT-PCR		
	Positive	No. of test	% of positive sample	Positive	No. of test	% of positive sample
0–3	2	6	33.3	4	6	66.7
4–5	11	11	100	10	10	100
6–12	5	18	27.8	16	17	94.1

PBMC

Days after fever onset	Virus isolation			RT-PCR		
	Positive	No. of test	% of positive sample	Positive	No. of test	% of positive sample
0–3	5	7	71.4	5	5	100
4–5	19	19	100	12	12	100
6–12	19	21	90.5	13	13	100

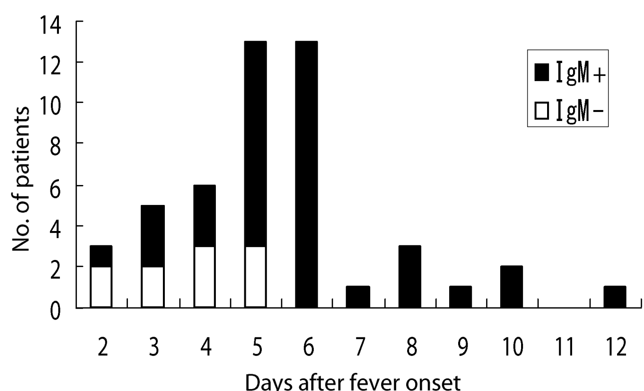

Fig. 1. The results of IgM positivity in plasma from measles patients by days after fever onset. Forty-eight plasma samples were collected from 48 cases with laboratory-confirmed measles cases.

Table 3. Comparison of diagnostic methods in measles cases

Sample	Method	No. of test	No. of positive sample	% of positive sample
PBMC	RT-PCR	31	31	100
	Isolation	48	44	91.7
Throat swab	RT-PCR	34	31	91.2
	Isolation	36	19	52.8[1)]
Plasma	IgM	49	39	79.6[2)]

49 cases were laboratory-confirmed measles cases. Number of test indicates the sample numbers tested by each method from 49 laboratory-confirmed measles cases.
[1)]: *P* value, 0.0004 compared with throat swabs by RT-PCR.
[2)]: *P* value: 0.007 compared with PBMC by RT-PCR.

collected from 0–3 days or 6–12 days after the onset of fever than those collected 4–5 days after the onset of fever. Thirty-nine of the plasma samples were IgM positive. Ten IgM-negative cases involving throat swab or PBMC were found to be positive by virus isolation or RT-PCR. All plasma samples collected 6 days after the onset of fever were IgM positive. In contrast, plasma collected within 5 days of the onset of fever showed both IgM positive and negative results (Fig. 1). Table 3 shows the percentage of positive samples according to diagnostic methods used in 49 laboratory-confirmed measles cases. One hundred percent and 91.7% of PBMC samples were found to be positive by RT-PCR and virus isolation, respectively. In contrast, the percentage of positive results in the throat swabs by virus isolation was significantly lower when compared with RT-PCR. The percentage of IgM-positive samples was significantly lower than that of PBMC by RT-PCR.

DISCUSSION

In this study, the numbers of samples used for virus isolation, RT-PCR, and measurement of IgM were different because throat swabs and peripheral blood were not collected from all patients. All laboratory-confirmed cases had symptoms such as fever, maculopapular rash, cough, coryza, and/or conjunctivitis. We consider that throat swabs and peripheral blood samples were collected from patients with cases of measles. We demonstrated that the sensitivity of RT-PCR was higher than that of virus isolation and that the virus isolation-positive ratio from throat swabs was lower than that obtained from PBMC. We have reported that the minimum amount of MV RNA detectable by SYBR Green real-time RT-PCR is 10 copies and that the sensitivity of nested RT-PCR is similar to that of SYBR Green RT-PCR (11). We have also previously detected MV RNA from virus stock containing 1 PUF/0.1 ml samples and virus isolation-negative throat swabs (11). It has been reported that MV isolation is less reliable as a diagnostic tool than serology or RT-PCR (7). The RT-PCR is able to detect MV RNA from samples containing noninfectious MV. We showed that the MV isolation rate from PBMC was higher than that from throat swabs. We kept the samples at 4°C and avoided freeze-thawing procedures. The lower virus isolation rate in throat swabs may have been caused by the loss of MV infectivity during handling or transportation. In addition, throat swabs were centrifuged and filtered through a membrane to remove bacteria. Therefore, the cell-associated MV in throat swabs might have been removed by the centrifugation or filtration before inoculation to B95a cells. There is no significant difference between the percentage of MV detection in PBMC by virus isolation and RT-PCR. Lymphocytes and monocytes associated MV is able to be isolated from B95a cells by cell-to-cell spreading. In this study, MV was detected in throat swabs and PBMC from all cases by either virus isolation or RT-PCR 4 to 5 days after the onset of fever. The virus load is considered to be greatest in this phase. The detection rate of MV in throat swabs decreased 6–12 days after fever onset. The infectivity of MV decreases after the appearance of a rash due to an increase in the level of IgG antibody. Therefore, the timing of specimen collection after the onset of fever can affect the detection rate of MV.

WHO recommends the detection of MV-specific IgM antibodies as the reference standard for laboratory diagnosis. It has been reported that the detection of measles-specific IgM is the standard test for laboratory diagnosis of measles (4). Single serum samples can be used to diagnose measles cases if collected between 72 h and 4 weeks after rash onset using an IgM capture enzyme immunoassay (EIA) (5). The negative result for IgM in the early stage of infection does not always imply negativity of MV infection. In this study, the measurement of IgM antibodies was insufficient to diagnose measles within 5 days of the onset of fever. WHO recommends that laboratories request a second sample for repeat IgM testing (4). In this study, we used throat swabs and peripheral blood as diagnostic samples. It has been reported that MV was isolated from urine in patients with measles for up to 5 days (12) and MV RNA was detected in urine from vaccine recipients (13). Urine samples are useful for diagnosis of measles because they are easy to collect. Therefore, a comparison of sensitivity and specificity using urine samples, throat swabs, and PBMC is necessary.

In conclusion, RT-PCR of PBMC is the most effective diagnostic method for the diagnosis of measles.

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