Marburgvirus Nucleoprotein-Capture Enzyme-Linked Immunosorbent Assay Using Monoclonal Antibodies to Recombinant Nucleoprotein: Detection of Authentic Marburgvirus

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SUMMARY: There have recently been large outbreaks of Marburg hemorrhagic fever (MHF) caused by Marburgvirus (MARV) in the Democratic Republic of Congo and Angola. The development of reliable diagnostic systems for MHF is urgently needed. An antigen-capture enzyme-linked immunosorbent assay (Ag-capture ELISA) using either of the two monoclonal antibodies (2A7 and 2H6) produced by immunizing mice with recombinant nucleoprotein of MARV was described (Journal of Medical Virology, 76, 111-118, 2005). In the present study, it was revealed that the Ag-capture ELISA specifically detected authentic MARV antigen and that the sensitivity of the Ag-capture ELISA was at a level similar to that of reverse-transcription polymerase chain reaction. These results suggest that the Ag-capture ELISA using the monoclonal antibodies, 2A7 and 2H6, is applicable to the diagnosis of MHF.

Lake Victoria Marburgvirus (MARV) infections cause Marburg hemorrhagic fever (MHF), one of the most severe forms of hemorrhagic fever, with a high mortality rate (1-3). MARV belongs to the genus Marburgvirus, family Filoviridae. The first documented outbreak of MHF occurred in the former West Germany and the former Yugoslavia in 1967 (4). After the first documented outbreak, 3 sporadic cases of MHF were reported in Zimbabwe (1975) and Kenya (1980 and 1987) (3,5-7). From 1998 to 1999, there was a large outbreak in the Democratic Republic of Congo, formerly Zaire (1). The largest outbreak of MHF to date started in 2004, in which more than 300 patients have been reported and with a mortality rate of over 90% in Uige Province in Angola (see WHO website: http://www.who.int/csr/don/2005_05_18a/en/index.html). Therefore, the development of diagnostics for MHF is urgently needed.

In our previous report, we characterized two monoclonal antibodies to the recombinant nucleoprotein (rNP) of MARV, 2A7 and 2H6 (8). These antibodies were useful in the antigen-capture enzyme-linked immunosorbent assay (Ag-capture ELISA). However, the efficacy of the Ag-capture ELISA using the authentic virus antigen was not evaluated. In the present study, to confirm the usefulness of the Ag-capture ELISA for the diagnosis of MHF, we examined the assay with each of the monoclonal antibodies to detect the authentic virus antigen (MARV Musoke strain). We also compared the Ag-capture ELISA with reverse-transcription polymerase chain reaction (RT-PCR) in terms of sensitivity for the detection of MARV.

MARV, Musoke strain (2.5 × 10⁷ plaque-forming units [PFU]/ml determined by a plaque-forming assay in Vero cells) was used. All procedures requiring the manipulation of infectious MARV were carried out in a maximum safety laboratory (Biosafety Level-4 laboratory) in INSERM (P4 Jean Merieux Inserm) in Lyon, France. Two monoclonal antibodies (2A7 and 2H6), which reacted with the carboxy-terminal part of the MARV-rNP, were used (8). The protein concentration of the purified monoclonal antibody solutions for 2A7 and 2H6 was determined to be 570 μg/ml and 470 μg/ml, respectively. The MARV solution was diluted with human serum before testing and mixed with the same volume of phosphate-buffered saline (PBS) containing 2% triton-X, and then subjected to Ag-capture ELISA. The Ag-capture ELISA using either of the monoclonal antibodies, 2A7 and 2H6, was carried out as reported previously (8). Purified monoclonal antibody was coated on microwell immunoplates (Falcon; Becton Dickinson Labware, Franklin Lakes, N.J., USA) at the designated concentration of monoclonal antibody in PBS (100 ng/100 μl/well) at 4°C overnight, followed by blocking with PBS containing 5% nonfat milk and 0.05% Tween-20 (PBST-M) for 1 h at room temperature (RT). After the plates were washed with PBS containing 0.05% Tween-20 (PBST), 100 μl of serially diluted samples was added and the plates were incubated for 1 h at 37°C. The plates were then washed with PBST, and 100 μl of rabbit polyclonal antibody raised against rNP of MARV diluted 1:500 with PBST-M was added to each well (9). After 1 h incubation at 37°C, the plates were washed with PBST and horseradish peroxidase-conjugated goat anti-rabbit IgG (Zymed Laboratories Inc., South San Francisco, Calif., USA) was added. The plates were incubated for 1 h at RT. After another extensive wash with PBST, 100 μl of ABTS substrate solution (4 mM 2,2'-azino-di-[3-ethylbenzthiazolin sulfonate (6)] (Roche Diagnostics, Mannheim, Germany) was added and the optical density (OD) was measured at a wavelength of 405 nm with a reference wavelength of 490...
nm after 30 min of incubation at 37°C. As a negative control, mock antigen-inoculated wells were also tested. The OD of the MARV sample showed high values in the Ag-capture ELISA with either of the monoclonal antibodies, 2A7 and 2H6, at dilutions from 1:8 to 1:2^{13} and from 1:8 to 1:2^{11}, respectively, in a dilution level-dependent manner, while the samples showed very low OD values in Ag-capture ELISA without the capture monoclonal antibodies (Fig. 1A and 1B). Negative control samples, human sera without MARV, also showed very low OD values in the Ag-capture ELISA with the monoclonal antibodies 2A7 and 2H6 (Fig. 1A and 1B). The newly developed Ag-capture ELISA could detect the authentic MARV nucleoprotein at a level equivalent to the concentration of MARV nucleoprotein from approximately 3 \times 10^5 PFU/ml to 1.2 \times 10^6 PFU/ml.

The minimal quantity of the MARV virions that can be detected by the Ag-capture ELISA was compared with that detected by RT-PCR. The partial L-gene of MARV was amplified by RT-PCR using the primer set of “Filo-A (5’-ATCGGAATTTTTCTTTCTATT-3’) and “Filo-B (5’-ATGTGGTGGGTTATAATAATCACTGACATG-3’)” as reported previously (10). Briefly, 100 µl of MARV solution serially diluted with human serum was treated with a QIAGEN viral RNA purification kit according to the manufacturer’s instructions for purification of MARV RNA. Ready-to-Go RT-PCR Beads™ (GE Healthcare Bio-Sciences, Piscataway, N.J., USA) was used for RT-PCR reaction. Five of 65 µl of the purified RNA solution was added to a PCR tube as a template as well as 50 pmole of each primer and water, the final volume of the reaction mixture being 50 µl per tube. The conditions of RT-PCR were as follows: 42°C for 30 min, 94°C for 5 min, 3 cycles of 94°C for 30 s, 72°C for 30 s and 72°C for 5 s, followed by 72°C for 5 min. Ten microliters of the reaction mixture was subjected to 2% agarose gel-electrophoresis, and the amplified DNA was visualized by the standard method using ethidium bromide. Although the data are not shown here, the RT-PCR using this primer set was the most sensitive among the RT-PCRs using either of the 3 primer sets, Filo-A/Filo-B, mbg1 (5’-ACTCTCCAGAAGACAGAAAGA-3’)/mbg2 (5’-AGCG ATGGGTTTTTCAAGGACA-3’), or mbg3 (5’-AGCGATGGG CTTTCAGGACA-3’)/mbg4 (5’-CGGTACATTGTGTTGGA GGC-3’). The MARV sample demonstrated positive reactions at dilutions from 1:10^1 to 1:10^4 in RT-PCR assay (Fig. 1C).

The minimal quantity of MARV virions that is detected by RT-PCR depends on the various factors such as RNA-purification methods, RT-PCR methods, PCR machines, visualization of the PCR products, etc., suggesting that the sensitivity of the Ag-capture ELISA cannot simply be compared with that of RT-PCR. Although the RT-PCR assays, especially the nested RT-PCR, are useful, false-positive and false-negative results must always be excluded. The data in the present study showing that the sensitivity of the Ag-capture ELISA was almost similar to that of the RT-PCR suggest that a combination of the RT-PCR with the Ag-capture ELISA make diagnosis of MHF more reliable. Antigen-detection ELISA has been used as a diagnostic procedure for Ebola hemorrhagic fever (11,12) but not for MHF. To control outbreaks of hemorrhagic fever including MHF, rapid and accurate diagnosis is essential. The newly developed MARV nucleoprotein-capture ELISA should be further

![Fig. 1](image-url)
evaluated for its efficacy using clinical samples collected from patients with or without MARV infections.

In summary, the MARV nucleoprotein-detection ELISA using monoclonal antibodies 2A7 and 2H6 detected the authentic MARV nucleoprotein, suggesting that the Ag-capture ELISA is applicable to the diagnosis of MHF.

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