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First Outbreak of Callitrichid Hepatitis in Germany: Genetic Characterization of the Causative Lymphocytic Choriomeningitis Virus Strains

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Callitrichid hepatitis (CH) is a highly fatal, rodent-borne zoonosis of New World primates (family Callitrichidae) caused by lymphocytic choriomeningitis virus (LCMV). It is unclear whether virulence in Callitrichidae is associated with specific genetic or phylogenetic markers of the virus as only a partial S RNA sequence of a single CH-associated isolate is known. In a period of 10 months, three pygmy marmosets (*Cebuella pygmaea*) and one Goeldi's monkey (*Callimico goeldii*) died from CH in a German zoo. LCMV was most likely transmitted by wild mice. Infection was associated with characteristic histopathological lesions in liver, brain, and lymphoid tissue. Virus sequences from all callitrichids and a captured mouse were ≥99.2% identical. LCMV strains from a pygmy marmoset and the Goeldi's monkey were isolated in cell culture and the 3.4-kb S RNA was completely sequenced. Both strains differed considerably in their genetic and phylogenetic characteristics from known LCMV strains, including the previously described CH-associated strain. These data show that CH is widespread and can be caused by distantly related LCMV strains.

Key Words: callitrichid hepatitis; pygmy marmoset; Goeldi's monkey; lymphocytic choriomeningitis virus; arenavirus; phylogeny; sequence; pathology; histology.

INTRODUCTION

Lymphocytic choriomeningitis virus (LCMV) is the prototype member of the family Arenaviridae. The natural reservoir of this virus are chronically infected mice and hamsters, which shed the virus in urine and other body excretions. Infection of humans may be asymptomatic or associated with meningitis or, in rare cases, meningoencephalitis. LCMV is also a teratogenic pathogen that causes a severe and often fatal syndrome with hydrocephalus and chorioretinitis (Barton *et al.,* 1995; Barton and Mets, 1999). Affected infants suffer from blindness, deafness, mental retardation, and paresis. Several recent reports from the U.S. and Germany indicate that congenital LCMV infection is an underdiagnosed disease (Wright *et al.,* 1997; Enders *et al.,* 1999; Barton and Hyndman, 2000; Mets *et al.,* 2000).

In addition to humans, LCMV is pathogenic for New World primates of the family Callitrichidae (marmosets, tamarins, and Goeldi's monkeys). After an incubation period of 1–2 weeks, the animals develop an acute and highly fatal disease, called Callitrichid hepatitis (CH), with elevated liver enzymes, jaundice, and sometimes hemorrhage (Montali *et al.,* 1989; Ramsay *et al.,* 1989; Montali *et al.,* 1993). In contrast to humans, in callitrichids LCMV displays a multiorgan tropism with involvement of liver, spleen, lymph nodes, adrenal glands, intestine pancreas, and central nervous system (CNS) (Montali *et al.,* 1995). Hepatitis is a consistent finding. Clinically and histopathologically, CH closely resembles Lassa fever of humans, a rodent-borne arenavirus disease endemic in West Africa. Lassa virus, the etiologic agent of Lassa fever, and LCMV are phylogenetically related. Both belong to the Old World complex of the Arenaviridae. A retrospective survey revealed several outbreaks of Callitrichid hepatitis in North American zoos since the early 1980s (Ramsay *et al.,* 1989). The association of the disease with LCMV infection has been recognized since 1991 (Stephensen *et al.,* 1991, 1995). A laboratory-confirmed common-source outbreak of CH was caused by unknowingly feeding primates with LCMV-infected, newborn mice (Montali *et al.,* 1993).

Arenaviruses are enveloped viruses containing a two-segmented RNA genome. The S RNA segment, which is 3.4 kb in length, codes for the glycoprotein precursor (GPC) and the nucleoprotein (NP). The large L RNA segment encodes the viral polymerase and a small zinc binding protein. Recent genetic analyses of isolates of Lassa virus (Bowen *et al.,* 2000; Günther *et al.,* 2000) and

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TABLE 1

Clinical, Epidemiological, and Histopathological Data of Callitrichids with Fatal LCMV Infection

Species, sex/age, animal no.	Enclosure, death, symptoms	Histopathology	LCMV detection and sequencing[a]
Cebuella pygmaea, m/2 yrs, 5625/99	A, 10 Sep 99	Liver: multiple foci of inflammatory cell infiltrate; mild to moderate periportal mononuclear infiltrate; diffuse activation of Kupffer cells. Lymph nodes: hyperplasia.	PA
Cebuella pygmaea, m/4 yrs, 1893/99	B, 15 Nov 99	Brain: mild encephalitis with perivascular mononuclear cell infiltration. Liver: hepatocellular necrosis and degeneration with few acidophilic (Councilman-like) bodies; moderate diffuse fatty change.	PA, LI
Cebuella pygmaea, m/4 yrs 5692/99	B, 18 Nov 99, jaundice	Brain: mild lymphohistiocytic meningitis and focal gliosis in the cortex; Liver: multiple foci of hepatocellular degeneration and necrosis; fatty change; few acidophilic (Councilman-like) bodies; moderate periportal mononuclear cell infiltration. Lymph nodes/spleen: depletion of germinal centers; follicular hyperplasia.	PA, SP, VC
Callimico goeldii, m/4 yrs, 5871/00	C, 14 Jun 00, jaundice	Brain: mild lymphocytic meningitis. Liver: multiple foci of hepatocellular necrosis and degeneration; fatty change; few acidophilic (Councilman-like) bodies; mild periportal and perivascular lymphocytic infiltration. Lymph nodes/spleen: depletion of germinal centers.	UR, SE, VC

[a] Method and specimen used for detection and sequencing of LCMV: PA, seminested PCR with liver and spleen tissue embedded in paraffin; LI and SP, single-round PCR with frozen liver and spleen tissue, respectively; UR and SE, single-round PCR with urine and serum, respectively; VC, virus culture with liver and spleen tissue (5692/99) or serum (5871/00).

of the New World arenaviruses Guanarito (Weaver *et al.,* 2000) and Junin (Garcia *et al.,* 2000) revealed considerable diversity among virus strains. Sequence information on LCMV is comparably rare and mainly restricted to the prototype laboratory strains Armstrong and WE, for which complete S and L RNA sequences are available (Romanowski *et al.,* 1985; Salvato *et al.,* 1988, 1989; Djavani *et al.,* 1998). LCMV strains differ in pathogenicity, as demonstrated by experimental inoculation of guinea pigs and cynomolgus monkeys (Dutko and Oldstone, 1983; Peters *et al.,* 1987). Whether high virulence of LCMV strains in Callitrichidae is associated with particular genetic or phylogenetic characteristics is unknown. So far, only a partial S RNA sequence of a single LCMV strain associated with CH has been reported (Stephensen *et al.,* 1995).

We report the first laboratory-confirmed outbreak of CH in Germany. Over a period of 10 months, three pygmy marmosets (*Cebuella* [*Callitrix*] *pygmaea*) and one Goeldi's monkey (*Callimico goeldii*) died with clinical and histopathological signs of CH. LCMV was detected by PCR and virus culture. Sequencing revealed that the outbreak was caused by highly homologous LCMV strains. Two strains were isolated in cell culture and the full-length 3.4-kb S RNA sequence was determined, permitting a detailed genetic and phylogenetic characterization of the pathogen.

RESULTS

Description of the outbreak

Between September 1999 and June 2000, three pygmy marmosets and one Goeldi's monkey died from CH in the zoo of Dortmund, Germany. Diagnosis was made by histopathology and virological testing. All animals were males aged 2 to 4 years. Deaths occurred at three time points: in September 1999, November 1999 (15th and 18th), and June 2000 (Table 1). Considering an incubation period of 1–3 weeks and a short infectious period of illness (Montali *et al.,* 1993), the time course of the outbreak indicates a primary infection in all cases. A common source is possible in the second cluster. Since the animals were not fed with mice, they most likely became infected by hunting or eating wild mice, or by contamination of their food with mice excretions. Remains of eaten wild mice were found in enclosure A during the outbreak. For this reason, rodent control was initiated in the surrounding of the enclosures. Two trapped mice were tested by LCMV PCR and one was found positive (see below). After the second cluster of deaths, six healthy callitrichids were tested by immunofluorescence for LCMV-specific antibodies to search for asymptomatic infections. One animal, a female pygmy marmoset which lived in enclosure A together with the infected male marmoset 5625/99 (Table 1), had serological evidence of a recent LCMV infection with an IgG titer of 1:10240 and an IgM titer of 1:160. LCMV-specific antibodies were not detected in 50 blood samples of veterinary staff and zookeepers exposed to the infected animals.

Clinical and histopathological data

Only Goeldi's monkey 5871/00 could be investigated before death. It was febrile and apathetic and had ataxia. Blood chemistry revealed hepatitis [aspartate amino-

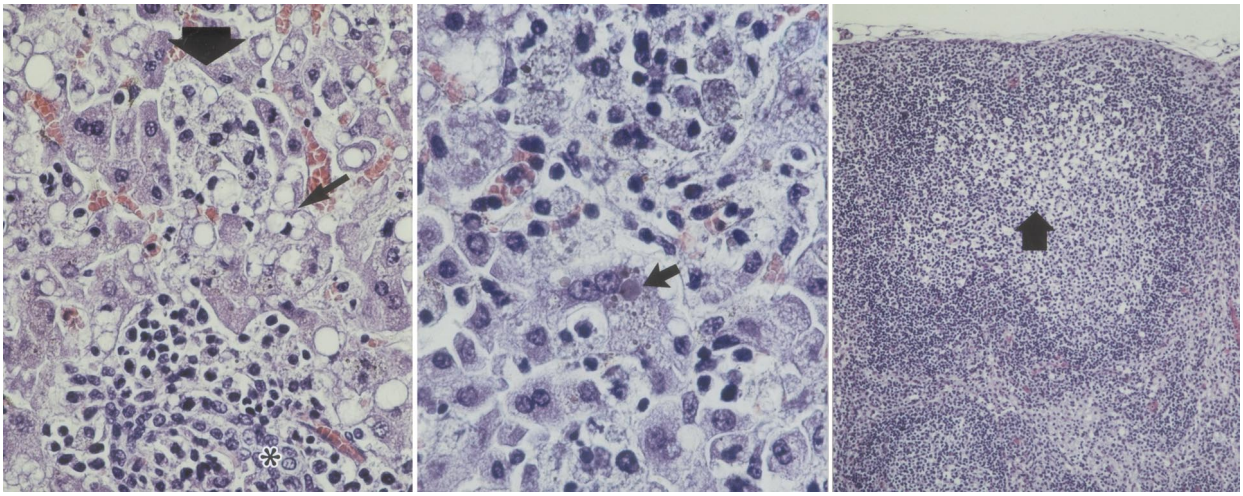

FIG. 1. Histopathological findings in pygmy marmoset 5692/99. (Left) Liver section showing necrotic foci (large arrow) with swelling and degeneration of adjacent liver cells (small arrow), infiltration of mononuclear cells in periportal areas (asterisk: bile duct), and diffuse fatty change (magnification ×376, H&E). (Middle) Typical acidophilic (Councilman-like) body (arrow) in the liver (magnification ×592, H&E). (Right) Lymph node showing hyperplasia of follicular and parafollicular regions with depletion of the germinal centers (arrow) (magnification ×94, H&E).

transferase above measurement range; γ-glutamyltransferase 9.3 × upper normal value (UNV); bilirubin 107 × UNV] and evidence of renal failure (creatinine 103 × UNV). At necropsy, few gross pathologic findings were observed in the pygmy marmosets and the Goeldi's monkey with LCMV infection: hepatomegaly (1893/99), enlargement of lymph nodes and spleen (5625/99; 5692/99; 5871/00), pulmonary edema (5692/99; 1893/99), or generalized jaundice (5692/99; 5871/00). As detailed in Table 1, histopathological findings characteristic for CH were seen in all animals without major differences between the two species. Hepatitis was characterized by multifocal degeneration and necrosis of hepatocytes, diffuse fatty change, and moderate infiltrates of mononuclear cells in periportal areas (Fig. 1, left). Acidophilic bodies resembling apoptotic (Councilman-like) bodies, which typically occur in CH, were observed in the liver of most animals (Fig. 1, middle). Lymph nodes showed follicular hyperplasia and depletion of germinal centers (Fig. 1, right). CNS involvement was evidenced by mild lymphocytic infiltration of the meninges or cerebral perivasculitis.

Detection of LCMV by PCR and virus culture

LCMV infection was demonstrated by PCR and virus culture using a variety of materials (Table 1). Frozen liver or spleen tissue, serum, and/or urine samples were available for pygmy marmosets 1893/99 and 5692/99 and for Goeldi's monkey 5871/00. The initial RT-PCR was performed with spleen tissue of pygmy marmoset 5692/99 using several primer combinations designed on the basis of known LCMV S RNA sequences. However, only primers LCMV-S 13^+ and LCMV-S 966^- yielded a specific fragment. Based on the sequence of this fragment, primer LCMV-S 322^- was designed for a short-range RT-PCR suitable for diagnostic purposes (primers LCMV-S 13^+ and LCMV-S 322^-). All of the above specimens were positive in this RT-PCR assay (as an example, see Fig. 2A, lane 1893 liver). The positive result with urine is characteristic for arenavirus infection and shows that urine represents a source for secondary transmission to both callitrichids and humans. LCMV was also detected in liver and spleen of one of two mice captured close to the monkey's enclosures (Fig. 2B).

For pygmy marmoset 5625/99 which was analyzed retrospectively, only paraffin-embedded liver and spleen tissue was available. These specimens were negative by single-round PCR. However, LCMV RNA could be detected using a seminested PCR procedure, consisting of RT-PCR with primers arena-1^+ and LCMV-S 322^- followed by reamplification with primers LCMV-S 13^+ and LCMV-S 322^- (Fig. 2C). This method was validated by testing paraffin-embedded liver and spleen tissue from monkeys without CH as a negative control and from pygmy marmosets 1893/99 and 5692/99 as a positive control. Similar to the specimen of pygmy marmoset 5625/99, the positive controls required seminested PCR before a signal was obtained (Fig. 2A, compare lanes 1893 PA in the left and right panels), while negative controls were always negative (Fig. 2C). All positive results were verified in replicate PCRs with independent RNA preparations.

In addition to PCR, virus was isolated from liver and spleen of pygmy marmoset 5692/99 and from serum of Goeldi's monkey 5871/00 by inoculation of L cells. Immunofluorescence with an LCMV-specific antiserum revealed a granular, cytoplasmic pattern characteristic of LCMV infection in cell culture (Fig. 2D). During these

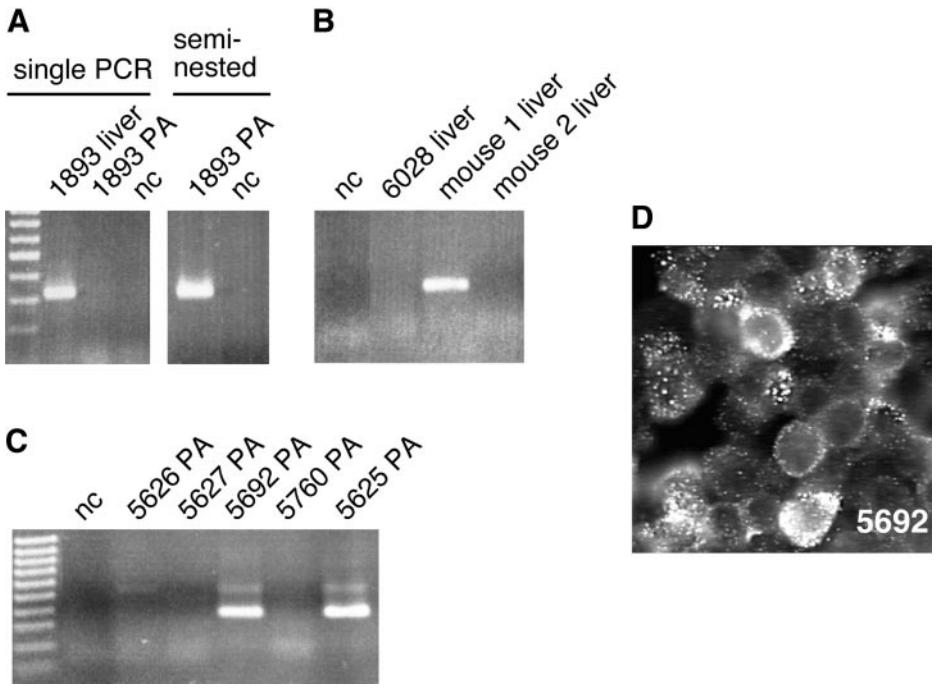

FIG. 2. Detection of LCMV by virus culture and PCR. (A) PCR with frozen and paraffin-embedded tissue of pygmy marmoset 1893/99. The frozen liver specimen was positive by single-round PCR (left), while the paraffin-embedded liver and spleen tissue required seminested PCR (right). (B) PCR with fresh liver specimens of captured mice. A liver specimen of monkey 6028/00 with an unrelated cause of death served as a negative control. (C) Detection of LCMV in paraffin-embedded tissue of pygmy marmoset 5625/99 by seminested PCR. The validity of the method was demonstrated using a specimen from monkey 5760/99 with an unrelated cause of death as a negative control, and a specimen of pygmy marmoset 5692/99 with CH as a positive control. In callitrichids 5626/99 and 5627/99, the cause of death was unclear. (D) Isolation of LCMV from spleen of pygmy marmoset 5692/99 after several passages in L cells. LCMV antigen was detected by immunofluorescence using LCMV-specific rabbit antibody WE3(KN)-12. nc, water negative control; PA, paraffin-embedded liver and spleen specimen.

studies, it became apparent that LCMV is fairly stable. Even though the specimens had been taken more than 24 h after death, were subjected to freeze-thaw cycles, or were shipped for prolonged periods of time at moderate to high temperature, isolation of the virus was successful after several passages.

Molecular and phylogenetic characterization of the causative LCMV strains

All PCR products were sequenced. Although the infections were spread over a period of 10 months, the 310-bp S RNA fragments from all callitrichids and the mouse were identical, except for a single nucleotide polymorphism at position 83. Pygmy marmoset 5625/99, which died in September 1999, had LCMV with a C at position 83; pygmy marmosets 1893/99 and 5692/99, which died in November 1999, had LCMV with a T at position 83, while Goeldi's monkey 5871/00, which died in June 2000, also had LCMV with a C at position 83. LCMV of liver and spleen of the mouse captured in May 2000 also had a C at that position. No differences were seen between sequences obtained with various specimens of the same animal (see Table 1 for tissues and body fluids analyzed). Taken together, epidemiologic and sequence data indicate that the outbreak was caused by primary transmissions of highly homologous strains.

To characterize the LCMV strains which caused the outbreak in detail, the 3.4-kb S RNAs of the L cell passaged isolates from spleen of pygmy marmoset 5692/99 and serum of Goeldi's monkey 5871/00, referred to as LCMV CH-5692 and LCMV CH-5871, respectively, were sequenced. To this end, we used a previously established long-range RT-PCR protocol (Günther *et al.,* 2000). It allows reverse transcription and amplification of the full-length S RNA of any arenavirus due to the use of primers binding to the highly conserved arenavirus RNA termini. Consistent with the analysis of the 310-bp PCR fragments, the full-length S RNA sequences of LCMV CH-5692 and LCMV CH-5871 were highly homologous (99.5%). Only 19 nucleotide differences including the abovementioned 83-T/C polymorphism were found, leading to six amino acid exchanges in GPC and NP (Fig. 3A). Ninety percent of the differences were transitions. The mutations were not evenly distributed, with the central part of the S RNA being most conserved. Passaging of the virus in L cells apparently introduced very few mutations. Within a 0.9-kb GPC gene fragment sequenced before (PCR with spleen tissue of pygmy marmoset 5692/99) and after cell culture passage (LCMV CH-5692), there

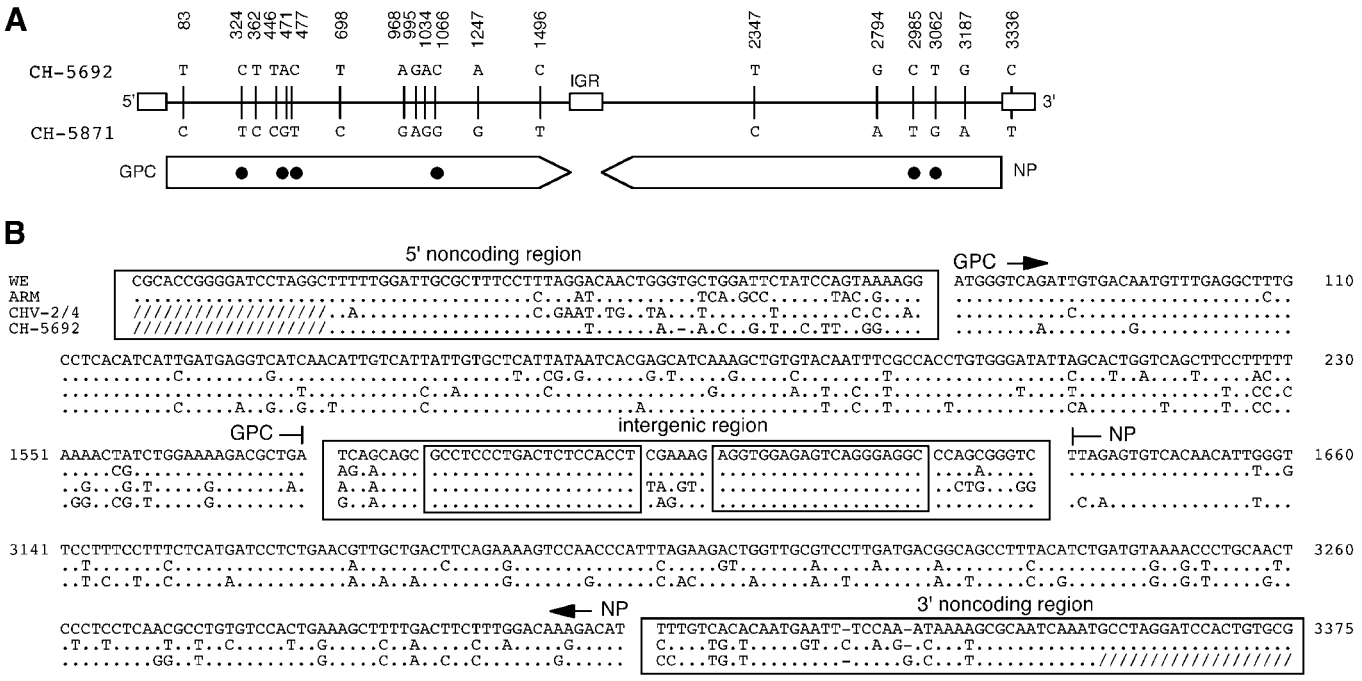

FIG. 3. (A) Schematic presentation of the nucleotide differences between LCMV CH-5692 and LCMV CH-5871. Amino acid differences are indicated by dots. Numbering is according to LCMV WE. (B) Comparison of selected S RNA nucleotide sequences of LCMV WE, LCMV Armstrong (ARM), LCMV CHV-2/4 (nucleotides 20–1638), and LCMV CH-5692. Noncoding regions are boxed, with additional boxes highlighting the complementary stem sequences of the RNA structure in the intergenic region. Primer binding sites used for PCR are indicated by slashes.

was only a single nucleotide exchange. The same region differed between LCMV CH-5692 and LCMV CH-5871 at seven positions. This suggests that the majority of nucleotide differences between both isolates does not result from cell culture passage. To compare the two LCMV isolates in more detail with LCMV of the captured mouse, a 0.9-kb GPC gene fragment of LCMV RNA from the liver of the mouse was amplified and sequenced. This sequence was 99.2% identical to LCMV CH-5692 and 99.8% identical to LCMV CH-5871. Taken together, analysis of large sequences indicates a high degree of homology, but not identity, among LCMV strains in the callitrichids and a local rodent.

The S RNA sequences of LCMV CH-5692 and CH-5871 were compared with the two known full-length S RNA sequences of LCMV WE (Romanowski *et al.,* 1985) and Armstrong (Salvato *et al.,* 1988), as well as with the partial S RNA sequence of the previously described CH-associated LCMV isolate CHV-2/4 (Stephensen *et al.,* 1995). LCMV CH-5692 and CH-5871 diverged distinctly from the two prototype strains and from LCMV CHV-2/4 (Fig. 3B). The coding sequences differed by 14.7–17.2% at the nucleotide level and 4.3–7.8% at the amino acid level, with a slightly higher divergence in the GPC gene compared with the NP gene (Table 2). The 5′ and 3′ noncoding regions just upstream of the GPC and NP start codons showed the highest level of variability among all strains. In contrast, sequences adjacent to the conserved 19-nucleotide RNA termini as well as the stem of the RNA stem-loop structure within the intergenic region were completely conserved (Fig. 3B).

An alignment of the GPC amino acid sequences showed that only a few residues were conserved among the three CH-associated LCMV strains and were not or rarely present in other LCMV strains (105-K, 177-A, 181-L, 477-S) (Fig. 4). Interestingly, at position 260, which codetermines tropism of LCMV and is involved in receptor binding (Ahmed *et al.,* 1991; Sevilla *et al.,* 2000), LCMV CH-5692 and CH-5871 had isoleucine instead of leucine (lymphotropism) or phenylalanine (neurotropism). This mutation was also found in LCMV RNA from the spleen of pygmy marmo-

TABLE 2

Nucleotide and Amino Acid Differences between LCMV Strains in S RNA Coding Regions

	% Nucleotide difference		% Amino acid difference	
LCMV strains[a]	GPC	NP	GPC	NP
CH-5692 vs WE	17.2	15.0	7.8	4.3
CH-5692 vs Arm	16.0	14.7	5.4	4.6
WE vs Arm	15.2	15.5	6.0	4.1
CH-5692 vs CHV-2/4	16.3	—[b]	6.0	—[b]

[a] Arm, strain Armstrong.
[b] NP sequence of LCMV CHV-2/4 is not known.

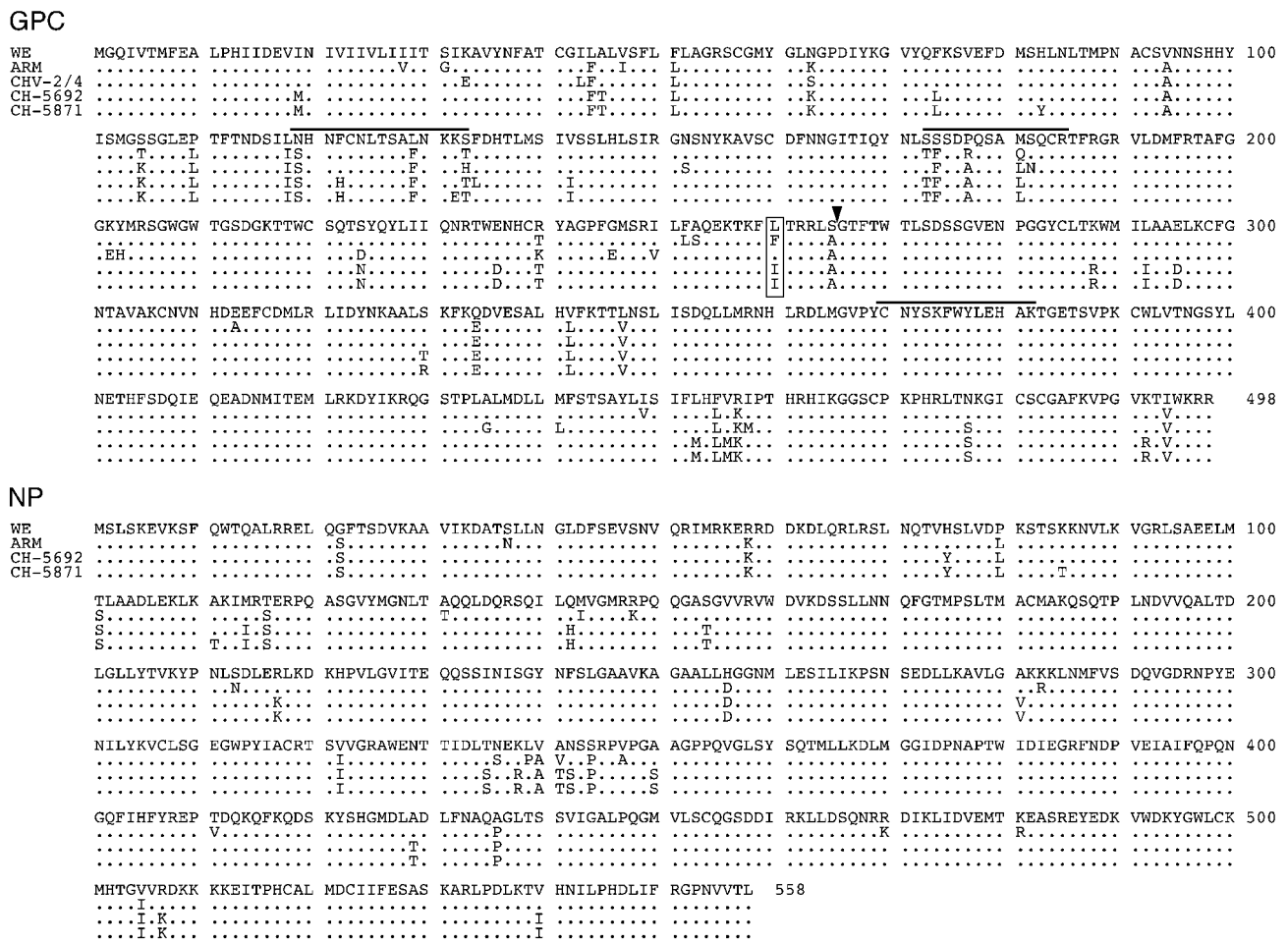

FIG. 4. Comparison of the GPC and NP amino acid sequences of LCMV strains WE, Armstrong (ARM), CHV-2/4 (only GPC), CH-5692, and CH-5871. Regions containing putative B cell epitopes (Weber and Buchmeier, 1988; Wright *et al.,* 1989; Seiler *et al.,* 1999; Ciurea *et al.,* 2000) are marked by bars above the sequence; the GP1–GP2 cleavage site (Burns and Buchmeier, 1993; Lenz *et al.,* 2000) is indicated by an arrow, and the GPC residues at position 260 implicated in receptor binding and tropism (Ahmed *et al.,* 1991; Sevilla *et al.,* 2000) are boxed.

set 5692/99 as well as in LCMV RNA from the liver of the captured mouse, indicating that isoleucine was most likely present in the virus transmitted to the callitrichids. The GP1–GP2 cleavage site and N-linked glycosylation sites were completely conserved. In NP, the CH-associated LCMV strains had 14 amino acid changes in common which were not present in the prototype strains (Fig. 4). A mutational hot spot involving proline and serine residues was evident between position 335 and 350 of NP.

Phylogenetic analysis was performed in a highly variable region of the arenavirus genome, namely in the 5′ end of the GPC gene (Fig. 5). This region was chosen because corresponding sequences are known for the largest number of LCMV isolates. The general topology of the tree corresponds fairly well with that of previous analyses of complete GPC or GP1 (Bowen *et al.,* 1997, 2000), demonstrating the validity of the method. LCMV CH-5692 and CH-5871 clearly segregated with the LCMV branch; however, there was neither a close relationship with any other known LCMV strain nor evidence for clustering of CH-associated strains.

DISCUSSION

This paper describes the first laboratory-confirmed outbreak of CH in Germany. The death of four callitrichids was caused by closely related LCMV strains. Infection was associated with characteristic histopathological lesions. Two LCMV strains were isolated in cell culture and the complete S RNA sequences were determined. The strains differed distinctly from known LCMV strains, including the previously described CH-associated strain. These data substantiate the relevance of LCMV as a zoonotic pathogen and indicate that CH can be caused by distantly related LCMV strains.

Although, or just because, LCMV has been known for more than 60 years, its pathogenic potential seems to be underestimated. This is emphasized by several recent reports indicating that congenital human LCMV infection with its devastating consequences is more common than

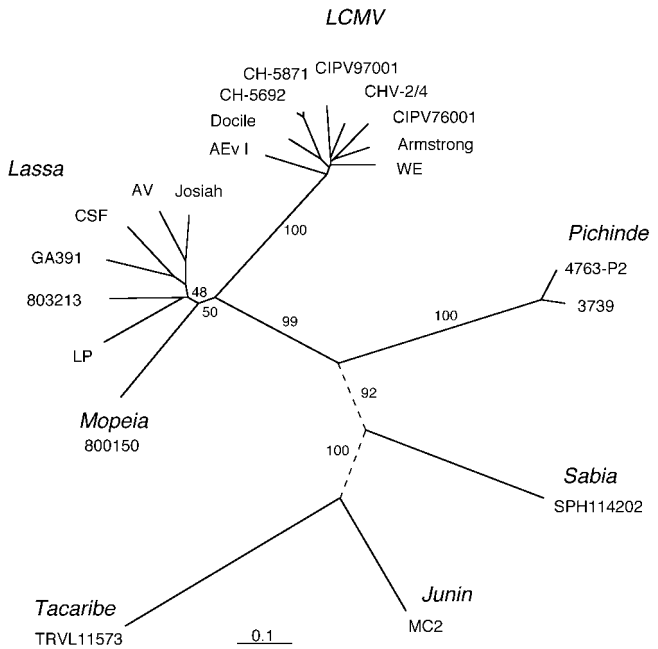

FIG. 5. Unrooted phylogenetic tree showing relationships among arenaviruses. The tree was computed with a 400-bp GPC gene fragment using the NJ method. Bootstrap support (in %) is indicated at the respective branch. The bootstrap values at the distal Lassa virus branches ranged from 38 to 85%, and the relationships within the Lassa virus taxon correspond fairly well to that reported previously (Bowen *et al.,* 2000). Bootstrap values within the LCMV taxon ranged only from 7 to 54%. Accordingly, when using the ML method the terminal LCMV branches showed a different topology, while the general topology of the NJ- and ML-based trees was identical. For space reasons, long branches have been shortened (dashed lines).

assumed (Barton *et al.,* 1995; Wright *et al.,* 1997; Barton and Mets, 1999; Enders *et al.,* 1999; Barton and Hyndman, 2000; Mets *et al.,* 2000). The present study describes the first laboratory-confirmed outbreak of Callitrichid hepatitis outside the U.S., showing that this zoonosis is widespread. It is tempting to speculate that many infections remain unrecognized due to the lack of postmortem histopathological and virological investigations. As has been pointed out previously (Montali *et al.,* 1995), CH closely resembles human Lassa fever and atypical LCMV infections have indeed been observed (Smadel *et al.,* 1942). Therefore, LCMV infection should be considered as a possible diagnosis in patients with severe febrile illness or acute hepatitis of unknown etiology.

Even though the histopathology of CH is relatively characteristic, the diagnosis requires confirmation by laboratory testing. We were not able to demonstrate LCMV infection by PCR (using frozen tissue specimens) in all cases showing a histopathology compatible with CH. The described LCMV PCR that has been established on the basis of several LCMV sequences and was evaluated using diverse clinical specimens may prove useful in the diagnostic of LCMV infection in callitrichids and humans.

The CH outbreak has been well characterized with regard to temporal pattern (separate transmissions during 10 months), spatial pattern (defined place of transmission), causative virus strains, and rodent strains of LCMV. Such information is hardly available in endemics or epidemics of human hemorrhagic fever caused by arenaviruses such as Lassa, Junin, or Guanarito virus. The LCMV data may allow some conclusions relevant to the human setting. A somewhat unexpected finding was the high degree of homology among all strains implicated in the outbreak, including the strain from the mouse. This suggests that a regional mouse population is infected by a relatively stable pool of nearly identical LCMV strains rather than by a collection of distantly related strains. The situation may be similar for Lassa virus and its rodent host, *Mastomys natalensis*. The isolation of identical Lassa virus strains (based on a 500-bp sequence) from two Mastomys trapped at the same site in Sierra Leone (Bowen *et al.,* 2000) support this view. Further evidence for the spatial and temporal stability of arenaviruses comes from phylogenetic studies on Lassa and Junin virus demonstrating a geographic, but not a temporal, pattern in the phylogenetic tree (Bowen *et al.,* 2000; Garcia *et al.,* 2000). In conclusion, our data and previous data suggest that highly homologous arenavirus strains circulate and may be transmitted at a defined place over long periods of time.

It is conceivable that only certain LCMV strains can cause CH. Clear differences in pathogenicity have been observed between LCMV strains in guinea pigs (Dutko and Oldstone, 1983) and in cynomolgus monkeys (Peters *et al.,* 1987), as well as between Lassa strains in guinea pigs (Jahrling *et al.,* 1985). However, no genetic or phylogenetic marker is currently known that could distinguish strains with different levels of virulence. In a large study of Junin virus sequences, neither phylogenetic clustering of hemorrhagic fever-associated strains nor common changes in GPC could be identified (Garcia *et al.,* 2000). Similarly, when compared with other LCMV strains, we found only a few amino acid positions that were relatively specific for the three known CH-associated strains and obtained no evidence for a specific phylogenetic origin of these strains. However, it should be taken into account that only part of the genome was analyzed and that the virulence of the other LCMV strains in Callitrichidae is not known. Nevertheless, the current data may indicate that virulence is determined by a variety of mutations which act largely independent of the phylogenetic background of an isolate.

Natural LCMV isolates usually have leucine at position 260 of GPC. The two CH-associated strains and the LCMV in the captured mouse are the exception with an isoleucine at this position. However, biological equivalence of leucine and isoleucine has very recently been demonstrated in a mouse model (Sevilla *et al.,* 2000). In contrast to brain-adapted strains with phenylalanine at

position 260, variants with leucine and isoleucine are selected in immune cells of persistently infected mice, show a high receptor-binding affinity, a tropism for dendritic cells, and eventually cause immunosuppression of the host (Sevilla *et al.,* 2000). Thus, the leucine-to-isoleucine change probably has no major consequence in persistently infected carrier mice; however, it may have some relevance after transmission to other species.

Including the two CH-associated strains described in this paper, four full-length S RNA sequences of LCMV are now known. This permits some conclusions regarding the genetic diversity in coding and noncoding regions of LCMV S RNA and a comparison with the closely related Lassa virus. As observed for Lassa virus (Bowen *et al.,* 2000; Günther *et al.,* 2000), the stem sequences of the RNA structure within the intergenic region were completely conserved among the LCMV strains, supporting the hypothesis that this structure has important virus species-specific functions. Differences in these sequences could, therefore, be used as a criterion, in addition to phylogenetic distance, to discriminate virus species within the genus arenavirus. As for Lassa virus (Bowen *et al.,* 2000; Günther *et al.,* 2000), the most divergent sequences were observed within the 5′ and 3′ noncoding regions, although the level of divergence was clearly lower. The diversity in GPC is slightly lower in LCMV than in Lassa virus (nucleotide: 16.1 vs 21.0%; amino acid: 6.4 vs 6.6%), while LCMV displays considerably less diversity than Lassa virus in NP (nucleotide: 15.1 vs 23.3%; amino acid: 4.3 vs 10.2%) [ref. for Lassa virus data (Bowen *et al.,* 2000)]. In conclusion, the location of variable and conserved regions within the S RNA seems to be identical in LCMV and Lassa virus, although the level of variability is higher among Lassa strains. In contrast to Lassa virus which shows an east → west pattern of evolution (Bowen *et al.,* 2000), phylogenetic analysis of LCMV revealed no clear evolution pattern within the LCMV taxon. The ancestral LCMV strains seem to be very closely related so that the relationships within the LCMV taxon could not be resolved unequivocally by the program. The availability of larger sequences may solve this problem.

Taken together, the study reveals novel insights into epidemiology, genetic features, and phylogeny of LCMV, with particular emphasis on strains associated with CH.

MATERIALS AND METHODS

Monkey colony

The outbreak occurred in the Amazonas building of the zoo of Dortmund, Germany, between September 1999 and June 2000. The Amazonas building has three levels. Pygmy marmosets and Goeldi's monkeys were housed at the third level in separate enclosures (enclosure B and C, respectively) at opposite sides of the visitor's corridor. The housing of the pygmy marmosets was separated from the corridor by panes of glass. Male pygmy marmoset 5625/99 lived together with a female marmoset in the separate enclosure A that was a few meters away and not accessible to the visitors. There was no contact between the animals of different enclosures. The callitrichids were not fed with newborn mice. Mouse trapping and poisoning was performed at all three levels of the building, but not within the enclosures and not outside the building.

Histopathological examination

Specimens of brain, kidney, liver, spleen, heart, lung, stomach, small and large intestine, pancreas, lymph nodes, muscle, adrenal glands, and reproductive tract were taken at necropsy and fixed in 10% buffered Formalin. Tissue samples from altered organs were frozen at −70°C. Fixed tissues were embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E) for microscopy according to standard procedures.

Serological testing

LCMV-specific antibodies in human and monkey sera were detected by indirect immunofluorescence using slides with LCMV strain Armstrong-infected Vero cells 48 h after inoculation as described (Enders *et al.,* 1999). Human and monkey IgG were detected by fluorescein isothiocyanate (FITC)-labeled anti-human IgG and anti-monkey IgG (Sigma), respectively. Human and monkey IgM were detected by FITC-labeled anti-human IgM.

RNA preparation

Specimens of frozen liver and spleen were homogenized in 600 μl lysis buffer of the RNeasy Mini Kit (Qiagen, Hilden, Germany) using a bead-mill Fast Prep FP 120 (Savant Instruments Inc., Farmingdale, NY) two times for 20 s at 6000 rpm. Cell debris were pelleted by centrifugation and RNA was isolated from the homogenate using the RNeasy Mini Kit according to the manufacturer's instructions (Qiagen). Paraffin-embedded liver and spleen tissues were cut into 20-μm-thick slices (note that both tissues were embedded in the same block and could, therefore, not be analyzed separately). Slices were heated in 600 μl buffer AVL (Qiagen) for 10 min at 62°C to melt the paraffin. The samples were centrifuged in a microcentrifuge for 10 min, allowing the paraffin to form a thin, solid overlay. The buffer phase was used for RNA preparation (QIAamp Viral RNA Kit, Qiagen). RNA was prepared without pretreatment from serum and urine samples as well as from cell culture supernatants using the QIAamp Viral RNA Kit (Qiagen).

Virus isolation

Frozen liver and spleen specimens were homogenized in 600 μl cell culture medium as described above. Cell

debris were pelleted by centrifugation and the cleared homogenate was used for virus isolation. L cells were plated at a density of 1.25×10^6 cells per 50-mm-diameter dish. Medium was removed 4 h later and cells were inoculated with 3 ml of tissue homogenate diluted 1:10 and 1:100, or serum diluted 1:50 and 1:100. The inoculum was replaced by medium after 40 min. The cells were passaged at regular intervals (3–4 days) and examined by immunofluorescence for LCMV infection. For immunofluorescence, cells were harvested, spread onto slides, air-dried, and acetone-fixed. Immunofluorescence was performed by using LCMV-specific rabbit antiserum WE3(KN)-12 (dilution of 1:750) and FITC-labeled anti-rabbit IgG diluted 1:100 (Dianova, Hamburg, Germany).

Diagnostic LCMV RT-PCR

A 310-bp 5′-fragment of the S RNA was amplified using the Platinum Quantitative RT-PCR Thermoscript One-Step System (Life Technologies, Karlsruhe, Germany) in a 20-μl assay containing 2 μl of purified RNA, 0.2 μM primer LCMV-S 13^+ (tatggcgcgcCTAGGCTTTTTGGATTGCGCTTT; heterologous *Asc*I site in lowercase; the primer number denotes the position of the 5′-nucleotide of the primer in the genomic sequence of LCMV S RNA, strain WE), and 0.2 μM primer LCMV-S 322^- (GACATRTCAAACTCCACTGA). For seminested PCR, 0.2 μM primer arena-1^+ (CGCACCGDGGATCCTAGGC) binding to the conserved terminus of arenavirus RNA was used instead of primer LCMV-S 13^+. The reaction was run in a Perkin–Elmer 9600 thermocycler as follows: reverse transcription at 50°C for 30 min; 95°C for 5 min; touch-down amplification for 10 cycles at 95°C for 10 s, 60°C for 5 s with a 1°C decrease every cycle, and 72°C for 25 s; amplification for 40 cycles at 95°C for 10 s, 50°C for 10 s, and 72°C for 30 s; final extension at 72°C for 5 min. If no PCR product was detected in ethidium bromide-stained gel, 1 μl of the arena-1^+–LCMV-S 322^--primed reaction was reamplified in a 25-μl seminested PCR assay containing 0.4 μM primers LCMV-S 13^+ and LCMV-S 322^-, and 1.5 u *Taq* DNA polymerase (Pharmacia). The reaction was run in a 9600 thermocycler with 95°C for 2 min; amplification for 25 cycles at 95°C for 10 s, 50°C for 10 s, and 72°C for 30 s; final extension at 72°C for 5 min.

RT-PCR of full-length S RNA

Full-length S RNA was amplified as described previously (Günther *et al.,* 2000). In brief, 6 μl of purified RNA were incubated with 20 pmol primer arena-1^+ in an 8-μl assay at 70°C for 15 min and quickly chilled on ice. A 19-μl reaction premix containing 8 μl RNA-primer mix, 50 mM Tris–HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, and 500 μM dNTP was incubated at 50°C for 2 min and subsequently 200 u Superscript II reverse transcriptase (Life Technologies) were added. The reaction was run at 50°C for 30 min; 55°C for 5 min; 50°C for 20 min; 60°C for 1 min; 50°C for 10 min. The enzyme was inactivated at 70°C for 15 min and RNA was removed by adding 2 u RNase H (Life Technologies) and incubation at 37°C for 20 min. cDNA was amplified by using the Expand High Fidelity PCR System (Roche Molecular Biochemicals, Mannheim, Germany) with a hot start. The 45-μl reaction premix contained 1 μl of cDNA, 1× buffer with 1.5 mM $MgCl_2$, 200 μM dNTP, and 0.2 μM primer arena-$1a^+$, 0.2 μM primer arena-$1b^+$, and 0.2 μM primer arena-$1c^+$ (tatggcgcgcCGCACCGAGGATCCTAGGCATT; tatggcgcgcCGCACCGGGGATCCTAGGCAAT; and tatggcgcgcCGCACCGGGGATCCTAGGCTT; respectively, heterologous *Asc*I site in lowercase). Alternatively, 0.3 μM of primers LCMV-S 13^+ and LCMV-S 966^- (TGAGCTCTGCAGCAAGGATCATCCA) were used. The premix was heated to 55°C, and 5 μl enzyme mixture, containing 2.6 u *Taq* and *Pwo* polymerase in 1× buffer, was added. The PCR was run for 40 cycles with 94°C for 1 min, 55°C for 1.5 min, and 72°C for 3 min with an increment of 2 min after every 10 cycles in a Robocycler (Stratagene, La Jolla, CA). To obtain sufficient material for sequencing, full-length S RNA amplification products were gel-purified and reamplified in several preparative PCRs.

Sequence determination

PCR products were purified using the QIAquick PCR Purification Kit (Qiagen) and directly sequenced on an ABI 377 automated sequencer using the BigDye Terminator AmpliTaq kit (Applied Biosystems). The diagnostic RT-PCR fragments were sequenced using primers LCMV-S 13^+ and LCMV-S 322^-. The full-length S RNA products were sequenced on plus and minus strand using the following primers: LCMV-S 13^+; LCMV-S 966^-; LCMV-S 301^+ (AGTCAGTGGAGTTTGAYATGTC); LCMV-S 437^- (GTGACTGATGATGGAGTCATTGGTG); LCMV-S 861^+ (AGGAGACTTGCAGGCACATTCAC); LCMV-S 1634^+ (GGGTCTTAGAGTGTCACAACATT); LCMV-S 1765^- (ACTGTGCACTCATGGACTGCATCAT); LCMV-S 1920^+ (AGTCCAGAAGCTTTCTGATGTCATC); LCMV-S 2325^+ (TCGTGTTCTCCCATGCTCTCCCCAC); LCMV-S 2390^- (CAGGTGAAGGATGGCCATACATAGC); LCMV-S 2814^+ (GAAGTGATGAGTCCTTCACATCCCA); LCMV-S 2886^- (GTTGGGATGAGAAGRCCTCAGCA); LCMV-S 3363^- (CCTAGGCATTTGATTGCGCWTTT). Each nucleotide position was sequenced at least twice. Overlapping sequences were identical and no sequence ambiguities were observed. S RNA sequences of LCMV strain CH-5692 and CH-5871 have been sent to GenBank and assigned the Accession Nos. AF325214 and AF325215, respectively.

Phylogenetic analysis

Phylogenetic analysis was performed with a fragment of the GPC gene (position 78-470 in LCMV WE), for which currently the largest set of LCMV sequence data exist.

GP gene sequences were initially aligned by the program Clustal X (1.8) (Thompson *et al.,* 1997). The alignment was manually refined based on amino acid similarity using the program Sequence Navigator (1.0.1) (Perkin–Elmer). In addition to LCMV strains described in this paper, the following arenavirus sequences were included (virus strain, accession number): Tacaribe TRVL11573, M20304; Mopeia 800150, M33879; Junin MC2, D10072; Sabia SPH114202, U41071; Pichinde 3739, K02734; Pichinde 4763-P2, AF081553; Lassa LP, AF181853; Lassa 803213, AF181854; Lassa GA391, X52400; Lassa CSF (Nigerian isolate, S. Günther, unpublished); Lassa AV, AF246121; Lassa Josiah, J04324; LCMV Armstrong, M20869; LCMV WE; M22138; LCMV AEv I, AJ233160; LCMV Docile, AJ249149; LCMV CHV-2/4, U10158; LCMV CIPV97001, AF079517; LCMV CIPV76001 Pasteur, AF095783. Phylogenetic analysis was performed using the PHYLIP 3.57c program package (Felsenstein, 1995) with default settings. Neighbor-joining (NJ) analysis was conducted using DNADIST and NEIGHBOR, and maximum likelihood (ML) analysis was conducted using DNAML. NJ analysis was performed on a bootstrapped data set (100 replicates).

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