Giant Viruses of Amoebae as Potential Human Pathogens

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Key Words

Mimivirus · Marseillevirus · Mimiviridae · Marseilleviridae · Megavirales · Amoeba · Humans · Pathogenicity · Disease

Abstract

Giant viruses infecting phagocytic protists are composed of mimiviruses, the record holders of particle and genome size amongst viruses, and marseilleviruses. Since the discovery in 2003 at our laboratory of the first of these giant viruses, the Mimivirus, a growing body of data has revealed that they are common inhabitants of our biosphere. Moreover, from the outset, the story of Mimivirus has been linked to that of patients exhibiting pneumonia and it was shown that patients developed antibodies to this amoebal pathogen. Since then, there have been several proven cases of human infection or colonization with giant viruses of amoebae, which are known to host several bacteria that are human pathogens. Mimiviruses and marseilleviruses represent a major challenge in human pathology, as virological procedures implemented to date have not used appropriate media to allow their culture, and molecular techniques have used filtration steps that likely prevented their detection. Nevertheless, there is an increasing body of evidence that mimiviruses might cause pneumonia and that humans carry marseilleviruses, and re-analyses of metagenomic databases have provided

evidence that these giant viruses can be common in human samples. The proportion of human infections related to these giant mimiviruses and marseilleviruses and the precise short- and long-term consequences of these infections have been scarcely investigated so far and should be the subject of future works.

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Introduction

At the origin of the assessment of the role of Mimivirus in pneumonia, a misunderstanding occurred. Tom Marie managed a collection of serum samples from patients presenting with pneumonia of unknown aetiology, and we had postulated that bacteria resistant to amoebae of the *Acanthamoeba* species, such as *Legionella pneumophila*, were potential causative agents of pneumonia [1–5]. We began this work with interesting results that showed serological reactions against new species of *Legionella* and *Parachlamydia* that we discovered through a collaboration with Tim Rowbotham, who had sent us a collection of *Legionella*-like amoebal pathogens. Interestingly, the microorganism that showed the most serological reactivity was Bradford coccus [6, 7]. In the course of analysing the results, it became clear that Bradford coccus

Fig. 1. Electron micrographs of the Mimivirus factory inside *Acanthamoeba* spp. (**a**; transmission electron microscopy), Senegalvirus particles (**b**; transmission electron microscopy; a particle is shown by the arrow), and LBA111 virus particle (**c**; negative staining).

was not a bacterium but the first amoeba-infecting giant virus. We named it Mimivirus for several reasons, including its mimicry of a bacterium by its size and appearance by Gram staining [6]. Therefore, while focusing on the remarkable features of Mimivirus, we postponed the publication of the results on Mimivirus seropositivity in patients with pneumonia [8, 9]. Thus, from the outset, while Mimivirus was still thought to be a coccus, we knew that there were antibodies to this amoebal pathogen.

The Mimivirus Discovery

Mimivirus was discovered in 2003 in the Rickettsia laboratory at the Marseille School of Medicine [7]. However, it was isolated 20 years ago in the centre of England, and its story was immediately linked to episodes of pneumonia [6, 7]. Indeed, Tim Rowbotham and his collaborators at the Public Health Laboratory in Leeds, UK, investigated a 1992 pneumonia outbreak in Bradford for which no causative agent was identified. These researchers showed the utility of culturing on *Acanthamoeba* spp., which are ubiquitous amoebae living in water and soil, to isolate agents of pneumonia, including *L. pneumophila*, from human samples [10–15]. These amoebae are phagocytic protists that have been described as true Trojan

horses because they can host multiple human pathogenic bacteria that survive and multiply within the amoebae and are protected from various external physical and chemical agents when the amoebae encyst [1, 16–21]. Therefore, to isolate the bacteria causing the pneumonia outbreak in Bradford, Rowbotham inoculated amoebae with water from cooling towers, which he thought might be the origin of the epidemic [7]. The presence of an amoebal pathogen was visualized by the formation of lysis plaques due to the multiplication of the microorganism. Several pathogens of amoebae were isolated by this strategy from environmental samples collected in Bradford. In 1995, Richard J. Birtles, a young English investigator, conducted a postdoctoral fellowship in our lab and brought this particular collection of microorganisms isolated by amoebal coculture. Several new *Legionella* species were characterized, including *L. rowbothamii*, *L. drozanskii*, *L. fallonii*, and *L. drancourtii* [4, 22]. Another of the amoebal pathogens had the appearance of a Gram-positive coccus and was named Bradford coccus [7]. Surprisingly, it resisted various strategies aimed at its identification, including the PCR amplification and sequencing of the 16S ribosomal DNA. These unexpected failures led to the observation of Bradford coccus using electron microscopy, which revealed that it had an icosahedral structure strongly suggestive of a virus (fig. 1a). Subsequently, the viral nature of Bradford coccus was confirmed by observations, including an eclipse phase dur-

Fig. 2. Timeline of evidence indicating that mimiviruses are causative agents of pneumonia and that marseilleviruses are present in humans. Giant virus detection by serological testing, PCR testing, culture on *Acanthamoeba* spp. and metagenomics are indicated by yellow, orange, green and blue backgrounds, respectively. Experimental findings are indicated by a purple background.

ing its replication cycle [6], and by genome sequencing and analyses [23]. Thus, Bradford coccus was renamed as Mimivirus [7].

Metagenomic, Serological and Epidemiological Findings Related to the Exposure of Humans to Mimiviruses, Marseilleviruses and Virophages

Several metagenomic studies have revealed the presence in environmental samples of sequences that are similar to genomes of amoebal viruses. Reads matching the DNA of mimiviruses and their virophages have been

detected from various environmental sources, including water from cooling towers, rivers, lakes and seas [24–32] (fig. 2). In addition, mimiviruses, Mimivirus virophages and marseilleviruses have been isolated from water or soil samples throughout the world [6, 33–41] (fig. 2). Also, Mimivirus-like particles were observed by light microscopy inside acanthamoebae in treated sewage sludge from a wastewater treatment plant in the West Midlands, UK [42]. This finding raised the question of whether dissemination of mimiviruses to agricultural land and surface waters may occur. Taken together, these data indicate that amoebae-infecting viruses are likely to be common inhabitants of our biosphere. Re-

Fig. 3. Schematic diagram of hypotheses and evidence regarding the transmission and detection of giant viruses of amoebae and their associated virophages in humans. * Contact lens storage case liquid. FISH = Fluorescence in situ hybridization.

cently, metagenomic reads matching the genomes of mimiviruses have been identified in various human samples, including faeces, respiratory samples and blood [32, 43–48] (fig. 2, 3). Moreover, it is worth underlining that the prevalence of giant viruses of phagocytic protists has likely been underestimated in metagenomic studies, because the majority of samples analysed over the last decade were passed through filters with 0.22- to 0.45- μ m pores before viral metagenomic investigations, which could considerably hamper the detection of the largest viruses [49, 50].

Three studies have used serology to assess the prevalence of infections with mimiviruses in patients living in France and Canada during the 2005–2010 period [8, 17, 51–53] (fig. 2, 3). Since then, two additional studies have been conducted in patients with chronic obstructive pulmonary disease in the Netherlands [54] and in intensive care unit (ICU) pneumonia patients in southeastern France [55]. In all these studies, Mimivirus serology was

tested in our laboratory by a microimmunofluorescence test. The prevalence of IgG ranged from 0% in 8 patients exhibiting aspiration pneumonia [55] to 25% in 8 patients at an acute phase of non-ventilator-associated pneumonia acquired at a hospital ICU [55]. In most cases, the IgG prevalence measured in pneumonia patients was approximately 10–20%. This prevalence was 2.5% among patients with chronic obstructive pulmonary disease in the Netherlands, and 6.7% during an exacerbation episode [54]. In addition, Bousbia et al. [55] showed additional data that support the role of mimiviruses in hospital-acquired pneumonia. Thus, a significant rise of prevalence of both IgG and IgM to Mimivirus was observed in the convalescent-phase serum samples compared to the admission serum samples. In this study, seroconversion to Mimivirus was described in 14 out of 71 patients (20%) with paired serum samples, and occurred 14 days after admission in 79% of these cases. As a comparison, 0% of intubated control patients without pneumonia in ICUs

and 2.3% of healthy control subjects in Nova Scotia [8] had IgG to Mimivirus.

Acanthamoeba spp. may play a significant role in the transmission of mimiviruses (fig. 3). These free-living amoebae are ubiquitous organisms that are predominant organisms in soil and water and can resist various unfavourable conditions as cysts [1, 16, 56]. As trophozoites, *Acanthamoeba* spp. are phagocytic protists that can host algae, fungi, yeasts, bacteria and viruses [1, 16, 56]. However, some bacteria, as well as mimiviruses and marseilleviruses, can resist and multiply into *acanthamoebae*, which, in such cases, have a role of replicative niche, reservoir, armour and vector [1, 16, 20]. Amoeba-resisting microorganisms live sympatrically in their host, with considerable opportunities to exchange genes, and such lifestyle has been shown to generate large genomes with a mosaic gene repertoire [57, 58]. Furthermore, three points are worth emphasizing with respect to the clinical relevance of giant viruses associated with amoebae. First, several amoebae-resisting bacteria, including *Legionella* spp. and *Mycobacterium* spp., are human pathogens, which supports the paradigm that describes *Acanthamoeba* spp. as Trojan horses [1, 16]. Second, it has been proposed that amoebae may be training fields for resistance to macrophages based on the fact that some of these bacteria also resist degradation by macrophages [1, 59]. Third, it has been previously pointed out that persons with a high risk of infection by Mimivirus likely were exposed to water contaminated by *Acanthamoeba* spp. [8, 52, 55]. This includes patients with ventilator-associated pneumonia that is associated with exposure to hospital water supplies [8, 17, 60]. More generally, risk factors for infection with mimiviruses may be similar to those identified for other microorganisms, such as *L. pneumophila*, which are resistant to amoebae and are human pathogens [8, 17] (fig. 2).

Evidence of Human Infection or Colonization with a Giant Virus

Mimivirus DNA has been detected only twice from the bronchoalveolar fluid of pneumonia patients [8, 61], although PCR testing has been performed in five studies that investigated the role of Mimivirus in respiratory infections [8, 62–65]. Nevertheless, it is worth noticing that only three PCR systems were used in these studies and they only targeted the Mimivirus genome obtained in 2004 [23]. The family Mimiviridae has expanded since 2004 and genetic diversity has been shown to be substan-

tial between mimiviruses [66, 67], and, therefore, these PCR assays should have had limited or no efficiency to detect other Mimivirus DNA than that of the first Mimivirus strain [68].

The first proven case of infection with a giant virus of amoebae involved one of our laboratory technicians who handled large amounts of Mimivirus to perform Western blot assays [69] (table 1; fig. 2, 3). This technician habitually made an annual check of his serologies to the microbes he manipulated. Mimivirus is known for its extreme resistance to degradation by physical and chemical agents [70], and amoebae can also protect them from degradation [71, 72]. The technician came for medical consultation with one of us (D.R.) for an unexplained respiratory infection, which was verified by a chest X-ray that showed bilateral basilar infiltrates suggesting viral pneumonia, and did not improve with 1-week administration of amoxicillin-clavulanic acid [69]. This technician concurrently seroconverted against Mimivirus. Using two-dimensional gel electrophoresis and Western blotting analyses on his 2 sera collected before and during pneumonia, we showed that the laboratory worker had seroconverted for total antibodies against 23 Mimivirus proteins, including 4 unique to Mimivirus. These findings established a true experimental model of human infection by Mimivirus, with specific serological reactivity and a disease that also appeared to be specific, as it was not cured by antibiotics, and no other potential agents of pneumonia were found.

A second association of a giant virus of amoebae with human was that of Lentillevirus, a new Mimivirus strain that was recovered from the contact lens storage case liquid of a 17-year-old myopic woman presenting with keratitis and for whom no bacteria or *Acanthamoeba* were detected in a corneal scraping [73]. *Pseudomonas fluorescens*, *Stenotrophomonas maltophilia*, *Mycobacterium chelonae* and *Acanthamoeba polyphaga* were detected from the liquid. Unexpectedly, we also isolated from this liquid Lentillevirus infecting the amoeba together with a new virophage of giant virus, which was named Sputnik 2, and previously undescribed bacterial symbionts of the amoeba [35, 73, 74].

The third evidence of human exposure to Mimivirus in association with clinical symptoms was provided by a young couple living in France who had travelled to Laos, their country of origin [31]. In Laos, the couple ate whole raw fish (a common practice in Laos) from the Mekong River. The 29-year-old woman and the 32-year-old man were admitted to Marseille University hospital on day 7 after the onset of unexplained symptoms that included

Table 1. Proven cases of infection with a giant virus

Refer- ence	Year	Virus	Viral classification	Case description	Clinical symptoms	Country where clinical samples were collected	Travel abroad	Positive diagnosis tests	Human sample testing positive
69	2006	Mimivirus	Family <i>Mimiviridae</i> , lineage A of Mimivirus of amoebae	Laboratory technician who handled large amounts of Mimivirus to perform Western blot assays	Unexplained respiratory infection with bilateral basilar infiltrates suggesting viral pneumonia; no improvement with 1-week administration of amoxicillin-clavulanic acid	France	-	Seroconversion to Mimivirus (against 23 Mimivirus proteins, including four unique to Mimivirus)	Serum
73	2011	Lentillevirus (Mimivirus of amoebae of lineage C); transpoviron	Family <i>Mimiviridae</i> , lineage C of Mimivirus of amoebae	17-year-old myopic woman with soft contact lenses	Keratitis, pain and redness of the left eye for 2 weeks; no intraocular reaction	France	-	Culture isolation on <i>Acanthamoeba</i> spp.	Contact lens storage case liquid
31	2012	Sputnik virophage, Mamavirus	Virophage; Family <i>Mimiviridae</i> , lineage A of Mimivirus of amoebae	29-year-old woman and her 32-year-old husband	Asthenia, nausea, myalgia, low-grade fever	France	Laos	Serology to the virophage; seroconversion for the virophage in the woman	Serum
32, 75	2012– 2013	Senegalvirus	Family <i>Marseilleviridae</i>	20-year-old Senegalese man living in rural Senegal	None	Senegal	-	Metagenomics, culture isolation on <i>Acanthamoeba</i> spp.	Stools
47	2013	Giant Blood Marseillevirus	Family <i>Marseilleviridae</i>	A blood donor	None	France	-	Metagenomics, PCR, culture isolation on lymphocyte T cells, transmission electron microscopy, fluorescence in situ hybridization	Serum
61	2013	LBA111	Family <i>Mimiviridae</i> , lineage C of Mimivirus of amoebae	72-year-old Tunisian woman	Unexplained pneumonia with fever, cough, dyspnoea and haemoptysis, hyperleukocytosis, inflammatory syndrome	Tunisia	-	Culture isolation on <i>Acanthamoeba</i> spp., serology	Broncho- alveolar fluid
Un- published	Shan		Family <i>Mimiviridae</i> , lineage C of Mimivirus of amoebae	17-year-old girl	Community-acquired pneumonia with fever (40°) for 15 days, productive cough, liquid diarrhoea, hyperleukocytosis, inflammatory syndrome	Tunisia	-	Culture isolation, serology	Stools

asthenia, nausea, myalgia and low-grade fever. We performed multiple serologies, and at that time, we were systematically testing serum samples for the presence of antibodies to the Sputnik virophage of Mimivirus [33]. Thus, we were able to discover that both patients had positive serologies for the virophage. In addition, because serum samples had been collected from the woman 5 months earlier during her pregnancy, we could demonstrate that she exhibited seroconversion for the virophage. Western blotting, two-dimensional gel electrophoresis and matrix-assisted laser desorption ionization mass spectrometry confirmed the specificity of the serological reactivity and identified that it targeted two virophage proteins. Moreover, we detected in both patients weak serological reactivity against Mamavirus, another strain of Mimivirus [33], and against *A. castellanii*. Various samples collected from the 2 patients during their follow-up were negative. Full recovery was observed for these patients, who received antiparasitic therapy due to positive serologies for *Toxocara*, *Trichinella* and *Fasciola* trematodes.

A fourth case consisted of the serendipitous isolation of a Marseillevirus from the stools of a young Senegalese man living in N'Diop, a rural area [32, 75], who exhibited no clinical symptoms. Indeed, while investigating the faecal microbiota through a new metagenomic approach that targets 16S ribosomal DNA but concurrently performs a full enzymatic digestion of the sample DNA, we generated many metagenomic reads that were initially discarded. Then, we searched these discarded reads in the 'trash' and found sequences matching the Marseillevirus genome, which led to the isolation of a new member of the family *Marseilleviridae*. This virus was named Senegalvirus and was actually the first Marseillevirus recovered from a human (fig. 1b). Subsequently, we searched for sequences related to members of the *Megavirales* in several human metagenomes, and we retrieved significant hits among reads recovered from saliva, oropharynx, lung and stools [32], which adds to the results of previous studies identifying sequences related to members of other families of the order *Megavirales* in human blood, nasopharyngeal samples or stools [43, 45, 76–79].

We recently described a fifth case that consisted of human infection by a giant virus of amoebae that was identified during a metagenomic study conducted on virally enriched blood fractions collected from blood donors [47]. In that study, the human virome was investigated using filters with a pore size of 0.45 µm but not 0.22 µm. This enabled the recovery of many sequences, represent-

ing 2.5% of the metagenomic reads, which were similar to Marseillevirus DNA. These sequences could then be assembled into two contigs with sizes of 13.6 and 10.2 kbp by mapping the reads onto the Marseillevirus genome. Marseillevirus DNA was detected by PCR in the blood from 1 of the 10 blood donors, and high-throughput sequencing generated reads that were assembled into a 357-kbp DNA sequence that was closely related to the Marseillevirus genome as confirmed by phylogeny reconstruction. This new giant virus was named Giant Blood Marseillevirus. In addition, the presence of the virus was confirmed by transmission electron microscopy and fluorescence in situ hybridization, and Giant Blood Marseillevirus virus was cultured from the blood sample on T lymphocyte cells. Finally, in the same study, antibodies to 26 Giant Blood Marseillevirus proteins were identified in the serum samples of the Giant Blood Marseillevirus-infected blood donor, while immunoglobulin G antibodies to this giant virus were detected in the sera of 3 out of 20 additional blood donors. In 2 of these cases, Giant Blood Marseillevirus DNA was concurrently detected, suggesting that infection with close relatives of Marseillevirus is relatively common among blood donors. Taken together, these findings suggest that this Marseillevirus may not cause clinical symptoms as persons in whom infection has been identified were asymptomatic. Nevertheless, whether primary or chronic infections may be associated with clinical manifestations is currently unknown.

In another recent work analysing 196 respiratory specimens collected from Tunisian patients [61], we diagnosed a case of infection with a new Mimivirus classified in lineage C and closely related to *Megavirus chiliensis* [39], whose genome is the largest that has been sequenced to date amongst mimiviruses. This new giant virus was named LBA111 (fig. 1c), as it was isolated from bronchoalveolar fluid collected from a 72-year-old Tunisian woman. This patient was hospitalized due to unexplained pneumonia, with fever, cough, dyspnoea and haemoptysis that was associated with an evocative chest X-ray, hyperleukocytosis and inflammatory syndrome; she also had an unfavourable clinical evolution with antibiotic treatment. LBA111 was isolated by amoebal culture. The genome of this new giant viral strain was then sequenced and was found to be 1.23 Mbp in length, the largest viral genome yet recovered from a human. The genome sequence was original, which excluded the possibility of contamination. In addition, the serum sample of the patient harboured total immunoglobulins to 9 LBA111-specific proteins identified by two-dimensional Western

blotting. Finally, a new Mimivirus tentatively classified within lineage C and named Shan was isolated from the stools of a Tunisian patient who exhibited pneumonia with poor response to antibacterial drugs [80].

Symptomatic and Asymptomatic Infections by Giant Viruses of Amoebae

The reports compiled here show, on the one hand, that marseilleviruses were detected in 2 asymptomatic cases. Marseilleviruses may induce symptomatic acute infections followed by a chronic carrier phase, or persons infected with these viruses may never exhibit symptomatic infections. This is the subject of a prospective study in our laboratory. On the other hand, there are clearly symptomatic infections with mimiviruses. It is noteworthy that two experimental studies were conducted with Mimivirus. First, inoculation of Mimivirus to macrophages led to an increase of the number of viral DNA copies [81]. Mimivirus appeared pathogenic for the macrophages, and Mimivirus particles recovered from these cells replicated within amoebae, which suggested that Mimivirus infected the macrophages and led to a productive cycle of viral infection. Secondly, an experimental murine model of infection was developed, which showed that mice infected intracardiacally with Mimivirus developed pneumonia, Mimivirus being re-isolated from and/or its antigens being detected in the lungs [82]. Although, clinical investigations have been scarce so far, overall, we have with Mimivirus all the criteria for pathogenicity of Koch's postulates linking the presence of this virus to pneumo-

nia. Thus, this virus has never been isolated outside a pathological context in human respiratory samples, it was isolated from pneumonia patients without finding any other causative agent of this pathology, and, in an experimental infection model in mice, it was able to recapitulate pneumonia and could be re-isolated from the lungs.

Conclusion

Giant viruses infecting phagocytic protists represent a major challenge in human pathology, as virological procedures implemented to date have not used appropriate media to allow their culture, and molecular techniques have used filtration steps that likely prevented their detection. The proportion of respiratory or other infections related to these giant viruses in humans is currently unknown and should be the subject of future work. However, there is an increasing body of evidence that mimiviruses might cause pneumonia and that humans carry marseilleviruses, and re-analyses of metagenomic databases have provided evidence that these giant viruses can be common in human samples. In conclusion, giant viruses infecting amoebae are emerging pathogens in humans, and their precise short- and long-term roles remain to be determined.

Disclosure Statement

The authors report no potential conflicts of interest or financial disclosures.

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