

First Isolation of *Mimivirus* in a Patient With Pneumonia

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Background. Mimiviridae *Mimivirus*, including the largest known viruses, multiply in amoebae. Mimiviruses have been linked to pneumonia, but they have never been isolated from patients. To further understand the pathogenic role of these viruses, we aimed to isolate them from a patient presenting with pneumonia.

Methods. We cultured, on *Acanthamoeba polyphaga* amoebae, pulmonary samples from 196 Tunisian patients with community-acquired pneumonia during the period 2009–2010. An improved technique was used for *Mimivirus* isolation, which used agar plates where the growth of giant viruses is revealed by the formation of lysis plaques. *Mimivirus* serology was tested by microimmunofluorescence and by bidimensional immunoproteomic analysis using *Mimivirus* strains, to identify specific immunoreactive proteins. The new *Mimivirus* strain genome sequencing was performed on Roche 454 GS FLX Titanium, then AB SOLiD instruments.

Results. We successfully isolated a *Mimivirus* (LBA111), the largest virus ever isolated in a human sample, from a 72-year-old woman presenting with pneumonia. Electron microscopy revealed a *Mimivirus*-like virion with a size of 554 ± 10 nm. The LBA111 genome is 1.23 megabases, and it is closely related to that of *Megavirus chilensis*. Furthermore, the serum from the patient reacted specifically to the virus compared to controls.

Conclusions. This is the first *Mimivirus* isolated from a human specimen. The findings presented above together with previous works establish that mimiviruses can be associated with pneumonia. The common occurrence of these viruses in water and soil makes them probable global agents that are worthy of investigation.

Keywords. *Mimivirus*; giant virus; pneumonia; human virus.

The importance of amoebae-infecting giant viruses in the case of pneumonia has already been examined. *Acanthamoeba polyphaga mimivirus*, the first isolate of the Mimiviridae family, was isolated from free-living amoebae (*Acanthamoeba polyphaga*) at a cooling tower in Bradford, England, and was sampled during the investigation of an unexplained pneumonia outbreak [1, 2].

These free-living amoebae have previously been used to grow *Legionella pneumophila* [2, 3]. We later discovered that this was the largest virus ever isolated [1], and we have previously described its 1.2-megabase genome [3]. Moreover, we found that mice inoculated with *Mimivirus* develop pneumonia and that the virus is detected in the lung tissues [4]. *Mimivirus* has also been shown to infect human macrophages [5].

We tested serologically for *Mimivirus* in several groups of patients with pneumonia. We found higher levels of *Mimivirus*-specific antibodies in the following groups: (1) pneumonia patients compared to controls [6, 7], (2) mechanically ventilated patients who have a poorer clinical outcome [8], (3) patients who were rehospitalized after discharge [6], and (4) patients with chronic obstructive pulmonary disease [9]. We also documented the case of a patient, our technician handling *Mimivirus*, who

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developed an unexplained pneumonia and concurrent seroconversion to 23 *Mimivirus* proteins [10]. Finally, we detected seroconversion against the *Mimivirus* virophage in patients from Laos, who ate raw fish from the Mekong River [11].

The detection of *Mimivirus* DNA in respiratory samples from pneumonia patients has been reported only once [6], and 4 other studies failed to detect *Mimivirus* DNA [9, 12–14]. We believe that the discrepancy between the serological and polymerase chain reaction (PCR) studies is due to the great genetic diversity of mimiviruses despite cross-reactivity among isolates [15]. Finally, our previous data suggested that *Mimivirus* could be an agent of pneumonia, but to fulfill the criteria of Koch postulates, isolation from a sick human was lacking. To further understand the pathogenic role of this virus, we cultured samples from a cohort of Tunisian patients with pneumonia.

PATIENTS AND METHODS

Patients

Respiratory samples, including 110 bronchial aspirations, 36 bronchoalveolar lavages, 38 lung biopsies, and 12 pleural samples, were collected from 196 Tunisian patients with community-acquired pneumonia during the period 2009–2010. Diagnosis of pneumonia was considered when a pulmonary infiltrate was present on a chest radiograph in combination with at least 2 of the following symptoms: cough, sputum production, a temperature $>38^{\circ}\text{C}$, auscultatory findings consistent with pneumonia, a C-reactive protein concentration $>15\text{ mg/L}$, and a white blood cell count $>12 \times 10^9\text{ cells/L}$ or $<4 \times 10^9\text{ cells/L}$ [16]. Respiratory samples were used primarily for standard bacteriological tests, and the remaining materials of the samples were used in this study. Written informed consent was obtained from the patients or family members. The project was approved by our ethics committee (12–018). One milliliter of remaining sample was stored at -20°C in Tunisia, and it was then shipped at 4°C to Marseille, where it was preserved at -80°C until the time of culture.

Isolation by Coculture With Agar-Grown *A. polyphaga*

The fastidiousness of our usual methods [17] led us to develop an agar technique that was inspired by a strategy previously used to isolate *Phycodnavirus* from an algae monolayer. In this strategy, the plaques detected were the result of the lysis of the host cells by the virus [18]. First, the mechanical breakdown of the cells of the respiratory samples was performed by passing them through syringes 0.33 mm in diameter (bioMérieux, Marcy l’Etoile, France). Samples were then inoculated, as previously described [15, 17], in an “enrichment” step with amoebae for 24 hours. A solution of 1 liter of PAS medium was mixed with 15 g agar (Research Organics). This solution was sterilized at 121°C for 20 minutes in an autoclave. An antibiotic mix

containing 10 μL of ciprofloxacin (4 $\mu\text{g/mL}$; Panpharma, Z.I., Clairay, France), 10 μL of vancomycin (4 $\mu\text{g/mL}$; Mylan, Saint-Priest, France), 10 μL of colimycin (500 IU/mL; Sanofi Aventis, Paris, France), 10 μL of rifampicin (4 $\mu\text{g/mL}$; Sanofi Aventis), and 10 μL of fungizone (100 $\mu\text{g/mL}$; Bristol-Myers Squibb, Rueil-Malmaison, France) was added to the medium before solidification. Fifty milliliters of the media was distributed in square Petri dishes of $23.5 \times 23.5\text{ cm}$ (Dominique Dutscher, Brumath, France). After solidification and cooling at room temperature, suspensions of 7 mL of *A. polyphaga* at a final concentration of 2.106 amoebae/mL were spread uniformly over the surface of the agar plate. Once dried, we added 5 μL of the above-described “enrichment” culture at regular 2-cm spaces on all plates. We can perform 100 tests per plate. The plates were incubated at 32°C in a moist chamber and examined at 12 hours for the detection of lysis plaques. This technique allows for the screening of only plaque-positive samples. One positive control (*Mimivirus* coculture supernatant) and 1 negative control (drop of PAS buffer) were used per plate (Figure 1).

Characterization of Virus Isolate

The peripheries of the plaques were used for amoeba cultures in liquid medium [15]. After the lysis of the amoebae, supernatants were used for virus cloning by end-point dilution [15], and 200 μL of the viral suspension was prepared for electron microscopy. The entire DNA of the *Mimivirus* was extracted from 200 μL of culture supernatant with a phenol-chloroform extraction method. DNA was prepared for whole-genome sequencing, as previously described [19]. The genome of the virus was initially pyrosequenced using a paired-end protocol on a Roche 454 GS FLX Titanium, as previously shown [20]. The genome was then sequenced on an AB SOLiD instrument (Life Technologies Corp, Carlsbad, California). Pyrosequencing reads were assembled de novo followed by mapping on the genomes with Newbler Assembly software [20]. SOLiD reads were mapped on the previously assembled genome with CLC Bio software (<http://www.clcbio.com/index.php?id=28>). Gene prediction was performed using GeneMarkS [21], Prodigal 2.5 [22], and Prokka (<http://bioinformatics.net.au/software.prokka.shtml>) software. Transfer RNA (tRNA) was predicted using Aragorn [23]. Genome annotation was performed by comparing the result to the closest *Mimivirus* genome available by the best bidirectional BLAST strategy. Comparisons were also made by BLAST searches against its proteome, the proteome of other Mimiviridae members, and the nonredundant NCBI protein sequence database. For the construction of the phylogeny tree, family B DNA polymerase sequences were aligned and then trimmed using TrimAl with automated parameters [24]. The resulting trees were subsequently verified manually. The phylogeny tree was built using PhyML set at the default parameters except for the bootstrap parameters, where the $-b$ value was set at 100.

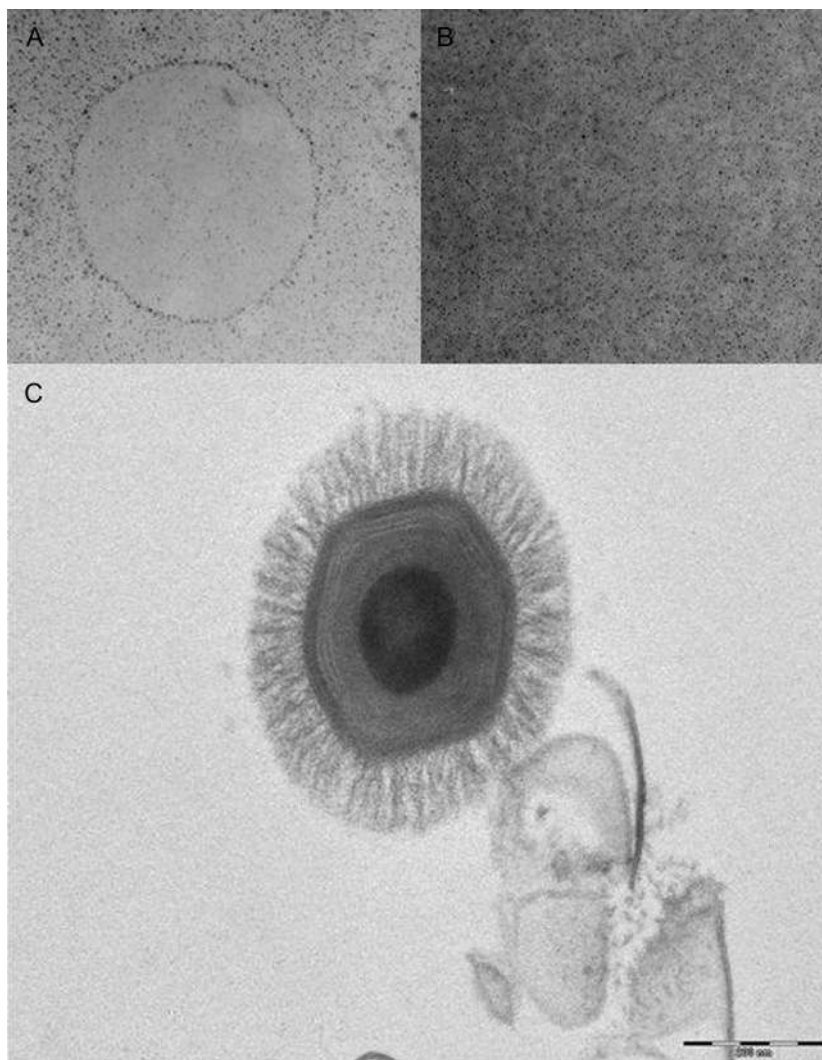


Figure 1. Visualization of lysis plaques on an agar plate coated with *Acanthamoeba polyphaga*, in the presence of LBA111 virus (A) and negative control (B). C, LBA111 staining with ruthenium red by transmission electronic microscopy.

Serologic and PCR Analysis

Serum from the positive case was obtained 23 months after infection. This serum was tested by microimmunofluorescence using LBA11 isolate as antigen, as were the serum samples of 50 blood donors. The serum was also tested by bidimensional immunoproteomic analysis as previously described [10], using both *Mimivirus* and the new strain to identify specific immunoreactive proteins. Retrospectively, we tested 179 remaining clinical samples with a PCR designed based on our new strain genome (using a primer-probe systems targeting capsid protein of the group C of Mimiviridae). Primers used were CE7-1675721_Left (5'CCA ATG ACC TAT CGT TGG-3') and CE7-1675721_Rig (5'TAT TTT ATA TTC AAC ACC AAG G-3'), and probe CE7_1675721_Pb1 (6FAM-CTTGGTCTAACAACCA AACACTA-TAMRA) and the remaining sample of the positive

patients with agents of atypical pneumonia (*Mycoplasma pneumoniae*, *Legionella pneumophila*, *Streptococcus pneumoniae*, *Coxiella burnetii*, *Mycobacterium tuberculosis*, *Chlamydiaophila psittaci*, and *Chlamydia pneumoniae*) as reported [25].

RESULTS

We tested 110 bronchial aspirations, 36 bronchoalveolar lavages, 38 lung biopsies, and 12 pleural punctures. After analyzing these samples, we managed to isolate, from a bronchial aspiration, a giant virus that generated a lysis plaque (Figure 1), which we named LBA111. Electron microscopy revealed a *Mimivirus*-like virion with a size of 554 ± 10 nm (Figure 1) and with a dense layer of fibrils.

The patient with LBA111 was a 72-year-old woman hospitalized with a history of a 3-day fever that was associated with cough, dyspnea, and hemoptysis. She was diabetic and suffered from hypertension and cardiac insufficiency. A chest radiography showed right lower lobe consolidation (Figure 2). She had hyperleukocytosis ($18\,400/\text{mm}^3$) and an increased sedimentation rate (57 mm first hour). Other etiological investigations by blood culture, fibroscopy, and standard culture of a bronchial sample displayed negative results. The PCR for agents of atypical pneumonia tested on our case were negative. Finally, she was treated with levofloxacin for 2 weeks, and she slowly showed signs of recovery.

The patient's serum, along with a collection of 50 healthy blood donors, was first tested by microimmunofluorescence using LBA111 as antigen. Reactivity was weak, with a titer of 1:32 for the patient's serum (Supplementary Figure 1); 1 blood donor had a titer of 1:16 and 5 donors had titers of 1:8. To confirm the patient's serum reactivity and to investigate the close relationship between the *Mimivirus* and LBA111, the immunoreactivity of the patient's serum was tested by performing a Western blot against both *Mimivirus* and LBA111 proteins. The patient's serum reacted strongly with 21 protein spots of LBA111, of which 17 were found to correspond to 9 LBA111-specific proteins (LBA_00225; LBA_00258; LBA_00266; LBA_00300; LBA_00442; LBA_00464; LBA_00547; LBA_00567; and LBA_00819). The serum also reacted with *Mimivirus* capsid protein (L425) and the putative GMC-type oxidoreductase (R135) (Figure 3). None of the tested samples, except that of the patient allowing the culture with LBA111, was positive with the PCR designed based on our new strain genome.

The genome size of LBA111 is 1 230 522 base pairs (Figure 4). It is closely related to *Megavirus chilensis* [26] (Figures 5 and 6). An approximately 11 000 nucleotide-long

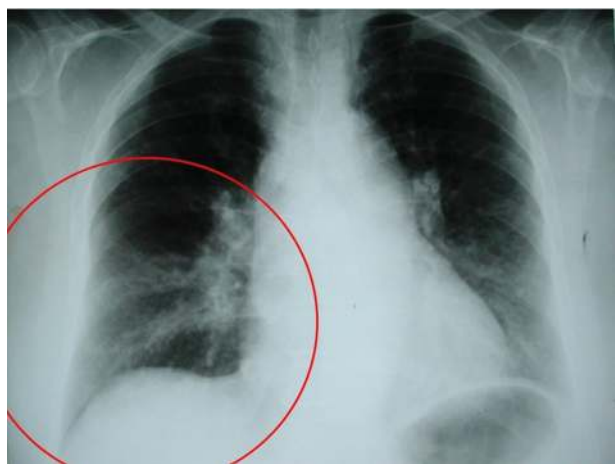


Figure 2. Chest radiograph of the patient displaying consolidation of the right lower lobe.

fragment located at the 3' end of the LBA111 genome is not collinear to that of *M. chilensis*. The 5' end of this fragment contains approximately 5000 nucleotides that are absent in *M. chilensis*, and the central part of this fragment matches the *Mimivirus* R8 gene. The 3' end matches genes that are located at the 5' end of the *M. chilensis* genome (mg4, 7, 8, 10). In addition, the first approximately 31 000 base pairs of the *M. chilensis* genome are partially found (in an inverted orientation) at the end of the LBA111 genome. A total of 1178 protein-encoding genes are predicted with 930 composing pairs of reciprocal best BLAST hits with *M. chilensis* genes. The mean length of these aligned pairs is 318 amino acids, and their mean identity is 98%. Overall, the best matches of 1141 (97%) and 14 (1%) LBA111 proteins were to *M. chilensis* proteins and *Mimivirus*/*Mamavirus* proteins, respectively. Of note, the LBA111 genome encodes 2 additional tRNAs (for histidine and cysteine) compared to that of *M. chilensis*. The GenBank accession number for the genome of LBA111 is JX885207.

DISCUSSION

In this work, we have isolated for the first time a *Mimivirus* (LBA111) from the bronchoaspiration sample of a human patient with pneumonia. The complete viral genome was sequenced and revealed that LBA111 belongs to the same clade C of the Mimiviridae as *M. chilensis* and Courdo11 [15]. Indeed, as its genome sequence is original, the possibility that it is the product of contamination from another virus grown in the laboratory is eliminated. LBA111 is the third-largest viral genome ever isolated, and it represents the largest viral genome ever isolated from a human patient. Moreover, serum samples showed reactivity against unique LBA111 proteins, confirming the patient's infection.

The causative role of giant viruses of amoebae in human pathology has remained controversial. A body of convergent arguments has suggested that these viruses may act as agents of some form of pneumonia. This makes sense because the giant viruses of amoebae live in the same ecological niche as *L. pneumophila*, which suggests that exposure to these viruses and *L. pneumophila* is comparable and that amoeba can act as a Trojan horse for giant viruses [2]. Currently, direct evidence for the pathogenicity of *Mimivirus* in pneumonia has only been shown in a single study, where *Mimivirus* DNA was detected in the bronchoalveolar fluid of a patient [6]. We tested whether the primers and probes used to detect *Mimivirus* in human respiratory samples in previous studies [6, 9, 12–14] could hybridize with the genome of LBA111 virus. We found that the median number of mismatches was 7 (Figure 4); thus, it is unlikely that these primers would amplify this virus. In contrast, LBA111 reacts with *Mimivirus* antibodies, and the patient was found to have antibodies to both *Mimivirus* and LBA111. This

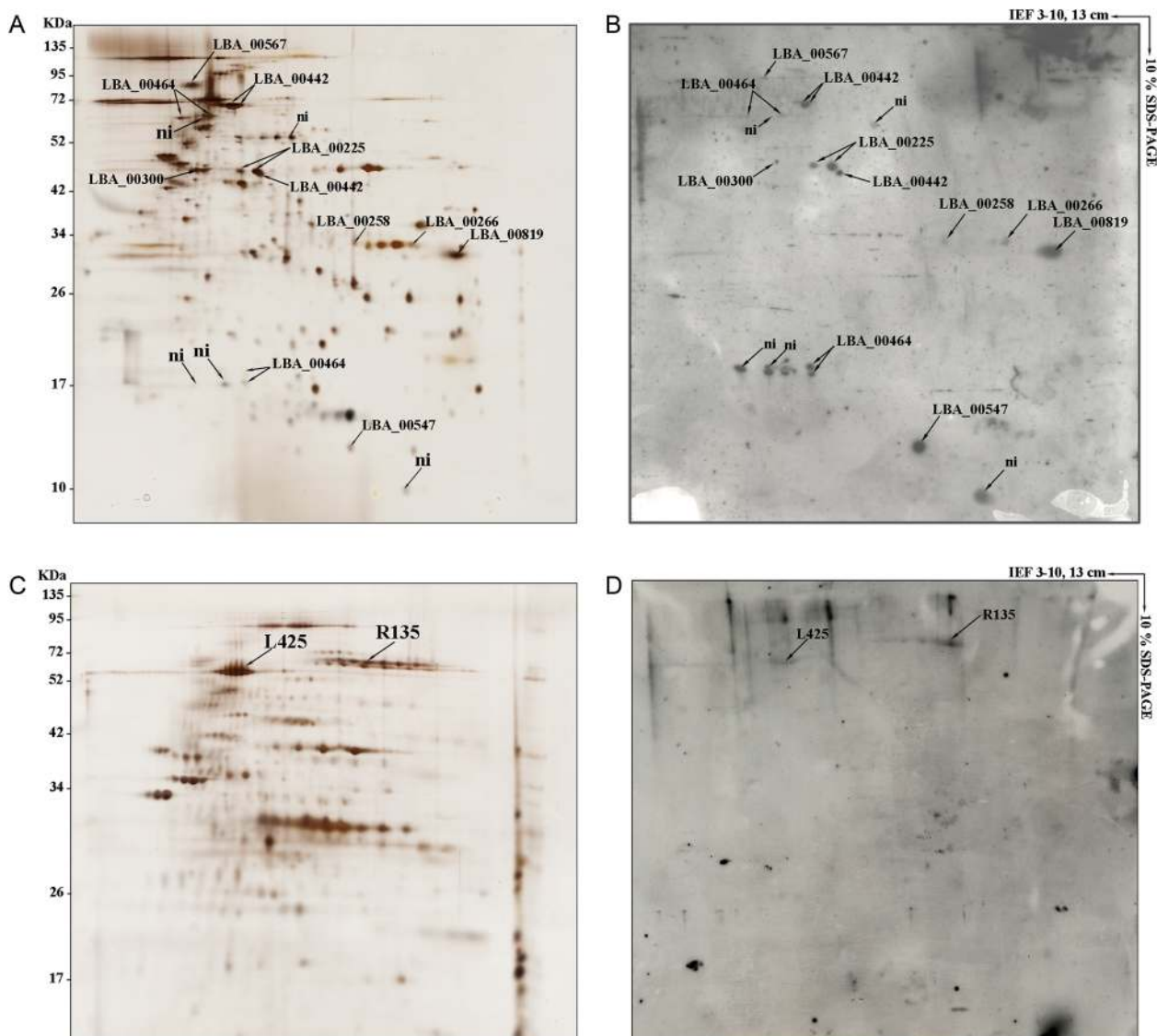


Figure 3. Two-dimensional immunoblotting patterns of the patient's serum against the LBA111 virus and *Mimivirus* proteins. Viral proteins (30 μ g) were resolved in *pI* values that ranged from 3 to 10 in the first dimension, followed by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis in the second dimension. Proteins were then detected by silver staining (A and C for LBA111 virus and *Mimivirus*, respectively) or transferred to a nitrocellulose membrane and probed with the patient's serum at 1:1000 dilution (B and D for LBA111 virus and *Mimivirus*, respectively). Immunoreactive protein spots are shown using arrows and the locus names (ni: not identified). The standard molecular weight sizes are indicated on the left in kilodaltons (KDa).

result suggests that the discrepancy between serological studies and PCR studies are caused by the genetic variability of *Mimiviridae* members despite a common antigenicity. Moreover, viral metagenomic studies commonly make up a first step of filtration that eliminates giant viruses and are, therefore, excluding these agents [27, 28].

The patient presented with a typical form of pneumonia that cannot be distinguished from bacteria-related pneumonia, except that it responded poorly to antibiotics. Clinical outcome

was spontaneously, albeit slowly, favorable, as in our index case [10]. However, the delayed recovery may or may not be dependent on antibiotic failure, given the considerable pneumonic infiltrates in an elderly lady with significant comorbidities. This study is preliminary and does not allow us to speculate further on the global frequency of *Mimivirus*-associated pneumonia. As it was already found to be associated with keratitis [29], we believe that the isolation of a *Mimivirus* from the bronchoalveolar fluid of one patient with pneumonia in the current study

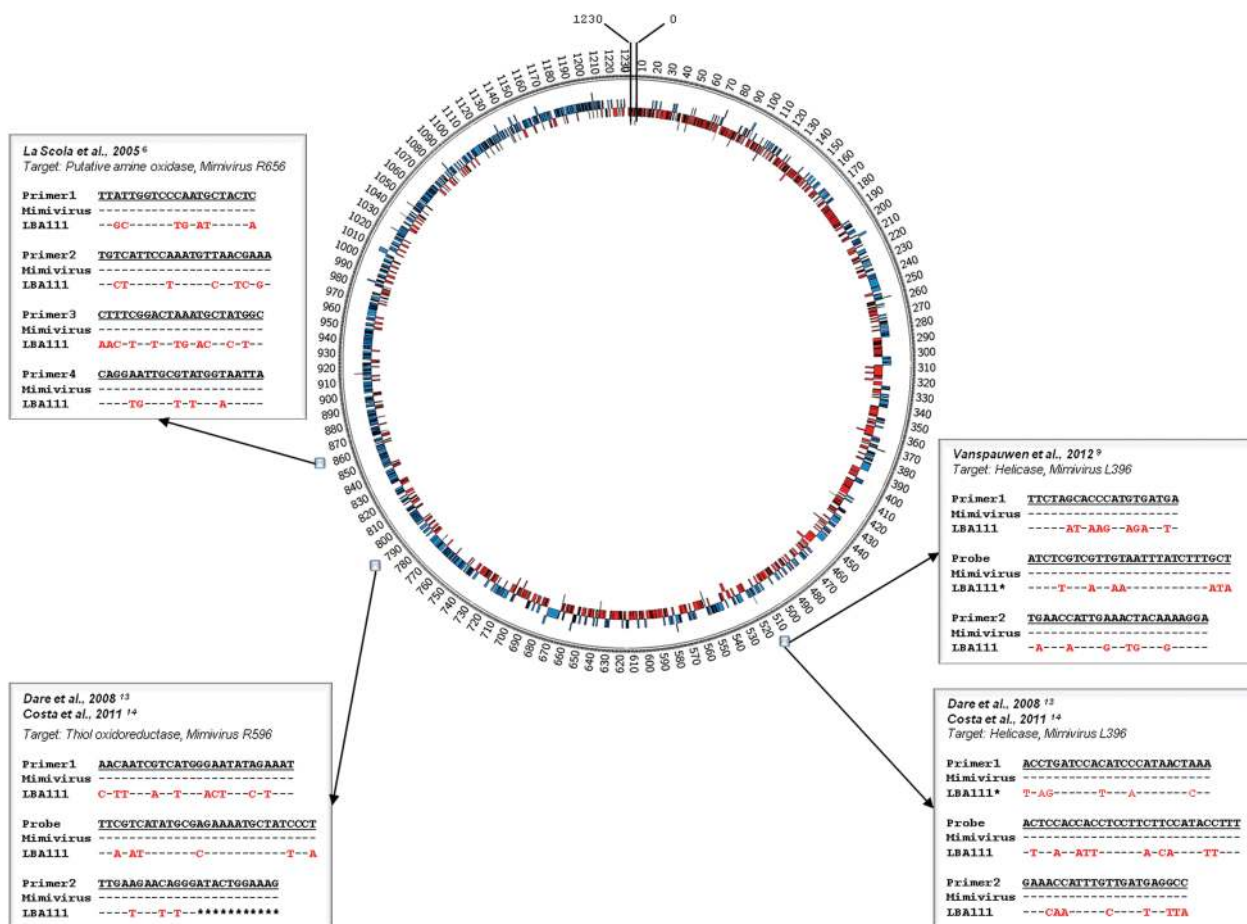


Figure 4. Map of the LBA111 virus chromosome. Rings starting from the outer to the innermost ring correspond to (1) genome coordinates in kilobases and (2) the predicted protein-coding genes, oriented in the forward (blue) or the reverse (red) strand. Boxes identify the regions in the LBA111 genome that correspond to *Mimivirus* fragments targeted by polymerase chain reaction assays in previous studies. Mismatches between the primers and probe used (top, underlined) and the genome sequences of *Mimivirus* (middle) and LBA111 (bottom) are shown (red font). Dashes indicate identical nucleotides in comparison with the primers or probe; asterisks indicate noncontiguous nucleotides in the LBA111 sequences.

suggests a role for mimiviruses as occasional agents of human infection. In previous articles, we described the identification of metagenomic reads similar to *Mimivirus* and *Marseillevirus*

DNA, followed by the isolation of a new *Marseillevirus* from the stools of a young healthy man living in rural Senegal [19, 30]. These findings indicated that giant viruses of phagotrophic



Figure 5. Comparison and gene alignment of the genomes of LBA111 virus and *Megavirus chilensis* using Mauve software (<http://asap.ahabs.wisc.edu/mauve/>). Blocks with colored outlines surround regions of the genome sequence that aligned to parts of the other genome. The colored bars inside the blocks illustrate the level of sequence similarity. Lines link blocks with homology between the 2 genomes. Regions that are inverted relative to the other genome are shifted below the center axis of the genome.

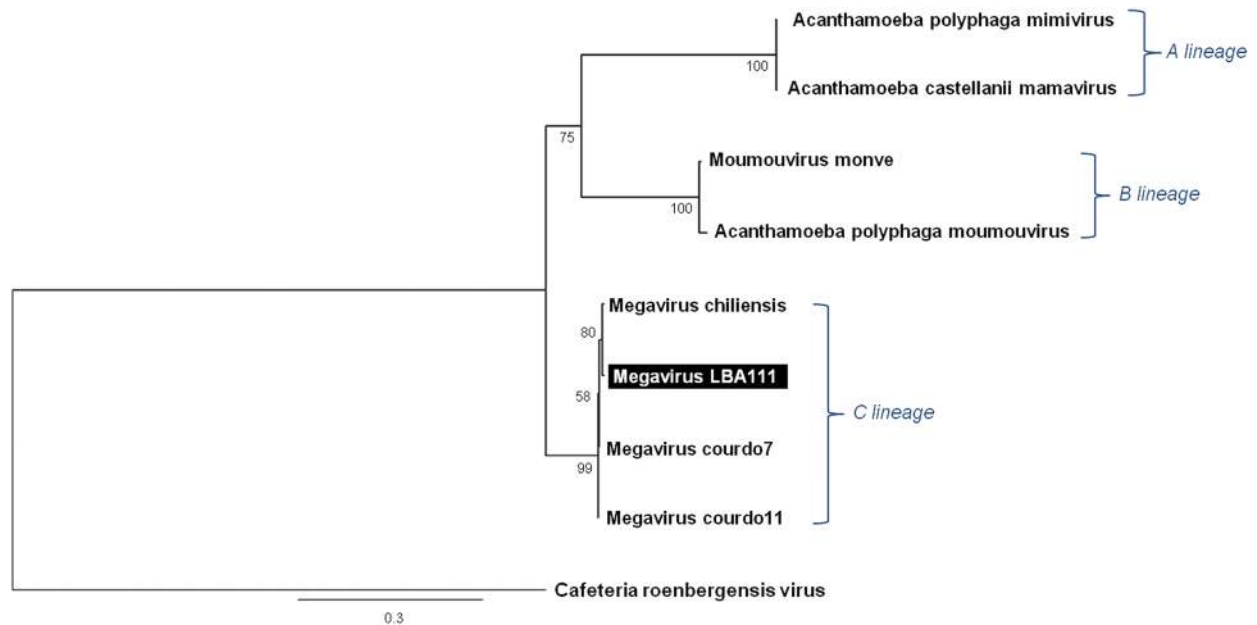


Figure 6. Phylogeny reconstruction based on the family-B DNA polymerase. LBA111 (white font, black background) is present within the *Mimivirus* C lineage, along with *Megavirus chilensis*24, Courdo7 [15, 17], and Courdo11 [15, 17] (gray background).

protists can be recovered from human samples. In addition, a review of the literature and additional searches by our team detected sequences similar to Megavirales DNA and homologs of the genes of these viruses in human metagenomes [30]. In a previous review published in this journal regarding the evidence of the putative role of *Mimivirus* in human diseases, we concluded that the next step to imply it in human pneumonia should be the isolation of a virus from a human sample [2]. This is what we report in the current study. Thus, together with the negative findings regarding culture and PCR for other pathogens and the specific antibody response, it strongly suggests that the patient suffers from *Mimivirus* pneumonia. After previous report of an infection associated with seroconversion in a laboratory technician [10] and a mouse experimental model of pneumonia with isolation of the virus from tissues of infected animal [4], the isolation of *Mimivirus* in a patient contributes to fulfill the criteria of Koch postulates and convince us that *Mimivirus* should be included as a potential human pathogen occasionally causing pulmonary infections. The present report, as increasing evidences of the common occurrence of these viruses in water and soil [17], supports their role as global agents that are worthy of investigation.

Supplementary Data

Supplementary materials are available at *Clinical Infectious Diseases* online (<http://cid.oxfordjournals.org/>). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted

materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

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Potential conflicts of interest. All authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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