Early Diagnosis of Lassa Fever by Reverse Transcription-PCR

AUSTIN H. DEMBY,[1]† JOHN CHAMBERLAIN,[2] DAVID W. G. BROWN,[1] AND CHRISTOPHER S. CLEGG[2]*

Virus Reference Division, Central Public Health Laboratory, London NW9 5HT,[1] and Division of Pathology, Centre for Applied Microbiology and Research, Porton Down, Salisbury, Wiltshire SP4 0JG,[2] United Kingdom

We developed a method based on a coupled reverse transcription-PCR (RT-PCR) for the detection of Lassa virus using primers specific for regions of the S RNA segment which are well conserved between isolates from Sierra Leone, Liberia, and Nigeria. The specificity of the assay was confirmed by Southern blotting with a chemiluminescent probe. The assay was able to detect 1 to 10 copies of a plasmid or an RNA transcript containing the target sequence. There was complete concordance between RT-PCR and virus culture for the detection of Lassa virus in a set of 29 positive and 32 negative serum samples obtained on admission to the hospital from patients suspected of having Lassa fever in Sierra Leone. Specificity was confirmed by the failure of amplification of specific products from serum samples collected from 129 healthy blood donors in Sierra Leone or from tissue culture supernatants from cells infected with related arenaviruses (Mopeia, lymphocytic choriomeningitis, Tacaribe, and Pichinde viruses). Sequential serum samples from 29 hospitalized patients confirmed to have Lassa fever were tested by RT-PCR and for Lassa virus-specific antibodies by indirect immunofluorescence (IF). RT-PCR detected virus RNA in 79% of the patients at the time of admission, comparing favorably with IF, which detected antibodies in only 21% of the patients. Lassa virus RNA was detected by RT-PCR in all 29 patients by the third day of admission, whereas antibody was detectable by IF in only 52% of the patients. These results point to an important role for RT-PCR in the management of suspected cases of Lassa fever.

Lassa fever (LF) was first described in 1969 as the cause of a nosocomial outbreak of hemorrhagic fever in the Jos Plateau region of northern Nigeria (7). The disease is caused by Lassa virus (LV), a member of the family *Arenaviridae* (17). The virus is enzootic in the multimammate rat *Mastomys natalensis*, a peridomestic rodent commonly found in west Africa. The rodents can become chronically infected at birth and excrete infectious virus in urine and other body fluids, with consequent transmission to humans (16). There is evidence of human-to-human transmission in both hospital and community settings (24). LF causes severe morbidity and mortality in many parts of west Africa, where LV is endemic. In two hospital studies in Sierra Leone, LF accounted for over 40% of febrile admissions and 30% of fatalities (13). It has been estimated that in west Africa as a whole there are 300,000 new cases of LF annually, with 5,000 deaths (15). On several occasions, patients have been transported from the area where LV is endemic to Europe, North America, and elsewhere. Although there have been no reported secondary cases under such circumstances, the potential for nosocomial outbreaks in areas where LV is not endemic is still considered to be a real possibility (2, 4, 10).

Ribavirin has been shown to reduce the fatality of severely ill patients with LF from 76 to 9%, provided that such patients receive treatment within 7 days of the onset of their illness (14). Laboratory diagnosis of LF currently relies on the demonstration of LV-specific immunoglobulin G (IgG) and IgM antibodies by immunofluorescence assay (IFA) or on virus culture. Diagnosis by IFA, especially the demonstration of IgM in patients in the acute phase of infection, is technically difficult, and patients often produce detectable antibodies only

after 12 to 14 days of illness (9). LV culture requires the use of high-level-security containment laboratories and takes 4 to 7 days for virus growth in susceptible Vero E6 cells. Diagnosis at this stage may be too late for patients to derive an optimum benefit from ribavirin therapy.

Attempts have been made to develop solid-phase immunoassays with the aim of improving the capability of the early diagnosis of LF (8), but the required level of sensitivity has not yet been achieved. In addition, methods of virus RNA detection based on reverse transcription (RT) and then PCR (RT-PCR) have been described by Lunkenheimer et al. (12) and Trappier et al. (22). A potential difficulty associated with this approach is the heterogeneity which has been demonstrated among different strains of LV at both the antigenic (18, 20) and the nucleotide sequence (5, 6) levels. Primer mismatch because of variations in target sequences could cause some LV infections to be missed. For the RT-PCR described here, we made use of the sequence data obtained from LV isolates from Sierra Leone, Liberia, and Nigeria (5) to design oligonucleotide primers with an enhanced capability of amplifying viral sequences of LV obtained from a wide geographical range. We describe here the development of an RT-PCR for LV and its evaluation for the diagnosis of LF in blood samples collected from patients in Sierra Leone.

MATERIALS AND METHODS

Serum samples and virus strains. Three sets of serum samples were used. The first group comprised 61 serum samples obtained on admission to the hospital from patients suspected of having LF and referred to the diagnostic center at the Segbwema Hospital in Sierra Leone in 1991. The second group consisted of 173 serum samples collected sequentially from 29 patients with untreated LF confirmed by LV antibody detection, virus culture, or both; these patients were also from the Segbwema Hospital. The third group comprised control sera collected from 127 healthy Sierra Leonean blood donors

* Corresponding author. Mailing address: Centre for Applied Microbiology and Research, Porton Down, Salisbury SP4 0JG, U.K. Phone: 44 980 612619. Fax: 44 980 612731. Electronic mail address: mbjsc@seqnet.dl.ac.uk.
† Present address: Division of Viral and Rickettsial Diseases, Centers for Disease Control and Prevention, Atlanta, GA 30333.

Origin	Strain	5	24	311	339
S. Leone	Josiah	-------------------------T-G-	-----C------------------------------------		
"	331	-------------------------T-G-	-----C---T------------------------------C----		
"	8189	-------------------------T-G-	-----C------------------------------------		
Liberia	523	-------------------------T-G-	-----C------------------------------------		
Nigeria	GA391	---------------------AG---	--G---A------------------------------------		
"	SA	---------------------AG---	--AT-G-----------------C---C------C--C---CC		
"	PA	----------------------G---	--CT-A---T--------A------------------------		
"	PB1	----------------------A---	------A------------A-----------C-----C----		
"	LP	---------------------AG---	--CT-A---A------C---------G------------C----		
	Consensus	GCACCGGGGATCCTAGGCATTTT-GAT	CCTCT-TCCTGCACAAAGAACAACAGTCATCATTATATAA		

Primers:-   (36E2) 5' ACCGGGGATCCTAGGCATTT 3'   (80F2) 3' ACGTGTTTCTTGTTGTCAGTAGTAATATA 5'
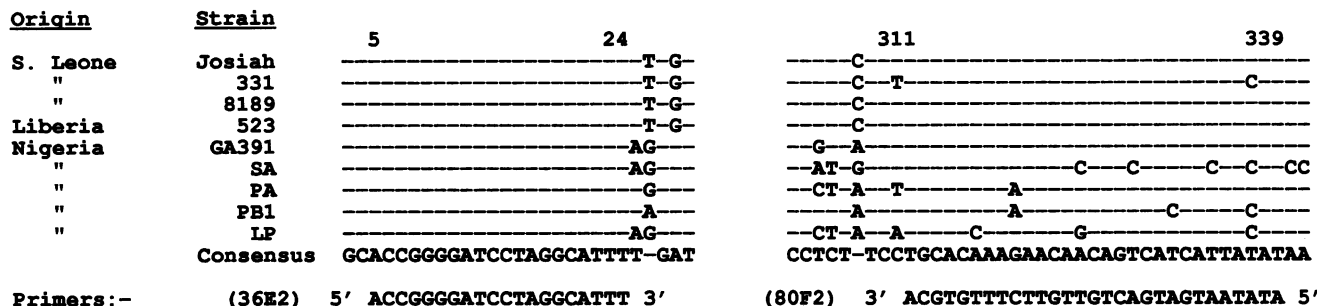
FIG. 1. Nucleotide sequence alignment of parts of the S RNA segments of LV isolates from patients in Sierra Leone, Nigeria, and a *Mastomys* sp. rodent in Liberia. The nucleotide coordinates shown correspond to those of strain Josiah from Sierra Leone (GenBank accession number J04324). The consensus sequence and the primer sequences used in this study are also shown.

living in Freetown, about 300 km from the region where LV is hyperendemic. Other virus strains examined included LV isolates in serum samples and infected culture supernatants from four Nigerian patients and Mopeia, lymphocytic choriomeningitis, Tacaribe, and Pichinde viruses in culture supernatants.

Indirect IFA and virus culture. All serum samples were tested by IFA for LV-specific IgG and IgM by the method described by Wulff and Lange (23); LV-infected E6 cells immobilized on a glass slide were used as the antigen source. This was incubated with patient serum before a fluorescein-labelled anti-IgG or anti-IgM conjugate was added. Positive samples had a characteristic fluorescence at a >1:16 dilution in phosphate-buffered saline.

All samples were cultured for LV in Vero E6 cells. Propagation of LV was confirmed by IFA. LV titers were estimated by the method of Reed and Muensch (19).

Optimized RT-PCR protocol. As a precaution against contamination of reaction components, separate laboratories were used for template preparation, reaction assembly, and analysis of amplification products. Nucleic acids were extracted from 100-μl serum samples by disruption in guanidinium thiocyanate, adsorption on silica particles, and elution into 50 μl of diethyl pyrocarbonate-treated water as described by Boom et al. (3).

First-strand cDNA was produced from LV genomic S RNA by RT by using a specific oligonucleotide primer 80F2 (5'-ATATAATGATGACTGTTGTTCTTTGTGCA-3'; see Fig. 1). The 20-μl reaction mixtures were prepared with buffer concentrates supplied by Promega for use with *Taq* DNA polymerase. Each reaction mixture contained 5 μl of template prepared as described above; 3.2 U of Moloney murine leukemia virus reverse transcriptase (Pharmacia); 1 mM (each) dATP, dCTP, dGTP, and dTTP; 20 U of RNAsin RNase inhibitor (Promega); 50 nM primer 80F2; 2.65 mM $MgCl_2$; 50 mM KCl; 2.5 mM dithiothreitol; 10 mM Tris-HCl (pH 9.0); and 0.1% Triton X-100. The reaction mixture was incubated for 1 h at 37°C, denatured at 95°C for 5 min, and cooled on ice before storage at −30°C.

For PCR, 5 μl of the RT product was supplemented with 10-times concentrated *Taq* DNA polymerase buffer (Promega) and 25 mM $MgCl_2$ to give final concentrations of 200 μM(each) dATP, dCTP, dGTP, and dTTP; 3.86 mM $MgCl_2$; 60 mM KCl; 12 mM Tris-HCl (pH 9.0); and 0.1% Triton X-100 in a volume of 20 μl. Each reaction mixture also contained 2 pmol each of primers 80F2 and 36E2 (5'-ACCGGGGATC CTAGGCATTT-3') and 0.5 U of *Taq* DNA polymerase (Promega). The cDNA was denatured at 92°C for 5 min, and

a 40-cycle PCR was performed with a denaturing temperature of 92°C for 45 s, annealing at 52°C for 45 s, and extension at 72°C for 45 s. The last cycle included a final extension time of 5 min at 72°C. The reaction was carried out in a Trio Thermocycler (Biometra). Amplification products were identified following electrophoresis on a 1.5% agarose gel stained with 1 μg of ethidium bromide per ml, with a 123-bp DNA ladder (Gibco-BRL) used as a marker. A presumptive diagnosis of LV infection was made when a band of the expected PCR product (about 340 bp) was seen.

The protocol described above was the result of systematic optimization of several variables in trial reactions with low concentrations of template. The most important of these were the magnesium concentrations in the RT and amplification phases of the reaction.

Hybridization of RT-PCR product. A fluorescein-labelled probe to confirm the identity of PCR products from clinical samples was made by an adaptation of previously described methods for labelling PCR products with ^{32}P or biotin (11, 21). Fluorescein-12-dUTP (Boehringer) replaced 25% of the dTTP in a 40-cycle PCR. The template used in the labelling reaction was derived by RT-PCR with RNA from cells infected with strain 808188, a recent isolate from Sierra Leone confirmed by sequencing to be LV. Labelled probe was stored at −30°C in the dark and was used without further purification.

Following electrophoresis, the PCR products in the agarose gel were transferred to a Zetaprobe membrane (Bio-Rad) in 0.5 M NaOH. The membrane was washed twice in 2× SSPE (1× SSPE is 0.18 M NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA [pH 7.7]) for 10 min, air dried for 1 h, baked for 30 min at 80°C, and then prehybridized in 7% sodium dodecyl sulfate (SDS)– 0.5 M sodium phosphate (pH 8.0)–1 mM EDTA at 60°C for 20 min.

Fluorescein-labelled probe was diluted 1:5 in TE buffer, denatured for 5 min at 92°C, and snap-cooled on ice before addition to the hybridization mixture. Hybridization was performed at 65°C overnight with gentle agitation. The membrane was washed twice in 0.1x SSPE–0.1% SDS for 10 min at room temperature and once at 60°C. The membrane was then blocked and incubated with antifluorescein-peroxidase conjugate, and the chemiluminescent signal was generated and detected with enhanced chemiluminescence reagents (Amersham International) as described by the manufacturer.

Construction of synthetic templates. By using the primers and conditions similar to those described above, an RT-PCR product was amplified from RNA from CV-1 cells infected with the Sierra Leone LV isolate 800134 (Josiah). The product was cloned into the vector pCRII by using the TA cloning

system (Invitrogen). Recombinant plasmid (pL134-1) was characterized by restriction enzyme digestion and sequencing. The plasmid DNA concentration was estimated by comparison with standards on ethidium bromide-stained agarose gels. To provide synthetic RNA template the virus-specific insert was transferred as an *Eco*RI fragment into the vector pTZ18R. The products were screened by restriction analysis, and a construct with the insert in the correct orientation relative to the T7 RNA polymerase promoter was designated pHD1. An RNA transcript was generated with T7 RNA polymerase (Pharmacia) by using *Xba*I-digested pHD1 as the template. The transcript consists of a 379-nucleotide (nt) sequence modeling LV genomic RNA and contained the downstream primer-binding site, the upstream primer sequence, and short flanking vector-derived RNA sequences. DNA was removed by digestion with RQ1 DNase (Promega), and RNA was purified by gel filtration on ChromaSpin-100 spin columns (Clontech). The RNA concentration was measured spectrophotometrically at 260 nm, and its integrity was assessed by agarose gel electrophoresis.

RESULTS

Primer Design. The complete nucleotide sequences of the S RNA segment of LV isolates from both Sierra Leone and Nigeria (1, 6) have been determined. These sequences are about 20% divergent from each other, with the differences most apparent in the third base positions of triplets encoding the envelope glycoproteins and nucleocapsid protein and in the nontranslated regions (apart from the well-conserved 5' and 3' termini). These differences place serious constraints on the choice of binding sites for PCR primers intended to amplify any LV strain circulating in west Africa. Further sequence data running from close to the 5' terminus of the S RNA segment into the glycoprotein gene have been obtained from seven strains of LV originating from human patients in Nigeria and Sierra Leone and from an infected *Mastomys* rodent from Liberia (4a, 5). These data were used to further define potential wide-range primer-binding sites. The primers used in the study described here are shown in Fig. 1, together with the alignments of the relevant parts of the sequences obtained from the different isolates. The upstream primer sequence (36E2) is located close to the 5' terminus of the S RNA (nt 5 to 24). The 17 nt of its 5' region form part of the characteristic conserved 5'-terminal sequence found in all arenaviruses examined to date. Its three 3'-terminal nucleotides are invariant in the nine available LV sequences. The downstream primer (80F2)-binding site is located in a part of the region coding for the G1 glycoprotein (nt 311 to 339), initially identified as invariant for 31 nt in a comparison of the complete sequences of the S RNAs of the Nigeria and Sierra Leone strains (1, 6). Subsequent sequencing of other strains confirmed that there was an unusually high degree of sequence conservation in this region (Fig. 1). Final refinements of primer sequences were made to avoid potential mismatch sites near the critical 3' end and to match optimum annealing temperatures as closely as possible. The size of the expected PCR product ranges from 335 bp for the strains from Sierra Leone and Liberia examined to 347 to 349 bp for the Nigerian strains.

Sensitivity and specificity of RT-PCR. We determined the sensitivity of the PCR using serial dilutions of a synthetic plasmid template containing a cloned copy of the target sequence (pL134.1). The specificity of the product was confirmed by agarose gel electrophoresis and Southern blotting; this was followed by hybridization with a full-length chemiluminescent probe. The limit of detection of the PCR was

determined to be between 1 and 10 copies of LV-specific DNA in each 20-μl reaction mixture. The efficiency of the RT phase of the reaction was determined by using dilutions of a synthetic RNA template. The combined RT-PCR was also capable of producing quantities of product visible by ethidium bromide staining from between 1 and 10 copies of the target RNA sequence per reaction mixture.

The standard method for the detection of LV involves virus culture. To determine the relative efficiency of the RT-PCR method we compared it with virus culture using 61 serum samples taken at the time of admission to the hospital from patients presenting with fever and other signs and symptoms consistent with LF. Of these, 29 (47.5%) were positive for LV by culture, with a mean virus load of 1,600 50% tissue culture infective doses per ml and a range of 39 to 100,000 50% tissue culture infective doses per ml. RT-PCR was performed on these samples (and all other samples) several months after virus titration and without reference to the culture data. The same 29 samples also gave positive results by RT-PCR; the remainder were negative and did not seroconvert to LV antibody positivity. There was thus complete concordance between RT-PCR and virus culture.

To further examine the specificity of the RT-PCR assay, we tested 127 serum samples from healthy blood donors from a region of Sierra Leone remote from the area endemic for LF. None was found to be positive by RT-PCR. Serum samples and culture supernatants derived from four patients from Nigeria that tested positive by culture were also detected by the RT-PCR assay. RT-PCR on culture supernatants from lymphocytic choriomeningitis virus-infected cells produced two products of 500 and 700 bp. These bands did not hybridize to the LV-specific probe. Culture supernatants containing Mopeia, Tacaribe, and Pichinde viruses produced no visible band by RT-PCR.

LV detection by RT-PCR in sequential serum samples from patients with confirmed infection. It is of evident interest to determine the utility of the RT-PCR procedure for the diagnosis of LF early in the disease process. For this purpose 173 sequential serum samples obtained from 29 patients with confirmed LF were tested by RT-PCR. The results for one such patient (patient 8424) are shown in Fig. 2. Six serum samples taken from the patient 5 to 18 days after the onset of illness were shown by RT-PCR to contain virus RNA; this was confirmed by hybridization with an LV-specific chemiluminescent probe. The same specimens contained infectious virus by culture. A positive result by RT-PCR was obtained as early as 5 days after the onset of disease (defined as the beginning of pyrexia) in this patient, whereas the diagnosis could not be confirmed by antibody detection until day 12 of illness, 1 week after the patient had been hospitalized. The LV RNA was still detectable at 18 days after the onset of illness. Similar results were obtained for samples from the other patients.

Of the 173 samples in this set, 150 (87%) were positive for LV by culture. In the initial examination by RT-PCR, when template was prepared from only 50 μl of serum, 142 serum samples showed positive amplification. When templates were extracted from additional 100-μl aliquots of the eight serum samples showing discordant results and retested by RT-PCR, all were then clearly positive, in agreement with the culture results. We thus recommend the use of 100-μl samples for template preparation in our optimized protocol.

The RT-PCR was most effective for diagnosis during the first 2 weeks after the onset of disease, when more than 90% of samples gave positive results (Fig. 3A). Notably, all five samples obtained 5 days or less after the onset of disease were positive. Later in the course of the illness LV RNA may be
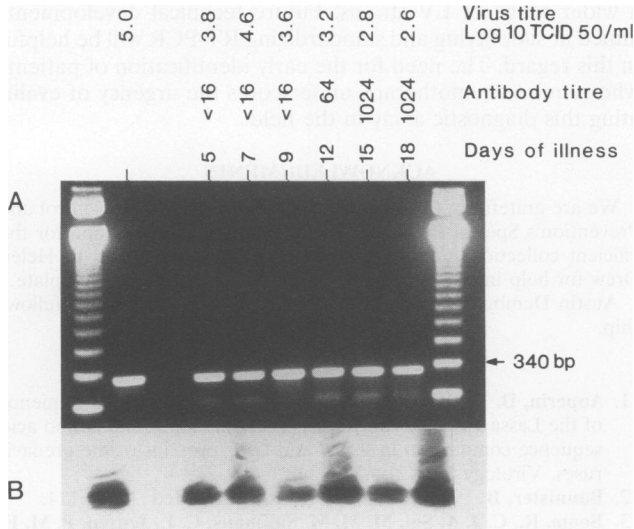
FIG. 2. Analysis of serum samples from patient 8424 with LF. (A) Ethidium bromide-stained agarose gel of RT-PCR products obtained from six sequential serum specimens taken on days 6 to 18 after the onset of disease. A positive control with culture supernatant and a negative control without added template were also analyzed. Size markers in the left and right lanes are the 123-bp ladder of Gibco-BRL. Titers of infectious virus determined by culture and LV-specific antibodies determined by IFA in each sample are shown above panel A. (B) Southern blot of the gel shown in panel A hybridized with fluorescein-labelled probe. $TCID_{50}$, 50% tissue culture infective dose.

detected less efficiently; none of the three samples taken after more than 30 days of illness were positive, but by this stage of illness LV-specific antibodies are reliably detected by IFA.

A measure of the effectiveness of diagnosis by RT-PCR in hospitalized patients in comparison with LV-specific antibody detection by IFA is obtained by analyzing these results at the patient level (Fig. 3B). On the day of admission, sera from 23 of 29 (79.3%) patients confirmed to have LF were positive by RT-PCR, whereas the sera of only 6 of 29 (20.5%) patients

were positive by IFA. By the third day of hospitalization a positive diagnosis had been made by RT-PCR in all 29 patients, whereas a positive diagnosis had been made by IFA in only 15 of 29 patients (χ^2 18.45; $P = 0.0000174$). Even after 10 days of hospitalization only 25 of 29 of the patients in this sample had detectable antibody. Of the remaining four patients, three died and the other one had recovered and left the hospital before a virus-specific antibody response was detected.

DISCUSSION

The early clinical diagnosis of LF is difficult. In west Africa the usual approach is for clinicians to try to exclude other common febrile illnesses such as malaria and typhoid fever that are amenable to treatment and that share clinical manifestations with early LF. By the time that LF is clinically obvious or the patient has developed demonstrable antibody levels, the window of opportunity for effective treatment with ribavirin may have passed. The RT-PCR assay described here takes 5 h to perform from RNA extraction to gel electrophoresis. Our results show that a reliable presumptive diagnosis of LF can be made early in the course of illness (often on the day of admission), allowing exclusion of illnesses with clinically similar presentations. Although PCR is often perceived as an expensive process, its costs must be balanced not only against those associated with failure to identify and treat patients who could benefit from ribavirin therapy but also against those of the unnecessary hospitalization and irrelevant treatment (with an expensive and potentially toxic drug) of patients with other diseases. A single course of ribavirin treatment costs about $1,000, with total costs of about $1,500 for a hospitalized patient in Sierra Leone. In countries outside of west Africa the assay provides the means for the rapid screening of pyrexic patients who have a history of recent travel in or near the areas endemic for the disease, so that the extraordinary costs associated with care in the high-level-security medical facilities usually used ($10,000 or more) can be restricted to patients for whom it may actually be required. RT-PCR can thus play a useful role in the more accurate targeting of therapy and cost-effective patient management.

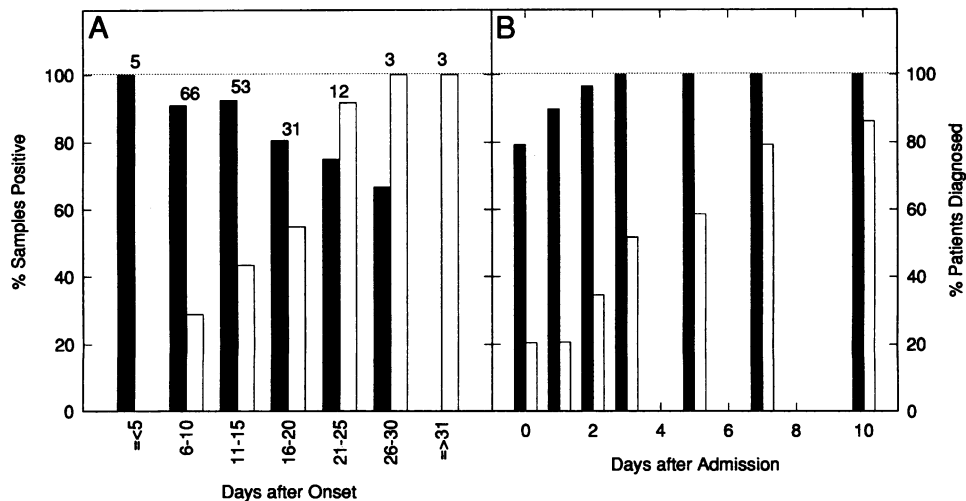The primers used in the RT-PCR described here have been

FIG. 3 Comparison of effectiveness of diagnosis of LF in hospitalized patients with LF by RT-PCR (■) and antibody detection by IFA (□). (A) Samples from all patients were stratified with respect to the time since the onset of disease. The number of serum specimens in each group is indicated. (B) Samples were analyzed on a patient-by-patient basis with respect to time since admission to the hospital.

designed with the intention of increasing the probability of detecting a wide range of LV strains by using sequence data from viruses isolated from different areas of west Africa. The present results show that the assay is as effective as virus culture with patient samples from Sierra Leone, confirming that no virus in the present sample had RNA which could not be amplified with the primer pair used in the study. All of a limited number of samples obtained from patients from Nigeria were also positive. However, further work to validate the assay with specimens of Nigerian origin is necessary because of the wider degree of sequence variation which has been observed (5). The primers used by Lunkenheimer et al. (12) and Trappier et al. (22) are based on the sequence of the Josiah strain of LV (1); they amplify parts of the nucleocapsid protein and envelope glycoprotein genes, respectively, for which sequence information from only single isolates is available. The failure to amplify certain LV isolates of Nigerian origin observed by Trappier et al. (22) may be due to sequence variation in the primer-binding sites.

The precise concordance between results of the RT-PCR assay and virus culture in the data presented here is striking. It might be expected that RT-PCR would amplify RNA contained in defective, damaged, or otherwise noninfectious particles. The absence of any indication of the existence of such RNA-containing yet noninfectious particles in the study described here may be due to the rapid clearance of such particles from serum or to the rapid degradation of the RNA associated with them through nuclease action.

The results suggest that detection of LV RNA by the RT-PCR assay provides a meaningful surrogate measure of the presence of infectious virus. It raises the possibility that the assay will provide information on virus clearance, not only for drug efficacy evaluation but also to help determine the prognosis for patients as the disease progresses. The quantitation of RT-PCR products may be useful in this. Another potential use of the assay, which follows from the close association of RT-PCR positivity with the presence of infectious virus, is the monitoring of the infectivities of rodent and environmental samples. A rapid and reliable assay capable of application to large numbers of samples would be of great value in the formulation of strategies to reduce transmission of the virus within hospitals and local communities.

Positive results of the RT-PCR assay described here and then LV-specific hybridization provide a definitive diagnosis of acute LV infection. LV RNA is reliably detected in samples collected during the first 2 weeks of illness. However, during the convalescent stages of illness, negative RT-PCR results are seen more frequently, presumably as virus is cleared. This does not represent a major shortcoming because the prognosis for the patient at this state of the disease tends to be good, and detectable antibody responses have usually been mounted by this stage of the illness.

The extreme sensitivity of RT-PCR is important in the diagnosis of LF for diagnostic purposes. The level of virus in the samples examined in the present study is lower than that described from patients with fulminant cases of infection and is unlikely to be reliably detected by immunoassay. The use of guanidinium thiocyanate in the RNA extraction process inactivates LV infectivity. This permits all subsequent manipulation of RNA outside of the high-level-containment laboratory, without hazard to staff or compromising biosafety regulations. The RT-PCR is easy to set up, and the use of a nonradioactive confirmatory hybridization probe makes it possible and desirable to transfer the methodology to west Africa, where the disease is endemic, to evaluate its performance under field conditions and to investigate its sensitivity and specificity with

a wider range of LV strains. Future technical developments aimed at simplifying and standardizing RT-PCR will be helpful in this regard. The need for the early identification of patients who require chemotherapy underscores the urgency of evaluating this diagnostic assay in the field.

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