Evaluation of Diagnostic Markers for Measles Virus Infection in the Context of an Outbreak in Spain

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A measles outbreak occurred from January to July 2003 in Spain, despite the fact that the Plan of Eradication of Measles and its surveillance program had been set up in 2001. Different diagnostic markers for measles virus infection were compared for 246 patients in tests of serum, urine, and pharyngeal exudate specimens. Measles virus immunoglobulin M (IgM) and IgG and rubella virus and parvovirus IgM levels in serum were assayed. Multiplex PCR was done on urine, serum, and pharyngeal exudates, and isolation of measles virus in the B95a cell line from urine was attempted. At least one positive marker for measles virus was obtained from 165 patients (67.1%; total number of patients, 246). A total of 136 cases (82.4% of the patients showing positive markers) were diagnosed by PCR and/or isolation and IgM detection methods. The results for 27 patients (16.4%) were positive only by direct methods. The results for two patients (1.2%) were positive only by IgM detection. In the case of the first group (136 cases), the time elapsed from appearance of the rash was significantly longer than in the case of the group which was only positive by PCR. Besides, 8 out of 27 PCR-positive IgM-negative cases showed specific IgG results, suggesting either secondary vaccine failure or reinfection. Numbers resulting from PCR performed with pharyngeal exudates proved to be significantly higher than those obtained with other specimens. Phylogenetic analysis showed the presence of genotype B3. The results strongly back the World Health Organization recommendation that detection of IgM should be supplemented by PCR and isolation for the diagnosis of measles virus infection.

Measles virus (MeV) is a highly contagious virus which produces a usually mild vaccine-preventable exanthematic disease in children. Nevertheless, the complications of this illness are a common cause of death in developing countries, where poor nourishment or immunodeficiency predisposes the patient to secondary infections such as bacterial pneumonia or other fatal diseases, for example, encephalitis or severe diarrhea (10).

Measles continues to be a menace to millions of children worldwide. Currently, the World Health Organization (WHO) has set up a number of programs starting in 1999 to reduce measles mortality worldwide by 50% by the end of 2005 and to reduce mortality as a whole by two-thirds by the year 2015 for children less than 5 years of age. Even though the rate of measles-related mortality decreased by 39% between 1999 and 2003, reducing deaths from 873,000 to 530,000, measles virus continues to be a leading cause of morbidity and mortality among children in developing countries (7).

Although the region of the Americas is near its goal of eliminating the virus, the remainder of regions are immersed in different stages of their programs, from an initial decrease of measles-related mortality in most African and southeast Asian countries to the more advanced situation of eradication pro-

grams in the European region. The WHO Regional Office for Europe has set the interruption of indigenous transmission of measles virus and the prevention of congenital rubella virus infection as objectives for 2010.

The vaccination policy in Spain differs between regions (autonomous communities). Generally, the first dose of measles, mumps, and rubella vaccination is given to children at age 15 months and the second at 3 to 6 years of age. In 1996 seroprevalence of measles virus antibodies was over 90% in all age groups, reaching 98% in patients over 20 years of age (1). According to the Spanish Measles Eradication Plan, surveillance should be conducted on every case, and every case must be reported and investigated immediately. Laboratory specimens should be collected and analyzed for measles virus infection markers in every suspected case.

Laboratory diagnosis of MeV infection is a basic tool for the surveillance program. This is mostly based on the detection of immunoglobulin M (IgM) in serum by use of different approaches, indirect enzyme immunoassays (ELISA) being the most widely used (15). On the other hand, viral isolation allows us to obtain the strain for epidemiological studies. However, the sensitivity of the isolation method is low and very dependent on the time of sample collection and transport conditions; the optimal time for virus culture sampling is very early after the onset of symptoms, when specific IgM is not detected (11, 14). Some reports show that genomic detection techniques, namely, PCR, notably improve the performance of the culture method (8) and should consequently be included in measles

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FIG. 1. Distribution of results of detection of IgM in serum (IgM), serum PCR results (PCR S), pharyngeal exudate PCR results (PCR Phar), urine PCR results (PCR Ur), and isolation of MeV (Cult Ur) in percent urine positivity per day after the start of the rash. The number of cases received per day is shown. *, number of specimens received per day.

surveillance protocols. However, there is little available data on the behavior of the PCR techniques as a diagnostic tool in the context of outbreaks.

The aim of this report is to evaluate different infection markers for acute measles virus infection in the setting of a measles outbreak. It compares the efficacy of PCR diagnosis versus classical techniques, such as IgM detection and virus cell culture isolation, in a real situation.

MATERIALS AND METHODS

Specimens. This report studies 246 patients with a complete set of three specimens, namely serum, urine, and pharyngeal exudate specimens. A total of 128 (52.1%) of the patients studied were men and 118 (47.9%) were women, with a mean age of 15.5 ± 12.5 years (mean age $\pm$ standard deviation) and a range of 0 to 60 years. A total of 116 (47.15%) patients with suspected cases of measles virus infection were 20 to 35 years old, and 44 (17.9%) were under 15 months of age. The number of samples collected in relation to the time elapsed from appearance of the rash is shown in Fig. 1. Specimens were collected and processed following WHO recommendations (3). Most of the specimens were sent as soon as collected without freezing, at a storage temperature 4°C, while other were sent in dry ice. All cases came from the same measles outbreak, which affected 317 people on the Almeria coast (Andalusia, Spain) from January to June 2003.

Serological assays. Sera were tested for the presence of MeV-specific IgM, rubella virus (RUBV)-specific IgM, and parvovirus B19 (B19V)-specific IgM and the presence of MeV IgG and for the avidity of MeV IgG. Commercial kits (Enzygnost, Dade Behring, Germany) based on indirect ELISA were used for the detection of MeV-specific and RUBV-specific IgM, and μ -chain capture ELISA was used for the detection of RUBV and parvovirus B19 IgM (respectively, Platelia Rubella IgM [Bio-Rad, Marseille, France] and Parvovirus B19 IgM EIA [Biotrin, Dublin, Ireland]). The tests were run according to manufacturers' instructions. Equivocal results obtained with MeV IgM were also considered positive cases by serology in the absence of a second serum sample.

MeV IgG avidity assays. An Enzygnost avidity kit was used following the manufacturer's instructions. The avidity indexes (AI) were calculated by assigning a 100% value to the mean value of reduction, after two determinations of the positive control treated with the avidity reagent in each assay. All determinations were compared with the corrected value, which was different for each assay. This value was the corrected AI. Hence, comparable interassay values were obtained.

Direct detection. Total nucleic acids were extracted from samples by use of a MagNA Pure LC external lysis protocol automatic extractor (ROCHE, Mannheim, Germany). As a part of the internal control system, a plasmid unrelated to the target viruses was included in the lysis buffer. A previously described multi-

plex reverse transcription-PCR (RT-PCR) method (12) for analysis of MeV, RUBV, and B19V was attempted with all three type of samples. Briefly, a coupled reverse transcription-amplification reaction was performed using an Access RT-PCR system kit (Promega, Madison, Wis.). The measles primers used for the retrotranscription and first reaction were Sar1F (5'CGGAGCTAAGAA GGTGGATAA3') and Sar1R (5'CTCCCATGGCATAGCTCCA3'), while the ones used in the nested reaction were Sar2F (5'CYAGGATTGCTGAAATGA TATG3') and Sar2R (5'AAYTTGTCTGAATTGAGTTCTC3') (12). A pair of primers specific for the internal control plasmid was also included. For the nested reaction, 1 μ l of the primary amplification products was added to 49 μ l of a new PCR mixture containing nested instead of primary reaction primers and internal-control-specific nested reaction primers. PCR products were visualized and sized by gel electrophoresis in 2% agarose containing ethidium bromide and were visualized under UV light.

Pharyngeal exudates from negative cases were tested for enteroviruses, herpes simplex virus, cytomegalovirus, Epstein-Barr virus, varicella-zoster virus, and human herpesvirus 6 by use of a multiplex PCR (4) as well as by PCR for adenoviruses (2). Two different aliquots of each sample were taken at the time of admission to our laboratory. One aliquot per specimen was tested. In the case of any positive result, the result obtained was confirmed with the second aliquot and only correlated results were considered positive. Unconfirmed positives were counted as negatives. Aliquot tests and PCR tests were run by different laboratories and technicians.

MeV isolation on the B95a cell line was attempted in urine specimens, following WHO recommendations (3); urine was centrifuged at 4°C, and 2 ml of medium was added to the sediment, decontaminated, and inoculated in a cell culture at 75 to 80% confluence.

RESULTS

Indirect and direct detection. A total of 165 cases out of 246 were positive for at least one MeV infection marker for 84 (50.9%) males and 81 (49.1%) females. The mean age in years for these MeV-positive patients was 16 ± 11.32 ; the patients ranged from less than 1 year of age to 44 years of age, although 58.2% (96) were 20 to 35 years old and 19.4% (32) were 15 months old or less (Fig. 2).

Most (136) of the 165 MeV-positive cases were positive by both direct detection and serology, while 27 were positive only by direct detection and 2 only by IgM detection (Fig. 3). The elapsed time between the onset of the rash and sample collection was significantly lower for the 27 patients which were

FIG. 2. Ages of measles virus-positive patients. A. Patients from less than 1 to 45 years of age. B. Patients less than 2 years of age.

positive only by direct detection compared to the 138 IgM-positive patients: 0.9 days (95% confidence interval, 0.4 to 1.4) versus 1.8 days (95% confidence interval, 1.5 to 2.1) ($P < 0.05$ by the chi-square method).

An MeV IgM-positive result or an equivocal result was obtained in 138 cases. A total of 105 serum, 161 pharyngeal exudate, and 152 urine specimens gave a positive result for MeV by multiplex PCR. Seventy-three urine specimens yielded MeV virus for the B95a cell line. PCR was significantly more effective than isolation as a diagnostic marker, as shown by the chi-square method, corrected by the Fisher exact two-tailed test P value ($P < 0.05$). PCR was also significantly higher ($P < 0.005$) when applied to pharyngeal exudates, in comparison

with PCR on urine. Moreover, PCRs using both urine and pharyngeal exudate specimens were significantly more effective than IgM detection (Fisher exact two-tailed test P value < 0.01).

The index case was a sailor who had come from an Algerian port and disembarked in Almería and whose genotype (B3) was determined retrospectively in a stored serum sample directly by PCR and sequencing (13).

MeV IgG avidity assays. To establish the characteristic of the assay, 22 MeV IgG-seropositive healthy adults were included as a control group. The results for 39 patients with positive PCR and IgM detection (9 with positive PCR results only, 2 with positive IgM results only, and 69 with no positive results) were compared with the control group results. From the 22 controls we obtained a mean corrected AI value of 99.5%. The mean corrected AI values were 65.2% for the 39 cases with positive PCR and IgM results, 73% for the 9 samples from cases with PCR-positive results only, and 83.6% for the 69 cases with negative results for both approaches.

IgM-negative cases. Twenty-seven cases which were positive in one or more specimens by direct detection were negative with IgM detection (Table 1). Seventeen cases were IgG negatives. For 9 out of the 10 remaining IgG-positive cases, IgG avidity was tested. Patient 1 showed an avidity index clearly higher than the mean values seen with recent infection cases, suggesting reinfection. The remaining IgG positives were probably false-negative IgM cases, since avidity values were below those obtained in the cases with PCR- and IgM-positive results. In case 10, the only positive finding was viral isolation, this being a probable PCR and IgM false-negative result, bearing in mind the relatively low IgG avidity. However, the possibility of a false positive resulting from the isolation method could not be dismissed.

In relation to IgG-negative results, in six cases there was a known antecedent of recent vaccination (1 to 13 days before onset; patients 11 and 17 to 21). Of these, the case of patient 18 only was confirmed, 10 days after vaccination, as having been caused by a genotype A, which includes the vaccine strains.

On the other hand, the samples collected more than 3 days after the appearance of the rash (patients 23 to 26) could be classified as probable IgM false negatives, since more than 88% of the samples taken at 4 days from onset gave IgM-positive results, as shown in Fig. 1. The remaining cases were

FIG. 3. Table of results obtained by the laboratory in the Almería outbreak.

TABLE 1. Results for IgM-negative cases

Patient	Age	Tsympt ^a	No. of Doses (time to rash) ^b	Result for ^c :					Measles virus IgG avidity ^d (%)	MeV genotype	Interpretation ^e
				Urine culture	PCR using serum	PCR using pharyngeal exudates	PCR using urine	IgG (UI/ml)			
1	27 yr	6	UNK	N	N	P	N	18,325	84	B3	Reinfection
2	30 yr	1	1 (9 days)	N	N	P	N	720	ND	B3	Reinfection/F- IgM
3	31 yr	3	1 (7 days)	P	N	P	P	700	25	B3	F- IgM
4	24 yr	0	1 (13 yr)	N	N	P	P	630	50	B3	F- IgM
5	22 yr	2	UNK	N	N	P	N	290	34	B3	F- IgM
6	21 yr	2	UNK	N	N	P	P	2,700	65	B3	Reinfection/F- IgM
7	22 yr	3	UNK	Cont	P	P	P	3,000	50	B3	Reinfection/F- IgM
8	25 yr	3	UNK	P	P	P	P	400	33	B3	F- IgM
9	34 yr	4	UNK	N	N	P	N	1,100	50	B3	F- IgM
10	5 yr	9	2 (20 mo)	P	N	N	N	1,297	54	B3	F+ culture/F- IgM
11	10 yr	0	1 (13 days)	P	P	P	P	N		B3	Early collection
12	10 yr	0	0	N	P	P	P	N		B3	Early collection
13	13 yr	0	1	N	P	P	P	N		B3	Early collection
14	20 yr	0	UNK	P	P	P	P	N		B3	Early collection
15	5 mo	1	0	P	P	P	P	N		B3	Early collection
16	20 yr	1	UNK	P	P	P	P	N		B3	Early collection
17	27 yr	2	1 (10 days)	N	I	P	P	N		B3	Early collection
18	34 yr	2	1 (10 days)	N	N	P	P	N		A	Vaccine
19	22 yr	3	1 (1 day)	Cont	P	P	P	N		B3	Early collection
20	26 yr	3	1 (1 day)	P	P	P	P	N		B3	Early collection
21	11 mo	3	1 (7 days)	N	P	P	P	N		B3	Early collection
22	27 yr	3	UNK	N	P	P	P	N		B3	Early collection
23	25 yr	4	0	P	P	P	P	N		B3	F- IgM
24	9 mo	4	0	N	N	P	N	N		B3	F- IgM
25	20 yr	4	UNK	P	P	P	P	N		B3	F- IgM
26	26 yr	4	UNK	Cont	P	P	P	N		B3	F- IgM
27	23 yr	UNK	1	P	P	P	P	N		B3	

^a Tsympt, time from onset of rash to specimen collection.^b Number of vaccine doses (time from administration to onset of rash). UNK, unknown.^c P, positive; N, negative; Cont, contaminated; I, inhibited.^d Result for MeV-specific IgG.^e F+, false positive; F-, false negative.

collected too early to obtain a positive result from the IgM assay.

DISCUSSION

This record shows the performance of direct and indirect diagnosis of measles in the setting of an outbreak in a restricted geographical area. The outbreak affected mostly either unvaccinated young adults over 20 years of age or children less than 15 months of age. The cause of this particular age distribution could be the fact that measles, mumps, and rubella vaccination at 15 months was introduced into Andalusia's general vaccination schedule in 1981, causing a decrease in the incidence of the illness in the following years as the number of the vaccinated children increased. This geographically restricted subset of susceptible young adults was not detected during a serological survey undertaken in 1996 in the whole region ("autonomous community") of Andalusia (9). This shows how an imported MeV genotype is able to produce an outbreak of more than 200 cases in areas with a high level of vaccine coverage and established surveillance programs, including serological surveys (5, 6).

Classically, measles diagnosis was based only on IgM detection, given the low sensitivity of the isolation. However, our results show that RNA detection by RT-PCR, using both urine and, especially, in pharyngeal exudate specimens, provides

more-sensitive markers. This could be due to early sampling in the cases studied. In this study most of the samples were taken at day 0 to 3 after onset of the rash and showed a higher detection rate for RNA in pharyngeal exudates (72.2%) than for specific IgM in serum (65.8%). It has been stated in publications that the IgM serum antibody level peaks within 2 days after onset of the rash (18). However, researchers in other studies found the RNA detection rate in throat swab specimens (98%) higher than that of MeV-specific IgM (83%) during the first 3 days after onset of the rash (17), in agreement with our results. Direct detection also enables diagnosis of cases of IgG-positive patients as reinfections after natural immunity or secondary vaccine failures which develop with no IgM production (Table 1).

A rate of 63.6% of our sera were positive by PCR, in contrast to another report that showed amplification in only 24% (16) of cases. Again, early sampling could account for this difference. Although RT-PCR performed with both pharyngeal exudate and urine specimens provides more-sensitive diagnostic markers, direct genomic amplification in serum must be attempted for genotyping, in the absence of more adequate samples. In our case, this made it possible to trace the index case of the outbreak.

RT-PCR was a more sensitive direct-detection technique than isolation in cell cultures, despite the high (44.2%) rate of

isolation from urine in comparison to the rates reported for other studies, 22% (17) and 18% (18). In spite of the fact that the genotype can be obtained through direct genomic amplification, viral isolation provides live virus, which is useful for a more extensive characterization of the strains involved in the outbreaks.

Most protocols for measles diagnosis included in active surveillance programs recommend urine and pharyngeal exudate sampling for virus recovery within the first 5 days after onset of the rash but also serum sampling for IgM diagnosis after the first week, to obtain optimal results for both viral isolation and IgM detection. Consequently, two patient visits are necessary to ensure a sensitive diagnosis. A single set of serum, pharyngeal exudate, and urine samples taken on the same day as the first clinical diagnosis is sufficient to obtain the most rapid and sensitive laboratory diagnosis, as well as full genetic characterization of the virus, if RT-PCR techniques are included in the protocols, in combination with IgM detection. Cases positive only by PCR should be confirmed by serology in a convalescent-phase serum if possible. Multiplex PCR will provide data on differential diagnosis with RUBV and B19V, without any additional effort. Finally, viral isolation will provide further characterization of the antigenic and phenotypic characteristics of the strain.

Thus, laboratory protocols used in the epidemiological surveillance of measles in the context of eradication programs should be reviewed in countries with access to genomic amplification technologies, considering the usefulness of RT-PCR.

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