Use of Reverse Transcriptase PCR in Early Diagnosis of Rift Valley Fever

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Reverse transcriptase PCR (RT-PCR) for diagnosis of Rift Valley fever (RVF) was evaluated by using 293 human and animal sera sampled during an RVF outbreak in Mauritania in 1998. Results of the RT-PCR diagnostic method were compared with those of virus isolation (VI) and detection of immunoglobulin M (IgM) antibodies. Our results showed that RT-PCR is a specific, sensitive tool for RVF diagnosis in the early phase of the disease and that its results do not differ significantly from those obtained by VI. Moreover, the combined results of RT-PCR and IgM antibody detection were in 100% concordance with the results of VI.

Rift Valley fever (RVF) is an arthropod-borne viral disease transmitted by mosquitoes that infects humans and animals, mainly livestock. In humans, infection can lead from a mild to a very severe clinical picture, including temporary and definitive blindness, encephalitis, and hemorrhagic fever syndrome with a fatal outcome (8). In animals, RVF virus (RVFV) induces almost 100% mortality among young animals and a high rate of abortion in gravid females. During the multiple outbreaks and epidemics which occurred in Africa and very recently in Saudi Arabia and Yemen (2), the virus exhibited an amazing flexibility to adapt to different ecological contexts and to take advantage of climatic change and environmental disruptions (dam building, land irrigation, etc.). Such an adaptive ability resulted in a high toll of deaths, ailments, and economic losses during its recent emergence episodes. Since livestock immunization against RVF has so far appeared difficult to implement efficiently in areas of endemicity, strengthened surveillance and early detection of cases seem to be among the best options to prevent extension of RVF epidemic foci. Success of such a surveillance strategy relies on efficient tools for early detection. For that reason, we developed a reverse transcriptase PCR (RT-PCR) assay to detect the viral genome in human and animal sera by targeting the NSs coding region of the smallest segment (S) of the tripartite negative-sense single-stranded RNA genome of RVFV (11). The primers used for that nested RT-PCR are listed in Table 1 and were shown to be very specific to RVFV, with a minimum detection limit of 0.5 PFU, reflecting approximately 500 to 1,580 RVFV RNA genomes (7). In 1998, the RVFV outbreak which occurred in Mauritania (10) provided human and animal clinical samples that were used to assess the relevance of RT-PCR as a diagnostic tool for RVFV. This paper compares the use of RT-PCR with the use of virus isolation (VI) and with the joint use of RT-PCR and anti-RVFV IgM antibody assay in RVFV early diagnosis based on human and animal blood samples. During the outbreak investigation, samples were obtained from humans and animals (camels, goats, sheep, and cows) suspected of having RVF. A patient suspected of having RVF was defined as someone with a fever occurring between 1 September 1998 and the date of investigation, whether or not the fever was associated with hemorrhagic signs, icterus, and/or neurological signs. Animals were suspected to have RVF when they had belonged to flocks for which abortions or stillbirths had been reported in 1998. Nucleic acids were extracted from 100-μl serum samples by disruption in guanidium thiocyanate, adsorption on silica particles, and elution in sterile distilled water, followed by nested RT-PCR as previously described (3, 11). As a precaution against contamination, aerosol barrier tips and separate rooms were used for template preparation, reaction assembly, and processing and analysis of the PCR products, on the one hand, and positive and negative controls were included among samples for the extraction and RT-PCR steps, on the other hand. For extraction, the three negative controls consisted of 100 μl of previously tested negative serum, sterile PCR-grade water, and Leibovitz 15 maintenance medium while positive controls included serum with 103 PFU of RVFV strain MP12. For the two steps of the nested RT-PCR, appropriate RT-PCR mixes were added to the negative and positive control tubes containing, respectively, sterile PCR-grade water and cDNA prepared from strain MP12 RNA that had previously tested positive. Moreover, each serum sample was independently processed twice to ensure confirmation of the results. Furthermore, VI from the samples was attempted with both Vero E6 cells and suckling mice. Confluent monolayers of Vero cells in 25-cm² flasks were infected with 0.5 ml of diluted serum samples (100 μl of serum and 400 μl of Leibovitz 15 medium with 5% fetal calf serum and antibiotics) and 4 ml of maintenance medium. When mice were used, VI was at-
tempted by intracerebrally inoculating 30 μl of serum sample into each mouse from a litter of 10. Cytopathic effects and mortality were examined, and identification and immunofluorescent tests using pools of hyperimmune ascitic fluid were performed 3 days postinfection by methods described previously (5). In addition, samples were screened for specific RVFV immunoglobulin M (IgM) antibodies using the ELISA method previously described by Digoutte et al. (4). Considering VI to be the “gold standard” method, we assessed the sensitivity and specificity of the RT-PCR. Comparison of the different methods was performed by using the Fisher exact test or chi-square values calculated using McNemar’s test (6).

Analysis of the 293 samples from humans and animals suspected of having RVF is summarized in Table 2. The low rate of detection of RVF cases by VI (detection in 17 of 293 samples) and RT-PCR (detection in 20 of 293 samples) may be explained by the low stringency criteria used to define a suspected RVF case. Indeed, the definition probably covers other diseases, such as malaria, which were circulating in Mauritania during the investigation. Concordant results were obtained for 12 positive and 268 negative sera by VI and RT-PCR, whereas divergent results, were obtained for 13 additional samples by the two methods. Despite these discrepancies, results obtained with RT-PCR and VI were not significantly different ($P > 0.5$ by McNemar’s test). Consequently, RT-PCR can be used reliably for RVF diagnosis early in the disease. If we consider VI to be the gold standard method, 12 samples were positive by RT-PCR and 17 were positive by VI, indicating that the sensitivity of RT-PCR is 70.6% (12 of 17 samples). The value of 97.1% (268 of 276 samples) was obtained for the specificity, taking into account the 268 sera which tested negative by RT-PCR out of a total of 276 negative samples. While the specificity of RT-PCR appeared to be high enough for diagnostic purposes, the sensitivity, even though acceptable, would need to be improved because of the explosive nature of RVF outbreaks. However, the apparent low value obtained for sensitivity may be due to the choice of VI as the gold standard. Indeed, among the 293 samples analyzed, if we consider any sample testing positive by VI and/or RT-PCR as indicative of RVF, we can consider 25 samples to be positive. Therefore, the sensitivity would be 80% (20 of 25 samples) for RT-PCR and 68% (17 of 25 samples) for VI.

As to the 13 samples out of 293 (4.4%) which led to divergent results, we tried to analyze the cause of this ambiguity. In eight samples, viral genome was detected but no virus was isolated. This observation might be explained either by the fact that storage and transport conditions were not adequate for the rescue of infectious virus although nucleic acid was demonstrable or by the persistence of RVF RNA after the clearance of the virus from the blood. This finding emphasizes a significant advantage of RT-PCR over VI, particularly in Africa, where RVF outbreaks occur in areas where cold chain is not always available and the duration of the transport is usually long. On the other hand, as to the five sera which tested positive by RT-PCR but negative for infectious virus, it is striking that they were all isolated from mice only and were positive for IgM antibodies. Nonetheless, RT-PCR using the same primers and protocol could be applied successfully on virus derived from mouse brain. Therefore, the discrepancy may be related to the fact that the volume of serum inoculated into a litter of mice, approximately 300 μl, is threefold greater than the volume used for RT-PCR or VI.

In order to investigate a possible influence of IgM antibodies on the detection of the viral genome, the number of IgM-positive or -negative sera is indicated in Table 3 for each of the four categories defined by the results obtained by RT-PCR and VI. If one considers the 17 samples from which RVFV was isolated, the proportion of PCR-positive to IgM-negative samples (9 of 9 samples) is significantly higher ($P = 0.009$ by Fisher’s test) than the proportion of PCR-positive to IgM-positive samples (3 of 8 samples), suggesting that RT-PCR works much better in the absence of IgM antibodies. This observation means that RT-PCR is more efficient in the very early phase of the disease, before the appearance of IgM antibodies and the decline of viremia, as shown previously for

| RT-PCR round | Primer | Nucleotide sequence | Map positions | Orientation |
|-------------|--------|---------------------|---------------|-------------|
| First       | NSca   | 5'-CCCTTAACCTCTAACATCAAC-3' | 841–824       | Antisense   |
|             | NSng   | 5'-TATCATGGATTATGTCTTTCC-3' | 31–48         | Sense       |
| Second      | NS3a   | 5'-ATGCCTGGGAAGTGATGAGCG-3' | 729–710       | Antisense   |
|             | NS2g   | 5'-GAATCGCAGAGTGCTGGC-3'    | 62–80         | Sense       |

| PCR result | No. of specimens with indicated VI result | Total |
|------------|------------------------------------------|-------|
|            | Positive | Negative |       |       |
| Positive   | 12       | 8        | 20    |
| Negative   | 5        | 268      | 273   |
| Total      | 17       | 276      | 293   |

| PCR result | No. of specimens with indicated VI result$^a$ | Total |
|------------|---------------------------------------------|-------|
|            | Positive | Negative |       |       |
| Positive   | 12 (3, 9) | 8 (0, 8) | 20    |
| Negative   | 5 (5, 0)  | 268 (56, 212) | 273 |
| Total      | 17       | 276      | 293   |

$^a$ Numbers in parentheses are numbers of samples that were IgM positive and negative, in that order.
other hemorrhagic fevers such as Crimea Congo hemorrhagic fever (1). Also, of the 268 samples which tested negative by VI and RT-PCR, 56 tested positive for IgM antibodies. These sera were probably sampled from patients after the clearance of the virus and RNA from the blood as witnessed by the detection of anti-RVFV IgG antibodies in all 56 samples (data not shown). Indeed, as demonstrated by Morvan et al. (9), anti-RVFV IgM can persist for 5 months in the presence of IgG antibodies after a natural infection. In light of this analysis, we assessed the suitability and relevance of IgM detection as a method complementary to RT-PCR for the diagnosis of RVF in the early phase of the disease. Interestingly, IgM antibodies were detected in the five sera which tested negative for the viral genome but positive for infectious virus, indicating that the results of the combination of RT-PCR and IgM detection exhibit a 100% concordance with the results of VI (Table 3). Since RT-PCR and IgM detection are more rapid than VI, we recommend using them in parallel as a first-line diagnostic method for RVF when an outbreak occurs. However, the efficiency of RT-PCR can be further improved with the use of a blood-collecting tube with a nucleic acid stabilizer to prevent the degradation of RNA, which occurs in developing countries mainly because of the interruption of cold chain during the transport of samples to the laboratory.

Overall, despite some limitations in terms of its sensitivity, its cost, and the level of expertise required of technicians, RT-PCR is a rapid, sensitive, specific, and reliable assay for early detection of RVFV infection. It should be used routinely in conjunction with IgM detection for the early diagnosis of suspected cases of RVF.

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