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Reverse ELISA for IgG and IgM antibodies to detect Lassa virus infections in Africa

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Abstract

Background: Anti-Lassa antibodies are detected by indirect immunofluorescence assay (IFA) or by enzyme-immunoassay (ELISA). Both methods have problems to detect low amounts of specific antibodies.
Objectives: We report here highly sensitive and specific reverse ELISAs to detect Lassa virus IgG and IgM antibodies. Due to the reverse techniques, serum samples could be applied at dilutions of 1:10 without increasing non-specific background reactions.
Study design: For IgM antibody detection microtiter plates were coated with anti-IgM antibodies and for IgG antibody detection with rheumatoid factor (RF) (Sachers M, Emmerich P, Mohr H, Schmitz H. Simple detection of antibodies to different viruses using rheumatoid factor and enzyme-labelled antigen (ELA). J Virol Methods 1985;10:99–110). In both assays a tissue culture antigen was used in combination with a labeled anti-Lassa monoclonal antibody (Hufert FT, Ludke W, Schmitz H. Epitope mapping of the Lassa virus nucleoprotein using monoclonal anti-nucleocapsid antibodies. Arch Virol 1989;106(3–4):201–12).
Results: The reverse ELISA turned out to detect virus-specific IgG and IgM antibody in all 20 samples of West African patients collected 2–8 weeks after onset of Lassa fever. Moreover, both IFA and reverse ELISA found IgG antibodies in 53 out of 643 samples of healthy West Africans (sensitivity of 100%). Six of the 643 samples were positive by reverse IgG ELISA only. Thus, the specificity compared to IIF was 99.0%, but it may be even higher, because compared to IFA the IgG ELISA was clearly more sensitive in detecting low antibody titers.
Conclusions: In Ghana 3% seropositives were found by IFA, but 4% by the reverse ELISA. The reverse ELISAs can be performed with high sensitivity and specificity under field conditions in Africa.

Keywords: Reverse ELISA; Lassa IgG; IgM; Guinea; Ghana

1. Introduction

Lassa fever is highly endemic in Sierra Leone, Liberia and Nigeria (Carey et al., 1972; Frame et al., 1970).

During the first days after onset the clinical diagnosis of Lassa fever is difficult (McCormick et al., 1987a; Schmitz et al., 2002). Therefore, a laboratory diagnosis is needed, although this is difficult to achieve in West Africa without high tech laboratories.

Acute Lassa fever is reliably diagnosed by virus isolation (Johnson et al., 1987; Jahrling et al., 1985a), by antigen detection (Bausch et al., 2000; Jahrling et al., 1985a,b) and by RT-PCR (Demby et al., 1994; Drosten et al., 2002; Lunkenheimer et al., 1990). RT-PCR has turned out to be most sensitive. While RT-PCR and virus isolation pose technical problems in West African field hospitals, antigen tests would be more appropriate. But they are less sensitive and cannot detect low amounts of virus (Bausch et al., 2000; Schmitz et al., 2002).

As an alternative, antibodies tests may help to diagnose an acute Lassa virus infection. Thus, the presence of anti-Lassa IgM antibody or a significant rise in titer of IgG antibodies can prove an acute Lassa virus infection (Johnson et al., 1987;

Abbreviations: RF, rheumatoid factor; SA, soluble antigen; IFA, indirect immunofluorecence assay

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McCormick et al., 1987b; Wulff and Lange, 1975). Besides, IgG antibodies to Lassa may be useful to study the epidemiology of Lassa fever infection (Lukashevich et al., 1993a; McCormick et al., 1987b).

The best-studied method for antibody detection is IFA (Knobloch et al., 1980; Wulff and Lange, 1975). Since IFA has been considerably observer dependent, we have improved the reading of slides by counterstaining the antigen inside the cells (Haas et al., 2003).

As an alternative to IFA, indirect ELISA techniques have been published to detect IgG and/or IgM antibodies using tissue culture (Bausch et al., 2000; Jahrling et al., 1985b; Niklasson et al., 1984) or by recombinant antigens (Barber et al., 1990; Gunther et al., 2001; ter Meulen et al., 1998). Anti-Lassa IgG antibodies were detected by indirect ELISAs. In contrast, IgM antibodies were also detected by a reverse system using anti-IgM coated plates (Bausch et al., 2000; Jahrling et al., 1985b; Niklasson et al., 1984).

Unfortunately, indirect ELISAs were not as sensitive as the IFA in detecting IgG antibody (Bausch et al., 2000). This may be explained by the high serum dilutions of about 1:≥100, which have to be applied to avoid non-specific background staining. Moreover, in the case of recombinant proteins (Barber et al., 1990; Gunther et al., 2001; ter Meulen et al., 1998) the loss of virus-specific epitopes may also contribute to a reduced sensitivity.

In contrast to indirect ELISA techniques, using labeled anti-immunoglobulin conjugates we have applied here a reverse ELISA technique not only for IgM but also for IgG antibodies. To detect IgG antibodies, we have used a solid phase coated with rheumatoid factor (RF) (Sachers et al., 1985), where the amount of bound antibody is measured using either a directly labeled antigen (Schmitz et al., 1980) or an antigen that is detected with a labeled monoclonal antibody (Schmitz and Gras, 1986). Coating of the solid phase with anti-IgG antibodies, similar to anti-IgM coating, would be too insensitive. Instead, we selectively bind Lassa antigen–antibody complexes to RF during simultaneous incubation of the serum with Lassa antigen. The bound antigen is finally detected using a biotinylated anti-Lassa mouse monoclonal antibody.

The reverse ELISAs both for anti-Lassa IgG or IgM antibody detection allowed to apply the sera undiluted without increased background staining. The sensitivity and specificity of the reverse IgG and IgM ELISAs were evaluated with samples of 20 acute Lassa fever patients and with more than 600 serum samples of healthy West Africans.

2. Material and methods

2.1. Serum samples

From studies in Nigeria (Omilabu et al., 2005), Liberia, Sierra Leone and Guinea late serum samples of 20 Lassa fever survivors were available. At onset of the disease all patients had had high fever and elevated aminotransferases. In the samples taken 2–30 weeks after onset, IgM and IgG antibodies to Lassa virus had be detected by IFA. In 11 of the 20 patients, of whom early samples were available, Lassa virus RNA was found by RT-PCR (Drosten et al., 2002).

Moreover, upon ethical clearance, 643 samples were collected from adult, healthy subjects in West Africa (age 18–53 years, m/f 1.2:1). Four hundred and thirty samples were from Ghana and 213 from Guinea. Serum samples of 200 healthy German blood donors were also tested.

2.2. IFA

IgG antibodies to Lassa virus were detected using acetone-fixed Vero cells infected with Lassa virus strain Josiah (Wulff and Lange, 1975). Cultivation of the viruses was carried out in a BSL 4 laboratory. Our own data have consistently shown that from acetone-fixed Lassa virus infected cells no virus can be cultivated upon inoculation of Vero E6 cells. Serum samples were tested in two-fold steps starting at a dilution of 1:20. Cell smears were routinely counterstained with an anti-Lassa nucleocapsid monoclonal antibody 2F1 (Hufert et al., 1989) using Rhodamine-anti-mouse as secondary antibody (Haas et al., 2003). Also cell smears of Vero E6 cells infected with Lymphocytic Choriomenigitis virus (strain Amstrong M20869) and with Mopeia virus (strain 800150, M33879) were prepared.

2.3. Preparation of soluble antigen (SA)

SA was obtained by sonification of Lassa virus (Josiah strain) infected Vero E6 cells of one bottle (50 ml) in 10 ml PBS containing 0.3% detergent (NP 40, Sigma–Aldrich, Munich, Germany). Cell debris was removed by low speed centrifugation (5000 × g, 20 min). The SA could be stored lyophilized at room temperature and was additionally inactivated by gamma irradiation (24 kGray) (Bausch et al., 2000).

2.4. Monoclonal antibody

Monoclonal antibody 2F1 (Hufert et al., 1989) reacts with the nucleoprotein of several Lassa viruses (Josiah isolate of Sierra Leone, AV of Ivory Coast, CSF of Nigeria; Gunther and Lenz, 2004) by IFA. It does not cross react with Mopeia virus or LCMV. It was purified to 1 mg/ml and labeled with NHS biotin (Sigma–Aldrich) according to standard procedures.

2.5. Antigen titration

The Sandwich ELISA has been described in more detail earlier (Schmitz and Wolff, 1986). The antigen was incubated on the antibody (2F1) coated plates over night at 4 °C and was detected using the biotinylated antibody 2F1 at a dilution of 1:4000 followed by strepavidine-peroxidase (see reverse ELISA).

2.6. Reverse ELISA for IgM and IgG antibodies

Anti-IgM coated microtiter plates were used to bind the IgM fraction of the serum samples (50 μl aliquots), diluted 1:10 in PBS + 0.5% Tween in two-fold steps. After incubation for 1 h and washing with PBS + 0.5% Tween, SA was employed for another 2 h at 20 °C.

For IgG antibody detection, 25 μl SA were mixed with an equal volume of the serum samples, which were diluted in two-fold steps starting with 1:5 (dilution in SA: 1:10) in PBS + 0.5% Tween and incubated over night at room temperature on RF-coated microtiter plates (Medac, Hamburg, Germany).

The following steps were identical for both reverse IgG and IgM ELISAs:

After washing, the bound antigen was detected by labeled monoclonal antibody 2F1, applied for 1 h at a dilution of 1:4000. After washing, streptavidin-peroxidase conjugate (1:8000; Sigma–Aldrich) was added for 30 min.
After washing the test was stained with TMB (Sigma–Aldrich). The reaction was stopped after 10 min by addition of 2 M sulfuric acid. The absorbance was measured at 450 nm. The cut off OD for positive results was calculated as mean OD of the negative samples + 3 standard deviations (s).

2.7. Improving test conditions

Upon infection of the Vero cells with a PFU of 0.01 and incubation at 37 °C for 4 days, SA titers of 1:≥256 were measured by the sandwich ELISA.

To optimize the reverse IgG ELISA, the positive/negative (P/N) ratios for different dilutions of the SA and the labeled monoclonal antibody were determined. A confirmed anti-Lassa positive serum at a dilution of 1:1000 (IgG antibody titer in IFA 1:5120) and a negative serum samples diluted 1:10 were applied. Highest P/N ratios were obtained with the undiluted SA (titer 1:≥256) and a dilution 1:4000 of the monoclonal antibody. Besides, the optimum incubation period of the SA-human antibody complexes was over night at 4 °C (Fig. 1).

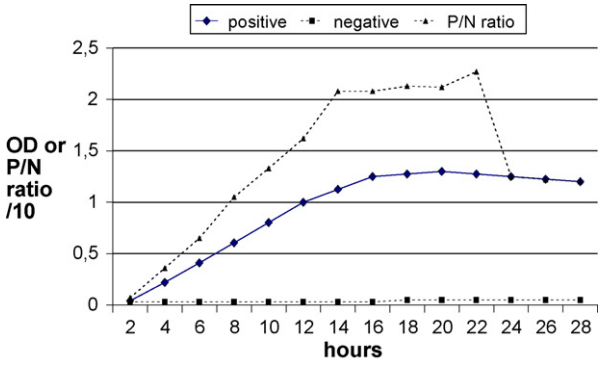

Fig. 1. Different incubation times of the antigen–antibody complexes on the RF-coated plates. Positive serum (titer 1:5120) and negative serum were used at a dilution of 1:1000 and 1:10, respectively. A maximum P/N ratio of about 20-fold was obtained after 12–24 h.

Table 1
Comparison of IFA with the reverse IgG Elisa using 643 serum samples of healthy donors of West Africa (Guinea, Ghana)

IIF	IgG Elisa		
	Positive	Negative	
Positive	53	0	Sensitivity 100%
Negative	6	584	Specificity 99%

3. Results

3.1. Sensitivity and specificity of the IgG ELISA

In 20 patients with recent Lassa fever IgM and IgG antibodies to Lassa virus had been found by IFA. The IgG IFA titers ranged from 1:320 to 10240. Using the reverse IgG ELISA, the same serum samples showed even higher antibody titers (range 1280–20480). Two hundred serum samples of German blood donors, were negative both by IFA and reverse IgG ELISA (mean OD + 3s ≤0.05).

Six hundred and forty three serum samples of healthy adults had been collected in West Africa (Guinea, Ghana). Fifty three out of the 643 serum samples showed IgG antibodies by IFA (Table 1). Likewise, the IgG ELISA detected all 53 samples, but 6 additional samples were positive by IgG ELISA only. Using IFA, the six sera did not have antibodies to Lymphocytic Choriomeningitis- and to Mopeia virus. The discrepant ELISA titers were rather low (between 1:20 and 1:80). Therefore, compared to IFA, the reverse IgG ELISA had a sensitivity of 100% and a specificity of 99% (predictive value positive: 89%, predictive value negative: 100%). A close correlation between the IFA and the reverse ELISA titers was found (correlation coefficient $r = 0.9$). Linear regression shows that titers detected by IgG ELISA exceed that obtained by IFA by about four-fold (Fig. 2A). But low titers of 1:≤80 in the IgG ELISA might not be detectable at a dilution of 1:20 by IFA. To test this possibility, three positive serum samples with a titer of 1:320 in IFA and 1:1280 by reverse IgG ELISA, were diluted 1:20 in negative serum. These mixtures should now have a titer of 1:64 in the ELISA and 1:16 by IFA. In fact, the residual anti-Lassa antibodies were not longer demonstrable by IFA using the mixtures at a dilution of 1:20, while the IgG ELISA detected a residual titer of 1:80 in all three mixtures.

3.2. Testing the reverse IgG ELISA in the field

Using IFA, the prevalence of antibody positives in Guinea was 18.8% (40 of 213 samples) and in Ghana 3% (13 of 430 samples). The prevalence data would be somewhat higher (4% for Ghana), if the data of the ELISA would be taken into account.

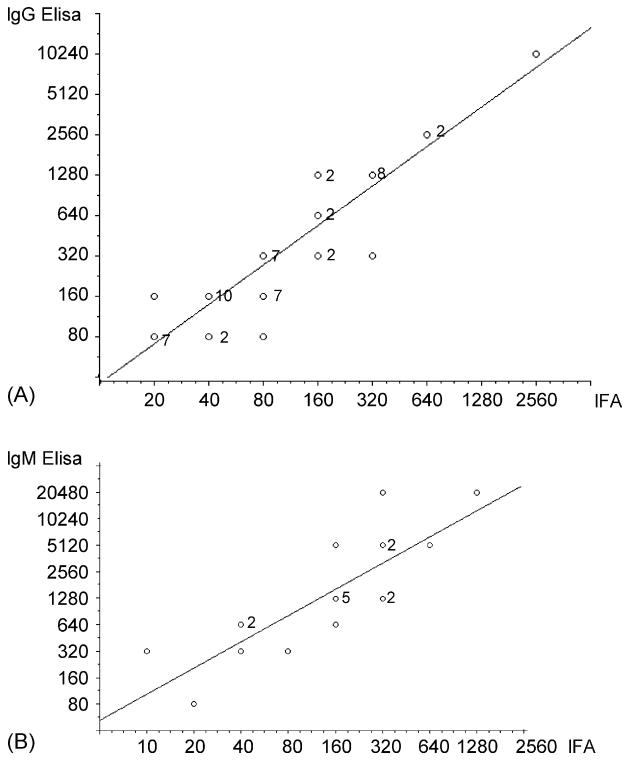

Fig. 2. (A) Correlation between IgG antibody titers detected by IFA (*x*-axis) and IgG Elisa (*y*-axis) in 53 serum samples of healthy West African blood donors. Correlation coefficient $r = 0.96$. (B) Correlation between IgM antibody titers detected by IFA (*x*-axis) and IgM Elisa (*y*-axis) in 20 serum samples of patients with recent Lassa fever. Correlation coefficient $r = 0.87$. On an average, Elisa titers exceed IIF titers by about four-fold as indicated by the linear regression lines. (Numbers indicate overlapping datum dots.)

3.3. Reverse IgM ELISA

In contrast to the reverse IgG ELISA, an incubation time of the sera of 1 h was sufficient.

Three hundred samples (including those 53 samples with IgG antibodies) of healthy people from Guinea (100) and Ghana (200) were negative both by IFA and reverse IgM ELISA. Furthermore, 100 samples of the German blood donors turned out IgM antibody negative with both tests. The reverse IgM ELISA showed the same low background staining (mean OD + 3s $\leq$0.05) as the IgG ELISA.

The 20 serum samples of patients with IgM antibodies by IFA were also positive using the reverse IgM ELISA. The titers in the IgM ELISA ranged from 1.80 to 1:20480 and exceeded that obtained by IFA by four- to eight-fold (Fig. 2B).

4. Discussion

Considering the high frequency of acute Lassa fever cases in West Africa (Lukashevich et al., 1993b; McCormick et al., 1987b; Schmitz et al., 1981), there is an urgent need for simple and reliable laboratory diagnostics. Antibody tests can help to confirm a Lassa fever suspect during an outbreak of hemorrhagic fever in West Africa. A positive IgM antibody result may give rise to isolation measures. The higher sensitivity of our reverse IgM ELISA compared to IFA suggests that it may detect specific IgM antibody early after onset of fever.

The reverse technique also improved the sensitivity of IgG antibody detection. Mixtures of positive and negative sera with a final titer of 1:80 by reverse ELISA were no longer detected by IFA. Since a continuous decline of IgG antibodies to Lassa virus has been reported in West Africans (McCormick et al., 1987b; ter Meulen et al., 2000), assays detecting low IgG antibody concentrations would be of value for antibody prevalence studies.

The high sensitivity of both reverse ELISAs may in part be due to the low background staining with an OD of <0.05 for negative samples. In contrast to indirect ELISAs or IFA, using labeled anti-globulin antibodies the reverse technique measures the amount of bound antibody via antigen. Therefore, it will not detect traces of non-specifically bound IgG or IgM immunoglobulin and parallel testing with a control antigen is not required. At least all 53 + 6 ELISA positive samples (Table 1) showed completely negative results (OD < 0.05) using a control antigen prepared of uninfected Vero C6 cells. Therefore, the control antigen could not provide additional information on false positive results.

The high specificity of the reverse IgG ELISA rests on two successive immune reactions: first, the anti-Lassa antibody has to find its counterpart, the SA, before a conformational change of Fc-portion will occur, which in turn is recognized by the RF (Sachers et al., 1985). Moreover, the application of a biotinylated monoclonal antibody to Lassa virus nucleoprotein contributes to the specificity of both tests. This monoclonal was chosen because the nucleoprotein induces a stronger immune response in humans than the Lassa virus glycoproteins (Barber et al., 1990; Hufert et al., 1989).

On the other hand, the long incubation period of the immune complexes may be problematic for a rapid diagnosis. But in acute Lassa fever, the less time consuming reverse IgM test has to be applied in the first place (Bausch et al., 2000) while the IgG ELISA may preferentially be used for confirmation.

Moreover, due to preformed immune complexes or RF in serum samples, the reverse IgG ELISA might give rise to false negative results. However, such interference has not been observed with similar reverse ELISAs for Cytomegalovirus (Schmitz et al., 1980) or flaviviruses (Sachers et al., 1985). Besides, compared to IFA, the reverse IgG ELISA did not produce any false negative results. Nevertheless, an initial dilution of 1:10 may be advisable to dilute possible immune complexes.

Both reverse ELISAs are currently applied in a field laboratory in Nigeria, where they may help to advance our understanding of Lassa virus infections.

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