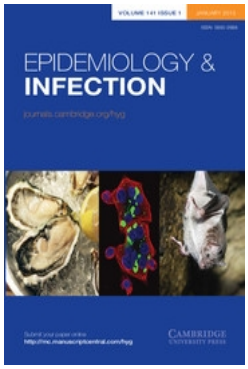

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Use of RT–PCR on oral fluid samples to assist the identification of measles cases during an outbreak

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SUMMARY

This study investigated the occurrence of mild modified measles cases during an outbreak in Niterói, RJ, Brazil by using RT–PCR on oral fluid samples. From August to December 1997 a total of 76 patients with rash were seen at the study sites. Confirmed diagnosis by serology was achieved in 47 cases: measles (39·5%), rubella (13·2%), HHV-6 (3·9%), human parvovirus B19 (3·9%), dengue fever (3%). For 19 of the 29 patients without a conclusive diagnosis paired serum and saliva samples were available for further tests. In four of them, measles virus RNA was detected by RT–PCR in saliva samples in the absence of specific IgM in serum samples. Vaccination histories obtained from three of the RT–PCR positive cases showed that individuals previously immunized can still be infected and contribute to the circulation of measles virus. This study demonstrated the usefulness of RT–PCR on non-invasive clinical samples for the investigation of measles cases.

INTRODUCTION

In Brazil, measles control had been achieved through mass vaccination campaigns, administered regardless of vaccination status, to pre-school and school-age children [1, 2]. However, in 1997 there was a re-emergence of measles with more than 53 000 confirmed cases reported from nearly all Brazilian states [2]. A shift in the age distribution of measles incidence towards older age groups was seen and it was more marked in states that had achieved better results in the control of the disease [3]. Co-ordinated efforts were implemented to raise vaccination coverage and to enhance measles surveillance, and the outbreak was controlled with success [2].

In the elimination programme for measles, the identification of cases is of utmost importance [1]. Moreover, it is also necessary to understand the clinical and epidemiological features of measles in highly immune populations [4]. Recent studies have indicated that measles virus can circulate in vaccinated people, causing mild symptoms or even asymptomatic infections [4–7]. Although some data have suggested that the occurrence of clinically atypical cases will probably not impede efforts to control and eradicate measles, further studies are necessary to define the role of those cases in measles transmission [4].

In January 1994 we set-up a study to define the aetiology of rash diseases in Niterói, state of Rio de

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Janeiro, Brazil. In that study, we searched for the viral infections most commonly causing rash diseases in Brazil: measles, rubella, dengue fever, human parvovirus B19, and among young children, human herpesvirus type 6 [8]. As reported for some states of the country, a measles outbreak occurred in this municipality during the second part of 1997 [8]. In a recent paper we described two cases of measles within the same household that were involved in this outbreak [9]. Measles diagnosis was confirmed serologically by specific IgM detection in one unvaccinated case who had presented with typical measles symptoms. The other case, with a history of two vaccinations with measles-containing vaccine had presented with mild symptoms (2 days of fever, myalgia and a maculopapular rash on face and neck for only 1 day); measles diagnosis was confirmed by raising specific IgG in the absence of IgM. Measles virus RNA was detected by reverse transcriptase polymerase chain reaction (RT–PCR) in saliva samples from both cases. The hypothesis of a mild modified measles case was suggested only by the knowledge of an epidemiological link to the index case who had presented with classical measles symptoms during the outbreak.

The detection of an atypical measles case in this epidemiological context raised the possibility of occurrence of other similar cases during the same outbreak. As at that time we were involved in a survey of the aetiology of rash diseases [8], we decided to search for other mild modified measles cases in the study group. This paper reports our findings and their implications for measles surveillance.

METHODS

Subjects and sample collection

From August to December 1997, during a measles outbreak in Niterói, RJ, 76 patients with acute rash, with or without fever were seen at a large primary healthcare unit and at a public general hospital in the city. A standard clinical examination was performed and blood and oral fluid samples were collected simultaneously. Doctors at these clinics were provided with kits containing instructions and equipment for the collection of blood and saliva samples.

A questionnaire was designed for the study and each case was interviewed regarding measured or reported fever, influenza-like symptoms, arthropathy, lymphadenopathy and other symptoms, complications and exposure to other cases of exanthematic diseases.

A clotted blood sample for serology collected in a sterile glass tube was obtained at the time of consultation. A commercial device (OraSure, Epitope, Beaverton, OR, USA) was used to collect the saliva specimen, as described elsewhere [9]. The oral fluid and serum samples were stored at $-20\,^{\circ}C$ until tested. Informed consent was obtained for all participants and from the parents or guardians of patients younger than 18 years of age. The study was approved by the hospital's Institutional Review Board.

Laboratory tests

All serum samples were tested for the presence of rubella virus IgM by a commercial enzyme immunoassay (EIA) (Rubenostika IgM, Organon), for measles virus IgM using an antibody capture EIA developed at the Centers for Disease Control (Atlanta, GA, USA) [10], and for dengue virus IgM by an in-house EIA [11, 12]. Specimens negative for rubella, measles and dengue virus IgM were also tested for human parvovirus B19 IgM using an antibody capture EIA (MACEIA) [13, 14]. An indirect immunofluorescence test for human herpesvirus type 6 (HHV-6) IgG [15] was also used to detect low avidity HHV-6 IgG (indicating recent primary infection) in children less than 5 years of age without an alternative diagnosis.

Those cases negative for all the viruses tested were further evaluated. Serum samples were tested for the presence of measles-specific IgG antibodies by a commercial EIA (Enzygnost® anti-measles-virus/IgG, DADE Behring, Germany). Oral fluid samples were tested for the presence of measles virus RNA by RT–PCR [16]. The usual precautions to prevent false-positive results due to cross-contamination in the laboratory were observed [17] including separate areas with a one-way work flow for the preparation of reagents, the extraction of nucleic acid from clinical samples, the addition of template to PCR reaction mixtures and the post-PCR analysis of amplified DNA: pipettes with plugged tips, reagents dispensed in small aliquots to minimize handling and negative and low-copy-number positive controls. Sequencing reactions were performed using the Taq Dye Deoxy terminator cycle sequencing kit in an ABI 373A automatic DNA sequencer (Applied Biosystems, Warrington, UK). Nucleotide and deduced amino-acid (aa) sequences were analysed with the SeqEd. V1.0.3 program and Clustal of the Megalign program, a multiple alignment program of the DNASTAR package.

Table 1. *Results of RT–PCR in saliva samples according to the onset of rash*

	Days of rash							
RT–PCR	1	2	3	4	5	6	7	8	Total
Positive	1 (1)*	1	—	—	—	1 (1)	1 (1)	—	4 (3)
Negative	2 (1)	1	—	4 (3)	1 (1)	4 (3)	2 (2)	1	15 (10)

* Number of cases with history of measles vaccination in the past within parentheses.

Table 2. *Distribution of the most common signs and symptoms observed in the study cases according to RT–PCR results**

Signs and symptoms	RT–PCR positive ($n=4$)	RT–PCR negative ($n=15$)
Fever	4	10
Cough	2	4
Coryza	1	4
Conjunctivitis	—	3
Lymphadenopathy	—	5
Arthropathy	2	5
Tonsilitis	—	2
Headache	1	1
Pruritis	—	3

* All patients presented with rash.

Table 3. *Day of onset of rash and frequency of measles virus-specific IgG in serum samples according to RT–PCR results of the study group*

	RT–PCR positive*		RT–PCR negative	
Day of onset of rash	IgG		IgG	
	Positive	Negative	Positive	Negative
1	—	1	2	—
2	1	—	1	—
3	—	—	—	—
4	—	—	2	2
5	—	—	—	1
6	—	—	4	—
7	—	1	1	1
8	—	—	—	1
Total	1	2	10	5

* In one case the IgG test was not performed.

RESULTS

There were no refusals to enrol in the study. From August to December 1997, 76 patients with an exanthematous rash were seen at the study sites. A laboratory-confirmed serological diagnosis was achieved in 47 (61·8%) cases investigated: measles (30 cases; 39·5%), rubella (10 cases; 13·2%), HHV-6 (3 cases; 3·9%), human parvovirus B19 (3 cases; 3·9%), dengue fever (1 case; 1·3%). No diagnosis was established in 29 (38·2%) cases and none of them developed symptoms consistent with classical measles [8].

For 19 of the 29 patients without a conclusive diagnosis, paired serum and saliva samples collected within 8 days of the onset of the rash were available for further tests (measles virus-specific IgG antibodies by EIA and RT–PCR, respectively) and these cases were the subjects of the study. No patient of the study group reported exposure to a suspected or a laboratory-confirmed measles case.

Overall, in 4 (21·0%) of the 19 cases measles virus RNA was detected by RT–PCR in oral fluid samples (Table 1). Measles virus RNA amplified was sequenced and the strain identified as genotype D6 in all four samples according to WHO designation [18].

Two of the four PCR-positive patients fulfilled the criteria of clinically suspected measles case used by the Brazilian Health Ministry [19], i.e. presence of a generalized maculopapular rash of $\geqslant 3$ days' duration, fever, and at least, one of the following: cough, coryza or conjunctivitis. The other two cases presented with fever and rash, without catarrhal symptoms (Table 2). One patient was 1 year old and the other three ranged from 18 to 24 years. The four PCR-positive cases were seen at the study sites in August ($n=1$), September ($n=2$) and October ($n=1$). None of them had a history of measles in the past. Vaccination histories were obtained from vaccination card ($n=1$) and self-report ($n=2$). Only one patient (1 year old) had a documented history of two doses of measles vaccine. One of the three cases tested for measles virus-specific IgG was positive (Table 3), but information about vaccination status was not available for this case.

Seven of the 15 PCR-negative patients met the criteria of a clinically suspected measles case as mentioned above [19]. The median age of the 15 cases was 11·9 years (range: 9 months to 47 years). Vaccination histories were obtained from vaccination card ($n=1$)

and self-report ($n = 9$). Six patients had a history of two doses of measles vaccine and the four remaining cases only one dose. Only one patient reported classical measles in the past. Overall, 10 of the 15 cases tested for measles virus-specific IgG were positive. Of them, seven cases had been vaccinated previously.

DISCUSSION

A fourfold or greater rise in specific IgG antibodies in paired serum samples or the presence of specific IgM antibodies in a single serum specimen have been the methods of choice to diagnose measles virus infections [6]. Whereas the collection of paired serum for IgG tests has low acceptability, field experience has demonstrated the usefulness and reliability of IgM EIAs for confirmation of suspected measles cases [10, 20]. Although highly sensitive and specific, laboratory diagnosis by IgM serology can vary depending on the timing of specimen collection. Helfand et al. [21], using an antibody-capture EIA, showed that the IgM was positive in 77% of specimens obtained within 72 h of rash onset and 100% of specimens obtained 4–11 days after rash onset. Moreover, failure to detect an IgM response in asymptomatic or non-classical measles infections among partly immune persons has been reported by some authors [4, 9, 22]. Although negative IgM results may occur in these conditions, a single specimen obtained at the time of the patient's first contact with the health-care system is considered adequate for measles surveillance [3, 20].

In the present study the early timing of specimen collection and previous contact with live attenuated measles vaccine are possible reasons for the failure to detect a specific IgM response in the four PCR-positive patients. In such cases, a week or more is required to complete the recommended, serological evaluation with paired serum samples. Laboratory diagnoses in most cases are retrospective and therefore too late for practical purposes concerning patient management or outbreak control [22]. On the other hand, RT–PCR may have missed other cases since the virus load drops rapidly after appearance of rash [23]. Moreover, the oral fluid device (Orasure) used in this study is designed to protect protein (especially antibody) rather than nucleic acid and may contribute to the degradation of any virus genome present. As suggested previously, the Oracol device (Malvern Medical Developments, Worcester, UK) should be used to collect oral fluid samples that may also be required for molecular studies of virus in addition to serology [24].

As measles becomes well controlled, the positive predictive value of clinical diagnosis becomes poor, and laboratory-based surveillance is of utmost importance [25]. Furthermore, in highly immune populations, non-classical measles infections can occur in at least 20% of previously immune persons with close exposure to a classical measles case [4]. In this context, detection of specific nucleic acids by PCR may be especially useful for diagnosing asymptomatic or non-classical measles cases [6, 22]. The detection of measles virus RNA by RT–PCR in a variety of clinical specimens, including throat swabs, urine and oral fluid, from acutely infected individuals has been reported as a feasible and non-invasive methodology for measles diagnosis [7, 9, 16, 22].

In this study we identified four patients who had measles virus RNA detected by RT–PCR in oral fluid samples in the absence of specific IgM. The measles virus strain identified was genotype D6 [18], the same genotype detected in classical measles cases in the municipality during the study period [9] and in different Brazilian states during the 1997 epidemic [26]. Although recommended, we were not able to demonstrate a rise in measles virus-specific IgG because second serum samples were not collected. As mentioned above, the collection of paired sera has a low acceptability which does not favour the effective investigation of the notified cases. Moreover, the non-classical clinical course of the study cases, and the concomitant occurrence of other rash diseases, which may be easily confused with measles, might also have resulted in misdiagnosis of the measles cases.

Although most vaccinated individuals continue to have detectable antibodies, vaccine-induced immunity can wane over time [27, 28]. Mild, non-classical measles has been described among patients who have been previously vaccinated with live attenuated vaccines [6, 9, 22]. In our study, three of the four PCR-positive cases had a history of measles vaccination in the past. The waning immunity could explain the failure to detect IgG antibodies by EIA in the vaccinated cases. However, as we were not able to retest the sera by another sensitive method (such as plaque-reduced neutralization test), this hypothesis could not be confirmed.

In such cases, where a specific IgG rise or seroconversion or a specific IgM cannot be demonstrated, PCR results afford useful information for the diagnosis of the non-classical course of measles infection, though the costs of PCR analysis and the technical skills required are higher than those associated with serological assays.

The four PCR-positive cases identified in this study occurred in the context of an outbreak of 45 measles cases in the city of Niterói, during a nationwide epidemic in 1997 [8, 29]. The population of the municipality was approximately 450 000 inhabitants in 1997 [30]. The low number of measles cases observed was not surprising, since there was a high level of immunity to measles in the population of the city due to high rates of vaccination coverage achieved during mass vaccination campaigns [1, 2]. Most of the measles cases of the outbreak occurred in young adults. This shift to older age groups has been reported as vaccine coverage increases, particularly in infants and children [31]. The PCR-positive cases also occurred in the age group most affected during the outbreak: one patient was 1 year old and the other three ranged from 18 to 24 years.

Although two of the four measles virus PCR-positive patients fulfilled the clinical case definition of the Brazilian Health Ministry [19], none of them was recognized and notified as a measles case. Moreover, none gave a history of exposure to a suspected or confirmed case. As measles becomes controlled, clinical features alone may not be sufficient to establish an accurate diagnosis [32]. Unrecognized exposure may occur, since measles is highly infectious and brief exposure can result in infection [33]. Cases that are not diagnosed through routine serological methods, such as those four RT–PCR-positive, also constitute an unrecognized source of exposure for their contacts.

The results of this study have public health surveillance implications beyond those of more accurate differential diagnosis. It demonstrated that individuals previously immunized through attenuated infection can still allow the circulation of the virus. We also found that IgM antibody testing for measles diagnosis failed to detect acute infection. Therefore, the use of RT–PCR in testing non-invasive clinical samples will play an important role in the investigation of measles cases and elucidating the molecular epidemiology of measles virus strains [24], even though there is an inherent disadvantage of false-positive results in this sensitive technique [22].

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