Combination of Reverse Transcriptase PCR Analysis and Immunoglobulin M Detection on Filter Paper Blood Samples Allows Diagnostic and Epidemiological Studies of Measles

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Received 20 July 2000/Returned for modification 29 September 2000/Accepted 18 October 2000

As measles control and elimination campaigns progress, laboratory confirmation of clinically diagnosed measles cases becomes increasingly important. However, in many tropical countries collection and storage of clinical specimens for this purpose are logistically complicated. In this study it is shown that blood samples spotted on filter paper are suitable for the laboratory diagnosis of measles using a combination of reverse transcriptase PCR (RT-PCR) analysis and immunoglobulin M (IgM) detection. First, it was shown that in vitro measles virus (MV)-infected cells diluted in human blood and spotted on filter paper can be detected by RT-PCR. Small amounts of infected cells remained detectable after 25 weeks of storage of the filter paper at room temperature, 4 weeks at 37°C, or 2 weeks at 45°C. Subsequently, this RT-PCR was applied to filter paper blood samples collected from 117 clinically diagnosed measles patients in Sudan in 1997 and 1998. Prior laboratory diagnosis had confirmed 90 cases as acute MV infections, while 27 proved to be nonmeasles rash disease cases. Positive RT-PCR signals were detected in filter paper blood samples of 43 of the 90 confirmed cases (48%) but in none of the 27 nonmeasles cases. In addition, MV-specific IgM levels measured in reconstituted filter paper samples correlated well with those measured in plasma samples. Measles diagnosis based on the combination of filter paper RT-PCR and IgM detection had a sensitivity and specificity of 99 and 96%, respectively. An advantage of this diagnostic approach is that sequencing of RT-PCR products allows phylogenetic analysis of the MV strain involved.

Measles continues to be a major childhood disease, resulting in an estimated 1 million fatal cases each year (2). Live attenuated measles virus (MV) vaccines have successfully been used to control measles morbidity and mortality in the industrialized world, but vaccination has been less successful in developing countries. This is thought to be the result of a combination of insufficient vaccination coverage, logistical problems related to cold chain maintenance, civil wars, and safety issues related to the current AIDS pandemic (4, 16).

The diagnosis of measles in developing countries is based almost exclusively on the evaluation of clinical symptoms. The World Health Organization (WHO) defines a clinical measles case as one in which the patient has a generalized maculopapular rash, a fever of 38°C or more, and at least one of the symptoms cough, coryza, or conjunctivitis (8). However, similar symptoms may also be caused by infection with other infectious agents. In a cohort study of almost 200 clinically diagnosed Sudanese measles patients, we recently found that in approximately 25% of these cases the clinical symptoms were not related to acute MV infection (6). Other studies reported between 12% and more than 50% falsely diagnosed measles cases (5, 7, 11, 13, 14), with an apparent inverse relationship with vaccination coverage.

As measles control and elimination campaigns progress, the laboratory confirmation of clinically diagnosed measles cases becomes increasingly important. The “gold standard” for laboratory diagnosis of measles is the demonstration of specific serum immunoglobulin M (IgM) antibodies (9). However, these may be low or absent in patients sampled in an early stage of the infection or in immunocompromised patients. We have recently shown the usefulness of reverse transcriptase PCR (RT-PCR) analysis as an additional tool to help in the diagnosis of these patients (6).

In many tropical countries collection and storage of samples for laboratory diagnosis are logistically complicated due to a limited infrastructure. While the usefulness of filter paper blood samples for the measurement of MV-specific serum antibodies had been demonstrated before (3, 12), recent publications have suggested that filter paper blood samples may also be suitable for RT-PCR analyses to diagnose certain virus infections (1, 15). In the present study we show that blood samples spotted on filter paper are indeed suitable for use in MV-specific RT-PCR analysis. In combination with IgM detection carried out on the same filter paper samples, this provides an adequate method for the retrospective laboratory diagnosis of measles in tropical countries.
MEASLES DIAGNOSIS USING FILTER PAPER BLOOD

TABLE 1. RT-PCR detection of MV in filter paper samples after storage under different conditions

<table>
<thead>
<tr>
<th>Storage conditions</th>
<th>Detection after the following storage time (wk):</th>
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<tbody>
<tr>
<td>20°C, dry</td>
<td>+ + + + + +</td>
</tr>
<tr>
<td>37°C, dry</td>
<td>+ + + − − NT</td>
</tr>
<tr>
<td>45°C, dry</td>
<td>+ + + − − NT</td>
</tr>
<tr>
<td>37°C, humidified</td>
<td>+ + NT NT NT</td>
</tr>
</tbody>
</table>

* Each filter paper (four used) was spotted with 30 in vitro MV-infected B-LCL cells per 25 μl of human blood with EDTA (20 identical spots) and stored dry at 20, 37, or 45°C or in a humidified 37°C incubator. Two 25-μl spots were collected from each filter paper at 1, 2, 4, 8, 12, 25, and 45 weeks of storage. RNA was isolated, and RT-PCR was carried out in duplicate. Results are shown as positive (+) or negative (−), results of duplicate measurements were in all cases concordant. Samples stored for more than 2 weeks at 37°C and humidified could not be processed due to fungal outgrowth.

** NT, not tested.

FIG. 1. RT-PCR detection of in vitro MV-infected cells diluted in human blood and spotted on filter paper. A human Epstein-Barr virus-transformed B-LCL was infected with a wild-type MV isolate from Khartoum, washed, counted, diluted in human blood with EDTA, and spotted on filter paper in 25-μl samples. After storage of the filter paper samples at room temperature for 6 weeks, RNA was isolated and RT-PCR was carried out with primers MV-N1 and MV-N2. The resulting amplicons were of the correct size as estimated on the gel using a 100-bp ladder as reference (not shown). The PCR products were botted and hybridized with 32P-labeled oligonucleotide probe MV-prN2. The autoradiogram is shown, with numbers of MV-infected cells per 25 μl indicated above the respective lanes. The positive (MV Edmonston) and negative (untreated human blood with EDTA) controls are indicated by + and −, respectively.

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sphere at 37°C, but after 2 weeks the samples were no longer suitable for analysis due to fungal outgrowth.

**IgM detection in Sudanese filter paper blood samples.** Subsequently, we tested filter paper blood samples collected from clinically diagnosed measles patients in Khartoum, Sudan. The samples were tested in an IgM capture ELISA, using peroxidase-labeled MV N as the conjugate. OD450, optical density at 450 nm. ●, laboratory-confirmed measles cases; ○, nonmeasles rash disease cases.

**FIG. 2.** MV-N-specific IgM levels in paired filter paper and plasma samples collected from clinically diagnosed measles patients in Khartoum, Sudan. The samples were tested in an IgM capture ELISA, using peroxidase-labeled MV N as the conjugate. OD450, optical density at 450 nm. ●, laboratory-confirmed measles cases; ○, nonmeasles rash disease cases.

**FIG. 3.** Frequency of RT-PCR-positive filter paper samples obtained from laboratory-confirmed measles patients in relation to the level of MV-specific IgM measured in the same filter paper samples. The cases correspond to the closed symbols in Fig. 2. The number of samples in each group is indicated above each bar.

Diagnostic value of combined RT-PCR analysis and IgM detection. While specific IgM measurement in plasma samples had a high sensitivity but low specificity (100 and 78%, respectively), IgM measurement in filter paper blood samples had a slightly lower sensitivity but higher specificity (95 and 96%, respectively) (Table 2). A diagnosis based on the combination of RT-PCR analysis and IgM detection on filter paper samples, defining a measles case as MV-specific RT-PCR positive and/or IgM positive, had a sensitivity and specificity of 99 and 96%, respectively (Table 2).

**DISCUSSION**

This study shows that combined RT-PCR analysis and IgM detection on filter paper blood samples allows a highly accurate diagnosis of measles. Proof of principle of the suitability of filter paper samples for MV-specific RT-PCR was obtained from studies with in vitro MV-infected cells diluted in human blood. When applied to clinical materials from a cohort of clinically diagnosed Sudanese measles patients sampled in 1997 and 1998, we found the combination of RT-PCR and IgM detection to have a sensitivity, specificity, and positive predictive value of 99, 96, and 99%, respectively. An additional advantage of this approach is that sequence analysis of the PCR product allows phylogenetic analysis of the MV strain involved.

The incubation time of measles is 9 to 19 days, with the peak of MV replication preceding the appearance of the rash (10).

**FIG. 3.** Frequency of RT-PCR-positive filter paper samples obtained from laboratory-confirmed measles patients in relation to the level of MV-specific IgM measured in the same filter paper samples. The cases correspond to the closed symbols in Fig. 2. The number of samples in each group is indicated above each bar.

**TABLE 2.** Sensitivity, specificity, and positive predictive values of laboratory diagnosis of measles using filter paper blood samples.

<table>
<thead>
<tr>
<th>Diagnostic method</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>Positive predictive value (%)</th>
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<tbody>
<tr>
<td>MV-specific IgM</td>
<td>95</td>
<td>96</td>
<td>99</td>
</tr>
<tr>
<td>MV-specific RT-PCR</td>
<td>48</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Combination of IgM and RT-PCR</td>
<td>99</td>
<td>96</td>
<td>99</td>
</tr>
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As the gold standard, a previously described method for the laboratory diagnosis of measles was used, which is based on measurement of MV-specific IgM and IgG antibody levels in plasma and, in doubtful cases, MV-specific RT-PCR with throat swab samples (6).
Several of the clinical symptoms of measles, including rash and conjunctivitis, have an immunopathological basis: they coincide with the appearance of MV-specific serum antibodies and specific T lymphocytes. As a result of the MV-specific immune response, the viral load decreases rapidly after onset of disease. In a previous study, we could isolate MV from PBMC of 17 out of 23 laboratory-confirmed measles cases tested, and we found numbers of infected cells between $10^{6.5}$ and $10^7$ cells per $10^6$ PBMC (6). Since these estimations are based on virus isolation, the frequency of in vivo MV-infected cells will probably be higher. With an estimated number of mononuclear leukocytes of between $10^4$ and $10^5$ per $25 \mu l$ of blood and a RT-PCR detection limit of three infected cells, about 0.03 to 0.003% of PBMC would need to be infected in vivo to give a positive RT-PCR signal in our assay. However, the rapidly decreasing virus load after the onset of rash (10) implies that measles diagnosis based on RT-PCR analysis alone may not be expected to be sensitive enough.

The IgM levels measured in filter paper samples were in most cases slightly lower than those measured in plasma samples, but in some cases the difference was more than threefold. Using a total IgM sandwich ELISA, we could demonstrate that these samples contained less than 0.2 $\mu g$ of total IgM per ml. In a diagnostic setup it would be advisable to always test the MV-specific and total IgM levels at the same time and to consider negative data from samples with less than 0.2 $\mu g$ of total IgM per ml to be a nonvalid.

As we have documented before, the diagnosis of measles based on serology alone has some shortcomings, especially when MV-specific IgM levels are low. This may be the case in patients sampled in an early stage of the infection, in patients with a secondary measles vaccine failure (who may mount a secondary immune response with low IgM and high IgG levels), and in immunocompromised patients. In these cases, the addition of RT-PCR analysis to IgM detection will reduce the number of false-negative diagnoses.

The Sudanese filter paper samples had first been stored frozen in Sudan, but after shipment to The Netherlands they were stored at $20^\circ C$ for a period of 5 months before the assays were performed. However, our longevity data using in vitro MV-infected cells demonstrated that small amounts of infected cells spotted on filter paper and stored for 25 weeks at room temperature can still be detected by RT-PCR. Although we do not have sufficient data for an accurate estimation of the half-life of the RT-PCR signal at this temperature, it is at least several weeks.

The WHO is organizing a global laboratory network for the diagnosis of measles, as a first step in the preparation of a plan for the eventual eradication of measles. This laboratory network is organized as a tiered system, with global reference laboratories, and regional, national, and subnational laboratories. The collection of whole blood samples on filter paper would fit well within such an approach. A limited number of drops of blood could be collected from each patient, and filter paper samples could be sent to the respective laboratories to be analyzed as described here. Our longevity data suggest that even in countries with high ambient temperatures a transportation time of several weeks would result in only a limited loss of signal. In the laboratory the filter paper samples would probably best be stored frozen.

In conclusion, the combination of RT-PCR analysis and IgM detection on filter paper blood samples was shown to result in a highly sensitive and specific diagnostic method. The ease of sample collection and transport makes it especially attractive for use in tropical countries. In addition, the option for phylogenetic analysis of the MV strains involved, based on sequence analysis of the RT-PCR products, will be important for molecular epidemiological studies during the final stages of the envisaged measles eradication program.

ACKNOWLEDGMENTS

This work was supported by INCO-DC grant IC18CT96-0116 from the European Commission.

We thank K. H. Siebelink, O. M. Mustafa, M. M. Mukhtar, E. E. Zijlstra, H. W. Vos, and C. Copra for their contribution to these studies and R. S. van Binnendijk for critical comments on the manuscript.

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