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Detection of Lassa Virus RNA in Specimens from Patients with Lassa Fever by Using the Polymerase Chain Reaction

K. LUNKENHEIMER, F. T. HUFERT, AND H. SCHMITZ*

Department of Virology, Bernhard-Nocht Institute for Tropical Medicine, D-2000 Hamburg 36, Federal Republic of Germany

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Suitable oligonucleotide primers and probes were synthesized to amplify Lassa virus (Josiah strain)-specific nucleoprotein and glycoprotein gene fragments by using reverse transcription combined with the polymerase chain reaction (PCR). Our primers did not amplify the related lymphocytic choriomeningitis virus. By using PCR, about 50 50% tissue culture infective doses could be detected in the supernatant of infected cells. Furthermore, in all five serum specimens and four of five urine specimens of patients with acute Lassa fever, viral RNA could be demonstrated. Negative results were obtained with all serum and urine specimens of healthy subjects. Our data suggest that PCR may be applied as an alternative to virus isolation in the rapid diagnosis of Lassa fever.

Lassa fever is a human disease which results in severe morbidity and mortality in West and Central Africa where several thousand deaths occur every year (11, 12). An extraordinarily high fatality rate seems to exist, particularly in pregnant women (14) and in children (13, 14). The virus is usually transmitted to humans by contact with persistently infected African rodents (*Mastomys natalensis*) living in or near human dwellings (12). Occasionally, nosocomial outbreaks have been reported, although human-to-human transmission is a rather rare event (11, 12, 17). An efficient therapy is available, particularly if the drug is administered during the early stages of the disease (8). Therefore, an early diagnosis would be of great value. It might also accelerate biosafety programs preventing further transmission.

Since the clinical symptoms in the initial phase of this disease are similar to those of influenza, a reliable diagnosis of Lassa fever is only possible by laboratory methods. The most convincing diagnosis can be made by virus isolation. Moreover, the level of viremia seems to be a good prognostic marker for the outcome of the disease (9). However, this procedure requires several days for viral cultivation and identification. Also, it may be difficult to use virus isolation in Africa because of problems with transportation and tissue cultivation. Since only half of the patients develop detectable levels of Lassa virus antibodies during the first week of the disease (9), seroconversion or specific production of immunoglobulin M antibody to Lassa virus may be too late for early isolation and therapy.

Therefore, in an attempt to develop an improved diagnostic method, we applied the polymerase chain reaction (PCR) to detect Lassa virus RNA. Previous investigations have demonstrated the sensitivity of this method, which allows the detection of proviral DNA molecules in as few as 100 human immunodeficiency virus-infected $CD4^+$ cells (6). In contrast to the direct amplification of human immunodeficiency virus DNA, the detection of Lassa virus RNA requires that it is reverse transcribed into cDNA prior to amplification.

Our specimens were collected in Liberia and Sierra Leone (18). Therefore, the virus in our material is likely be closely related to the Josiah strain, which also originates from Sierra Leone (4). The complete S genome RNA of this strain has been published recently (1), and these sequence data were used for the selection of suitable primers and probes.

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MATERIALS AND METHODS

Samples. The samples used in this study are listed in Table 2. The serum and urine samples from patients with acute Lassa fever were obtained from Liberia and continuously maintained at −70°C. In two early serum samples of these patients, antibodies to Lassa virus were not yet detectable. Virus was isolated from all specimens, except for one urine sample collected on day 62 after the onset of clinical symptoms. The exact time after the onset of the illness had been documented for all specimens. Five serum and urine samples from healthy African subjects of Liberia and 10 serum and urine samples from healthy European subjects, without antibodies to Lassa virus, served as negative controls.

Synthesis and purification of the oligonucleotides. The oligonucleotides were synthesized by using the β-cyanoethyl method on an Applied Biosystems 381A DNA synthesizer. The purification was carried out by high-performance liquid chromatography by using ion-exchange chromatography (Mono-Q column; Pharmacia, Uppsala, Sweden).

Preparation of the sera, urine specimens, and tissue culture supernatants for PCR. The human specimens were thawed and diluted in H_2O (serum, 1:4; urine, 1:2.5). Tissue culture supernatant was applied undiluted. All specimens were boiled for 5 min, briefly centrifuged to remove cellular debris, and finally cooled on ice. Then 5 μl of each sample was used immediately for reverse transcription. Alternatively, the boiled specimens could be stored at −70°C. In some experiments, the RNA from infected and noninfected Vero cells was used. RNA was extracted with guanidinium isothiocyanate and purified by CsCl ultracentrifugation (16).

Reverse transcription. For the detection of the Lassa virus RNA genome in the specimens using PCR, different sequences were chosen from the genes encoding the nucleoprotein (N) and the glycoprotein (GPC). The oligonucleotide

* Corresponding author.

TABLE 1. Josiah strain-specific primers (N1, N2, GPC1, and GPC2) and probes (N3 and GPC3) used for Lassa virus detection[a]

Primer, probe, or virus	Nucleotide no.	Reference	Gene	Sequence	Product length (bp)
Primer N1	554–578	1	N	5′ GGGGCTCGGGCTGGGAGAGATGGAG 3′	123
Primer N2	676–652	1	N	5′ CTGCCCCTGTTTTGTCAGACATGCC 3′	
Probe N3	602–634	1	N	5′ AATGCAGAGTTGCTCAATAATCAGTTCGGGACC 3′	
Primer GPC1	2625–2649	1	GPC	5′ ATAACCGATGGGAGATGGTCTCGAG 3′	146
Primer GPC2	2770–2746	1	GPC	5′ GGATGGCTTGGGGTGGGAGCTACAT 3′	
Primer GPC3	2654–2688	1	GPC	5′ GGCAGTGATCTTCCCAGGTTGTATTTTGGATTATC 3′	
Nigeria strain N1	556–580	3	N	5′ GGGGGAAAGGGTGCCAGTGATGGCA 3′	
Nigeria strain N2	678–654	3	N	5′ TTGCCCCTGTTTGGTCAGGCATGCT 3′	
Nigeria strain N3	604–636	3	N	5′ AATGCAGAGTTACTCAACAATCAGTTCGGAACA 3′	
LCMV N2	2752–2776	15	N	5′ CTGTGACTGTTTTGCCATGCAAGCC 3′	
LCMV N3	2826–2794	15	N	5′ GACTCATCACTTCTGAACAATCAGTTCGGCACA 3′	
LCMV GPC1	822–798	2	GPC	5′ TCTAGACATCCCAAAAGGGCCTGCA 3′	
LCMV GPC2	677–701	2	GPC	5′ GAAAGTACATGAGAAGTGGCTGGGG 3′	
LCMV GPC3	793–759	2	GPC	5′ TACAGTGGTTTTCCCAAGTCCTGTTTTGTATGATT 3′	

[a] Primers N1 and GPC1 were applied for reverse transcription.

primers which amplify a portion of these genes are shown in Table 1.

For the reverse transcription reaction, 5 μl of the sample was added to 15 μl of a mixture containing 10 pmol of the N1 or GPC1 primer (Table 1); 1 mM (each) dATP, dCTP, dGTP, and dTTP; 2 μl of 10× PCR buffer (500 mM KCl, 15 mM $MgCl_2$, 100 mM Tris [pH 8.3], 0.1% gelatin); 1 μl of 50 mM $MgCl_2$; 15 U of RNasin (Boehringer, Mannheim, Federal Republic of Germany); 100 U of Moloney murine leukemia virus reverse transcriptase (Gibco-BRL, Berlin, Federal Republic of Germany); and distilled H_2O up to 15 μl. This mixture was overlaid with 100 μl of mineral oil (Sigma Chemical Co., St. Louis, Mo.), incubated for 30 min at 37°C followed by 5 min at 96°C, and then placed on ice.

Amplification. The cDNA generated by the reverse transcription reaction described above was amplified by directly adding 20 μl to 80 μl of the amplification mix. This mix contained 40 pmol of N1 or GPC1 primer, 50 pmol of N2 or GPC2 primer (Table 1), 8 μl of 10× PCR buffer, and 2 U of *Thermus aquaticus* polymerase (Perkin-Elmer Cetus Instruments, Norwalk, Conn.) in 80 μl of solution.

The specific amplification conditions used were as follows: denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and elongation at 60°C for 120 s, for a total of 35 cycles. The amplified DNA fragment was detected by liquid hybridization with a ^{32}P-end-labeled oligonucleotide. The hybridization was done by using 10 μl of the amplified DNA sample and 2×10^5 cpm of the labeled oligonucleotide complementary to one of the amplified strands in 0.15 M NaCl solution at 62°C for 30 min. The samples were loaded onto a 20% polyacrylamide gel (mini-electrophoresis chamber; Bio-Rad, Munich, Federal Republic of Germany), and electrophoresis was run for 25 min at 200 V. The hybrid molecule between the labeled probe and the amplified target was detected by exposure on Kodak X-Omat AR film (Eastman Kodak, Rochester, N.Y.) for 3 h at −70°C.

In addition, in all of the experiments the amplified DNA was directly applied to a 2.5% agarose (Sigma) gel. After ethidium bromide staining, the length of the amplified material was determined. Marker DNA (Boehringer, Mannheim, Federal Republic of Germany) served as a size standard.

Controls used for the reverse transcription and for the PCR. Supernatants from infected and from noninfected Vero cells served as positive and negative controls. These controls were included in all amplification experiments. To prove that our reagents (amplification buffer, oligonucleotide primers, distilled water, and PCR buffer) were not contaminated with amplified DNA, a PCR buffer control was also run in every amplification experiment. Moreover, different rooms were used for the handling of the samples, for the amplification, and for the preparation of the oligonucleotide primers and PCR buffers.

Virus propagation and isolation. Lassa virus strain Josiah was cultivated in Vero cells and titrated according to routine procedures. Virus in infected cells was detected by immunofluorescence (IF) staining by using a mixture of monoclonal antibodies to Lassa virus NP produced in our laboratory (7). Usually NP synthesis was demonstrable in about 30% of the cells. For the isolation of virus from urine or from serum, three tissue culture bottles (10 ml) were inoculated with 100 μl of the material. After adsorption for 1 h, the medium was changed and the cells were grown at 37°C for 5 days. Virus was identified after fixation of the cells in the bottles by IF staining.

Virus titration was done by using the tissue culture Lab Tek system on slides (Miles Scientific, Naperville, Ill.). Each dilution (from 10^{-1} up to 10^{-8}) was run in four parallel tests. The tests were read 5 days after inoculation by indirect IF by using a mixture of monoclonal antibodies.

Antibody detection. Antibodies to Lassa virus were detected by indirect IF as previously described (7, 9).

RESULTS

Two oligonucleotide primer pairs (each 25 nucleotides long) were chosen for the reverse transcription and amplification of Lassa virus genomic RNA (Table 1). The primers and the corresponding probes (35 and 33 nucleotides long) were based on the sequences coding for the glycoprotein (GPC) and for the nucleoprotein (N) of the Lassa virus Josiah strain (1). Comparison of other sequenced arenaviruses (Lassa virus Nigeria strain [3], lymphocytic choriomeningitis virus (LCMV) WE strain [1, 15]) with the Josiah strain revealed that the homology of N1, N2, and N3 to the Nigeria strain was 60, 84, and 88%, respectively. The homology of N2, N3, GPC1, GPC2, and GPC3 to the genome

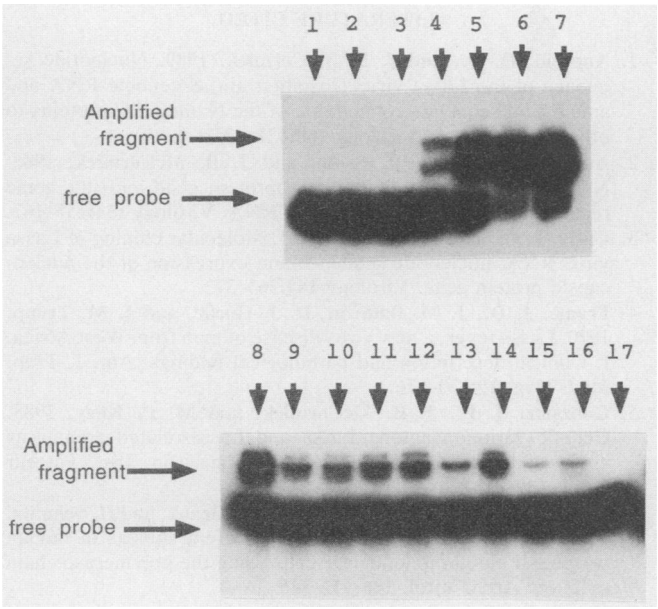

FIG. 1. Lassa virus PCR using primers N1 and N2. The amplified human material had been probed with a ^{32}P-labeled oligonucleotide (N3). Autoradiography of the 20% acrylamide gel. Lanes: 1, negative control; 2 to 6, different dilutions of supernatant containing 10^6 $TCID_{50}$ of Lassa virus per ml (10^{-4}, 10^{-3}, 10^{-2}, and 10^{-1} for lanes 2, 3, 4, and 5, respectively; lane 6, undiluted); 7, positive control; 8 to 12, positive serum samples 1 to 5 (Table 2); 13 to 16, positive urine samples 1 to 4 (Table 2); 17, negative urine sample 5 (Table 2).

of LCMV was 72, 64, 36, 36, and 69%, respectively (Table 1). Sequences related to N1 were not found within the LCMV genome.

Various dilutions of RNA, extracted from Lassa virus-infected Vero cells (5 μg), were amplified by using the primers N1 and N2. Analysis of the amplified DNA fragments on an agarose gel demonstrated that after ethidium bromide staining the appropriate 123-bp fragment was visible when RNA from infected Vero cells was used up to a dilution of 10^{-4}. Control RNA from noninfected Vero cells did not produce the 123-bp band, but a faint DNA fragment with a higher molecular weight could frequently be identified. Because of occasional nonspecific amplification products in the controls, we concluded that clear detection of Lassa virus sequences may not always be possible without specific hybridization.

Therefore, to increase the specificity and also the sensitivity of Lassa virus detection, we decided to probe our amplified material with the ^{32}P-end-labeled oligonucleotide N3 (Table 1). The hybridization with oligonucleotide N3 improved the detection of the viral RNA considerably. While 0.5 μl (5 μl diluted 1:10) of supernatant (10^6 50% tissue culture infective doses [$TCID_{50}$] per ml) could be detected in the stained agarose gel, 10-fold less was demonstrable after hybridization (Fig. 1, lane 4). Thus, an infectious dose of approximately 10^2 $TCID_{50}$ could be detected by PCR. Supernatants of infected and noninfected Vero cells served as positive (Fig. 1, lane 7) and negative controls (Fig. 1, lane 1). A buffer control was always run in parallel to exclude any possibility of contamination of our specimens by DNA fragments, which may be present in significant amounts in the vicinity of the thermocycler as a result of earlier amplification experiments. In the lower part of Fig. 1 (lanes 8 to 17), the results obtained after amplification of 5 μl of serum or urine are shown. The specimens were diluted in water (1:4 to 1:2.5, respectively) and then simply boiled to liberate the viral RNA and to destroy any residual infectivity. Positive reactions were found with five serum samples (Fig. 1, lanes 8 to 12) and with four of five urine specimens (Fig. 1, lanes 13 to 16) of patients with Lassa fever.

TABLE 2. PCR results obtained with serum and urine specimens of 10 patients with acute Lassa fever and 15 healthy seronegative control subjects[a]

Sample	No. of days after onset	Virus isolation	Antibody titer (IF)	N PCR	GPC PCR
Serum					
1	5	+	16	++	++
2	5	+	<8	++	+
3	6	+	<8	++	+
4	7	+	256	++	++
5	11	+	256	+	+
Urine					
1	12	+	ND	++	++
2	15	+	ND	++	++
3	32	+	ND	++	+
4	40	+	ND	+	+
5	62	0	ND	0	0
15 negative sera		ND	<8	0	0
15 negative urine samples		ND	<8	0	0

[a] The amplification was carried out by using primer pair N1 and N2 and primer pair GPC1 and GPC2. The amplified material was probed with the ^{32}P-labeled oligonucleotides N3 and GPC3. ND, Not done; ++, strong positive; +, positive; 0, negative result.

In Table 2, the various results obtained with the serum samples of the five patients with Lassa fever and with urine specimens of five other patients are listed. The specimens, which had been kept frozen at −70°C, had been collected in West Africa from patients 5 to 62 days after the onset of clinical symptoms. In all samples obtained during the acute phase of the Lassa fever, viral RNA could be detected with both primer pairs. In two of the patients (Table 2, patients 2 and 3) antibody to Lassa virus was not detected by IF staining. In a urine sample collected two months after the onset, a negative PCR result was found. In contrast, in 15 control sera and 15 control urine specimens of seronegative healthy Europeans (10) and Africans (5), only negative PCR results were obtained.

To exclude the possibility of cross-reactivity with other arenaviruses, tissue culture supernatant of the closely related LCMV containing 10^8 $TCID_{50}$ was amplified by using the primer pair N1 and N2 and primer pair GPC1 and GPC2. Amplified viral fragments were not detected by agarose gel electrophoresis nor by hybridization with the corresponding probes.

DISCUSSION

Our experiments suggest that PCR can be used to reliably detect Lassa virus in serum and urine samples. In two samples from newly infected individuals, the detection was possible before antibodies to Lassa virus could be demonstrated by IF staining. However, more extended studies are

required to provide reliable data on the sensitivity, specificity, and predictive value of Lassa virus PCR.

In a tissue culture supernatant containing 10^6 $TCID_{50}$ per ml, Lassa virus RNA could be detected in 5 μl diluted up to 10^{-2}. Thus, a minimum of about 50 infectious particles might be detectable by PCR. From these data, a higher sensitivity of virus isolation with PCR might be deduced. On the other hand, the situation in Africa, where appropriate conditions for tissue culturing may not be available, has to be considered. Also, compared with infectivity, viral RNA seems to be relatively stable in most specimens. Thus we were able to detect viral RNA in a urine specimen that had been kept frozen for more than 10 years. During this period, the material had been thawed and refrozen at least three times, whereupon reisolation was no longer possible. We have shown earlier (6) that the amount of viral nucleic acid can be quantified, if endpoint dilutions of the specimens are tested by PCR. Quantitation of virus particles in serum samples may be important, as it has been shown that patients with fatal Lassa fever had higher virus concentrations in their blood than patients with nonfatal cases (9).

At present, the Lassa virus PCR is a rather complicated technique requiring high-quality reagents and special technical equipment. However, radioactive labeling can be substituted by nonradioactive procedures. Under such improved test conditions, the PCR might be carried out even in poorly equipped laboratories, provided a reliable source of energy is available. Thus, Lassa virus PCR might not only help to accelerate the diagnostic procedure in highly developed countries but may also be applicable to Africa.

With regard to the specificity and sensitivity of Lassa virus PCR, some technical problems have to be mentioned. First of all, contamination with previously amplified sequence fragments has to be avoided, since it may lead to a false-positive reaction. In PCR assays, we therefore recommend an additional buffer control in which contaminating nucleic acids can be easily detected. Contaminating DNA fragments may be found in enormous quantities in the vicinity of the thermocycler as a result of earlier amplification experiments.

The primers used in our experiments probably do not detect all Lassa virus strains. The amount of nucleotide conservation among different Lassa virus isolates needs to be studied in more detail. For example, our N primers will not amplify the Nigerian strain GA 391 (3), since the homology of our N1 primer used for reverse transcription is only 60%. It seems to be difficult to find primers fitting all isolates, but mixtures of several primer pairs may be applied to solve this problem.

By using PCR, the transmission of Lassa virus from rodents to humans may be studied in more detail. Furthermore, it should be possible to study the distribution of the virus in different human tissues, especially in peripheral blood cells, as it had been described previously that the virus replicates in human monocytes (10). Thus, more information on the pathogenesis of this important hemorrhagic fever may be acquired.

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LITERATURE CITED

1. **Auperin, D. D., and J. B. McCormick.** 1989. Nucleotide sequence of the Lassa virus (Josiah strain) S genome RNA and amino acid sequence comparison of the N and GPC proteins to other arenaviruses. Virology **168**:421–425.
2. **Auperin, D. D., D. R. Sasso, and J. B. McCormick.** 1986. Nucleotide sequence of the glycoprotein gene and intergenic region of the Lassa virus S genome RNA. Virology **154**:155–167.
3. **Clegg, J. C. S., and D. Oram.** 1985. Molecular cloning of Lassa virus RNA: nucleotide sequence and expression of the nucleocapsid protein gene. Virology **144**:363–372.
4. **Frame, J. D., J. M. Baldwin, D. J. Gocke, and J. M. Troup.** 1970. Lassa fever, a new virus disease of man from West Africa. I. Clinical description and pathological findings. Am. J. Trop. Med. Hyg. **19**:670–676.
5. **Gonzalez, J. P., J. B. McCormick, and M. P. Kiley.** 1988. Genetic variation among Lassa and Lassa-related arenavirus analysed by T1-oligonucleotide mapping. Ann. Inst. Pasteur Virol. **139**:405–420.
6. **Hufert, F., D. V. Laer, C. Schramm, A. Tárnok, and H. Schmitz.** 1989. Detection of HIV-1 DNA in different subsets of human peripheral blood mononuclear cells using the polymerase chain reaction. Arch. Virol. **106**:341–345.
7. **Hufert, F., W. Lüdke, and H. Schmitz.** 1989. Epitope mapping of the Lassa virus nucleoprotein using monoclonal anti-nucleocapsid antibodies. Arch. Virol. **106**:201–212.
8. **Huggins, J. W.** 1989. Prospects for treatment of viral hemorrhagic fevers with ribavirin, a broad-spectrum antiviral drug. Rev. Infect. Dis. **4**:750–761.
9. **Johnson, K. M., J. B. McCormick, P. Webb, E. S. Smith, H. Elliott, and I. J. King.** 1987. Clinical virology of Lassa fever in hospitalized patients. J. Infect. Dis. **155**:456–464.
10. **Lewis, R. M., T. M. Cosgriff, B. Y. Griffin, J. Rhoderick, and P. Jahrling.** 1988. Immune serum increases arenavirus replication in monocytes. J. Gen. Virol. **69**:1735–1739.
11. **McCormick, J. B., I. J. King, P. A. Webb, K. M. Johnson, R. O'Sullivan, E. S. Smith, S. Trippel, and T. C. Tong.** 1987. A case-control study of the clinical diagnosis and course of Lassa fever. J. Infect. Dis. **155**:445–455.
12. **McCormick, J. B., P. A. Webb, J. W. Krebs, K. M. Johnson, and E. S. Smith.** 1987. A prospective study on the epidemiology and ecology of Lassa fever. J. Infect. Dis. **155**:437–444.
13. **Monson, M. H., A. K. Cole, J. D. Frame, J. R. Serwint, S. Alexander, and P. B. Jahrling.** 1987. Pediatric Lassa fever: a review of 33 Liberian cases. Am. J. Trop. Med. Hyg. **36**:408–415.
14. **Price, M. E., S. P. Fisher-Hoch, R. B. Craven, and J. B. McCormick.** 1988. A prospective study of maternal and fetal outcome in acute Lassa fever infection during pregnancy. Br. Med. J. **297**:584–587.
15. **Romanowski, V., Y. Matsuura, and D. H. L. Bishop.** 1985. Complete sequence of lymphocytic choriomeningitis virus (WE strain) compared to that of Pichinde arenavirus. Virus Res. **3**:101–114.
16. **Sambrook, J., T. Maniatis, and E. F. Fritsch.** 1989. Molecular cloning, 2nd ed., p. 7.19–7.22. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
17. **Schlaeffer, F., Y. Bar-Lavie, E. Sikuler, M. Alkan, and A. Keynan.** 1988. Evidence against high contagiousness of Lassa fever. Trans. R. Soc. Trop. Med. Hyg. **82**:311.
18. **Schmitz, H., J. Knobloch, and M. Dietrich.** 1981. Prevalence of antibodies to Lassa virus in Liberia. Tropenmed. Parasitol. **32**:200–201.