

## The role of giant viruses of amoebas in humans.

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**Key words:** Giant virus, human, *Megavirales*, pathogenicity, amoeba, nucleocytoplasmic large DNA virus, TRUC, pneumonia

**ABSTRACT (119 words)**

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Since 2003, dozens of giant viruses that infect amoebas (GVA), including mimiviruses and marseilleviruses, have been discovered. These giants appear to be common in our biosphere. From the onset, their presence and possible pathogenic role in humans have been serendipitously observed or investigated using a broad range of technological approaches, including culture, electron microscopy, serology and various techniques based on molecular biology. The link between amoebal mimiviruses and pneumonia has been the most documented, with findings that fulfill several of the criteria considered as proof of viral disease causation. Regarding marseilleviruses, they have been mostly described in asymptomatic persons, and in a lymph node adenitis. The presence and impact of GVA in humans undoubtedly deserve further investigation in medicine.

36 **TEXT (2,625 words)**

37

38 **The emergence of giant viruses of amoebas**

39 The story of giant viruses that infect amoebas (GVA) began with the isolation of the  
40 Mimivirus in 1992 [1;2]. This was made possible by using a strategy that consisted of  
41 inoculating samples on an axenic culture of *Acanthamoeba* spp. and was implemented to  
42 isolate amoeba-resisting microorganisms such as *Legionella* spp. [2]. The first mimivirus  
43 isolate was obtained from cooling tower water while investigating a pneumonia outbreak in  
44 England. It took a decade to identify that one of the amoeba-resistant microbes was a giant  
45 virus, which was visible on light microscopy and looked like a Gram-positive coccus. This  
46 was eventually revealed in 2003 in Marseille by using electron microscopy [1;2]. Thus, the  
47 investigation triggered in 1992 by pneumonia cases serendipitously led to discovery of the  
48 largest viruses known so far, which strongly challenge the concept and definition of viruses  
49 [1;3;4]. Moreover, it suggested the link between these GVA and humans and their possible  
50 pathogenicity.

51 Dozens of additional mimiviruses, which were classified in the family *Mimiviridae*,  
52 were isolated in amoebas from environmental water samples collected in various geographical  
53 areas worldwide [5;6]. In addition, these studies led to the discovery of the first viruses of  
54 viruses, named  $\varphi$ -virophages, which replicate in the viral factories of mimiviral hosts and can  
55 impair their replicative cycle and morphogenesis [7;8]. Moreover, other GVA have been  
56 discovered since 2008 [4;9]. Some were classified in the family *Marseilleviridae* and others  
57 include pandoraviruses [10;11], *Pithovirus sibericum* [12], faustoviruses [13] and *Mollivirus*  
58 *sibericum* [14], which represent new putative virus families [9]. All these GVA cultured in  
59 amoebas display many unique characteristics that put them on the edge of the virus definition,  
60 and warrant proposing their reclassification as representatives of a fourth  $\varphi$ -TRUC (an

61 acronym for Things Resisting Uncompleted Classifications) of microbes [15] (reviewed by V.  
62 Sharma et al. [4]). They have been proposed for classification in a new viral order,  
63 *Megavirales*, alongside other double-stranded DNA viruses [16].

64 GVA appear to be common in our biosphere; they have been isolated from marine  
65 water, freshwater and soil samples collected in several countries worldwide  
66 (<https://www.google.com/maps/d/edit?mid=zA3X4ljz-uM.kFSrbnCtoBLc>) [5;17;18]. This  
67 has been corroborated by metagenomic studies that detected sequences matching these viruses  
68 in similar environmental samples collected in highly diverse geographical areas [19;20]  
69 (reviewed by S. Halary et al. [21]). In addition, their hosts, *Acanthamoeba* spp. (for most of  
70 these viruses) or *Vermamoeba vermiformis* (for faustoviruses) are ubiquitous organisms that  
71 are common in human environments, very resistant and described as "Trojan horses" for their  
72 parasitic pathogens [22;23]. Moreover, GVA prevalence was probably underestimated  
73 because 'viral' fractions analyzed were most often obtained by filtration through a 0.2 µm-  
74 large pore size, which neglects gigantic virions [20]. Taken together, these findings strongly  
75 suggest that humans are exposed to GVA. Noteworthy, 12% of 242 samples collected from  
76 inanimate surfaces in a Brazilian hospital were positive for Mimivirus DNA by PCR, the  
77 incidence being significantly greater in respiratory isolation facilities, and amoebal lysis was  
78 obtained from 83% of these samples [24].

79 Other studies have reported the isolation of mimiviruses from oysters [25] and a leech  
80 [26], and their detection by PCR in monkeys and cattle [27]. In addition, a Marseillevirus was  
81 isolated from a diptera [26] and a faustovirus was cultured from culicoides [28]. Moreover,  
82 mimivirus-like sequences were identified in metagenomes generated from bats, rodents,  
83 dromedaries and culicoides, and faustovirus- and pandoravirus-like sequences were detected  
84 in metagenomes generated from culicoides [20;21;28] (reviewed S. Halary et al. [21]).

85

## 86 **Evidence for a causative role of giant viruses of amoebas in pathogenicity**

### 87 **Causality criteria**

88 An increasing body of data supports the presence of GVA in humans, and in addition,  
89 the question of the putative pathogenic role of these viruses has been addressed and  
90 documented, mainly for mimiviruses, and more recently for marseilleviruses. Establishing a  
91 causative role of viruses in diseases has been a long journey. Criteria developed since 1840 by  
92 Henle, Loeffler and Koch to prove the etiologic association between an infectious agent and a  
93 specific disease have been deemed less and less appropriate over time [29]. Other criteria for  
94 causative relations were proposed [30], including some specifically applied to viruses in 1937,  
95 1957 and 1976 (Box 1) [31-33]. However, newly discovered viruses challenge existing  
96 postulates, as, for instance, with viruses determining chronic or latent infections. Thus, with  
97 the advent of new technologies and improved knowledge in microbiology and virology,  
98 criteria considered for suspecting or establishing a causality link have drifted considerably.  
99 Notably, sequence-based criteria were introduced in 1996, and metagenomic Koch's  
100 postulates were finally proposed in 2012 [34;35]. Since 2003, the presence and possible  
101 pathogenic role of GVA has been serendipitously observed or investigated using a broad  
102 range of technological approaches including culture, electron microscopy, serology and  
103 various techniques based on molecular biology, including metagenomics (Table 1). The  
104 findings fulfill several of the criteria considered as proofs of viral disease causation.

### 105 **Host cells other than phagocytic amoebas for giant viruses of amoebas**

106 All GVA have been isolated on cultures of *A. castellani*, *A. polyphaga*, or *V.*  
107 *vermiformis* [13;36]. Numerous cell lines have been tested for their permissivity to  
108 mimiviruses or marseilleviruses. In experimental inoculation tests, Mimivirus was capable of  
109 entering professional phagocytes, among which various human myeloid cells including  
110 circulating monocytes, monocyte-derived macrophages and myelomonocytic cells, and also

111 mouse myeloid cells [37]. Further experiments conducted with mouse macrophages showed a  
112 significant increase in Mimiviral DNA load during a 30-hour period of incubation; in  
113 addition, only approximately one quarter of the macrophages were viable after 30 hours, and  
114 macrophage extracts led to Mimivirus replication within amoebae and to amoebal lysis. These  
115 findings indicated productive infection of macrophage by Mimivirus post-internalization. In  
116 addition, Mimivirus was demonstrated to replicate in total human peripheral blood  
117 mononuclear cells (PBMC), as measured by the tissue culture infective dose method [38].  
118 Furthermore, Mimivirus was revealed to induce type I IFN production in infected human  
119 PBMC and to inhibit interferon stimulated genes expression in these cells. These findings  
120 question if amoebae are the exclusive hosts for the giant Mimivirus. Moreover, inoculation of  
121 Jurkat cells, which are immortalized human T lymphocyte cells, with a serum sample positive  
122 for Giant blood Marseillevirus (GBM) DNA led to detection of this virus by PCR in the  
123 culture supernatant, and viral DNA and virions were detected within Jurkat cells 21 days post-  
124 infection by PCR, fluorescence *in situ* hybridization, or transmission electron microscopy [39].  
125 Although GBM was not propagated, these results indicated productive infection of these cells.  
126 It should be considered that the host barrier may be far more limited for GVA than for other  
127 viruses, because GVA infect their hosts by phagocytosis [37]. This was exemplified by the  
128 capability of Mimivirus to enter human macrophages through phagocytosis, and this closely  
129 resembled Mimivirus entry in amoebas [37]. In addition, mimiviruses, marseilleviruses or  
130 faustoviruses have been isolated from different phagocytic protists, including amoebozoa and  
131 chromalveolata, and also mammals, including humans, and also insects [26;48;49].

## 132 **Mimivirus**

### 133 ***Serological-only evidence***

134 Concomitantly with the initial attempts to identify the giant Mimivirus, serological  
135 testing of sera from patients with unexplained pneumonia showed that the strongest

136 reactivities were against this amoeba-resisting microbe [40]. Subsequently, the prevalence of  
137 antibodies to Mimivirus was assessed using microimmunofluorescence in several studies, in  
138 most cases in pneumonia patients hospitalized in intensive care units (ICU) (Table 1). IgG  
139 prevalence was most often  $\approx$ 10-20% in pneumonia patients, ranging from 0% to 25% [41-44].  
140 In contrast, it was 0% and 2.3% in intubated control patients without pneumonia and healthy  
141 controls, respectively [41]. Moreover, IgG and IgM elevations or seroconversions were  
142 observed in patients with hospital-acquired pneumonia [44]. The first strong evidence of  
143 infection with a GVA was in a laboratory technician who handled large amounts of Mimivirus  
144 and developed unexplained pneumonia [45]. He exhibited seroconversion to 23 Mimivirus  
145 proteins, as assessed by 2-dimensional gel electrophoresis (2DGE) and Western blotting,  
146 among which 4 proteins were unique to this virus. Interestingly, this story is very similar to  
147 the one that linked Epstein-Barr virus (EBV) to infectious mononucleosis. In 1968, a  
148 laboratory technician who worked with EBV developed infectious mononucleosis and  
149 concurrently exhibited seroconversion to this virus [46]. Positive serology to the Sputnik  
150 virophage was also observed in two patients of Laotian origin who exhibited fever while  
151 returning from Laos [47]. Serological reactivities were obtained by Western blot, 2DGE and  
152 mass spectrometry and targeted two virophage proteins. In addition, one seroconversion could  
153 be shown. The serological detection of Sputnik in humans suggests the exposure of humans to  
154 this virophage, and the concurrent exposure to mimiviruses, which are the Sputnik hosts [7;8].  
155 Thus, in this study, serological reactivities were also observed to Mamavirus and  
156 *Acanthamoeba*. No virus was isolated. In addition, a significant association was reported  
157 between antibodies to Mimivirus L71 protein, which harbors collagen-like motifs, and  
158 rheumatoid arthritis in patients [48].

### 159 **PCR**

160 Detection by PCR of GVA in humans has only been conducted to date in clinical



161 specimens evaluated for mimiviruses and marseilleviruses [49]. Mimivirus DNA was  
162 screened for in respiratory samples and was first found in 1 of 32 patients with ICU-acquired  
163 pneumonia (Table 1) [41]. Then, mimiviruses were detected by conventional PCR in a  
164 Tunisian patient presenting unexplained pneumonia, concurrently with mimivirus isolation  
165 [50]. Other studies have reported negative PCR testing in human respiratory samples [43;51],  
166 which may mean that mimiviruses are uncommon in this setting, or present at a low titer, but  
167 the main reason may be the substantial genetic diversity within the family *Mimiviridae*, which  
168 prevents implementation of universal PCR assays [49].

### 169 ***Culture isolation***

170 Two mimiviruses have been isolated to date from clinical samples, in Tunisian  
171 patients with unexplained pneumonia (Table 1). In the first case, LBA111 virus was cultured  
172 from the bronchoalveolar fluid of a 72-year-old woman [50]. The patient was admitted to the  
173 hospital for a 3-day fever with cough, dyspnea and hemoptysis; chest X-ray revealed right  
174 lower lobe consolidation and the white blood cell count was elevated. Concurrently,  
175 antibodies to 9 LBA111 virus proteins were detected by 2D Western blotting. The second  
176 case was a 17-year-old girl admitted for fever (40°C) and cough for 15 days, with lower left  
177 lung opacity, diarrhea, and leukocytosis [52]. In this case, Shan virus was isolated from the  
178 stool; no respiratory sample was available. In addition, another mimivirus, named  
179 Lentillevirus, was isolated from the contact lens storage liquid of a keratitis patient [53].  
180 Interestingly, its *Acanthamoeba* host was isolated and revealed to be infected with two  
181 amoeba-resisting bacteria and a virophage, Sputnik2.

### 182 ***Experimental evidence***

183 Histopathological features of pneumonia, including thickened alveolar walls,  
184 inflammatory infiltrates and diffuse alveolar damage were observed in an experimental mouse  
185 model following intracardiac Mimivirus inoculation (Table 1) [54]. No other experiment

186 model of inoculation to animal has been conducted to date for another GVA. Such approach is  
187 of strong interest but questions on the most appropriate inoculum and inoculation route.

### 188 **Marseilleviruses**

189 The first hint of the presence of a marseillevirus in humans was serendipitously  
190 obtained during a metagenomic study that targeted bacterial sequences generated from the  
191 stools of a healthy Senegalese young man, and consisted of sequences best matching  
192 Marseillevirus among trashed metagenomic reads (Table 1) [20;55]. Subsequently, a close  
193 relative to Marseillevirus was isolated from this sample in *Acanthamoeba* and named  
194 Senegalvirus. Another metagenomic study identified reads matching the Marseillevirus  
195 genome in the blood of healthy blood donors [39]. This was confirmed by positive serology to  
196 Marseillevirus using immunofluorescence and Western blotting, and positive fluorescence *in*  
197 *situ* hybridization (FISH) and PCR on the blood and infected human lymphocytes. The  
198 presence of Marseillevirus was further detected by serology and PCR in other blood donors in  
199 France (IgG prevalence, 13-15%; DNA prevalence, 4-10%) [39;56], in Switzerland (IgG, 1.7-  
200 2.5%) [57], and in polytransfused thalassemic patients in France (IgG, 23%; DNA, 9%) [56].  
201 The detection of Marseillevirus DNA in blood donors and recipients has been a controversial  
202 issue, as it has not been observed in other studies [58-61]. However, the body of data  
203 supporting the presence of Marseillevirus in humans has continued to grow. In 2013, an 11-  
204 month-old child was found to exhibit a very high level of IgG to Marseillevirus [62]. He  
205 presented an unexplained adenitis, and Marseillevirus DNA was detected in his blood, while  
206 the virus was visualized in the lymph node by immunohistochemistry and FISH.

### 207 **Other giant viruses of amoebas**

208 GVA other than mimiviruses and marseilleviruses, including pandoraviruses,  
209 faustoviruses and *P. sibiricum* and *M. sibiricum*, have been discovered during the past three  
210 years, which has prevented extensive investigation of their presence in humans until now

211 [13;17]. However, *Pandoravirus inopinatum* was isolated from the contact lens storage liquid  
212 of a keratitis patient [11] and sequences related to faustoviruses have been detected in  
213 metagenomes generated from human serum [13].

#### 214 **Metagenomic data**

215 Metagenomics has emerged during the same period as GVA, representing a new  
216 technological approach and powerful tool, although it may lack sensitivity and may allow  
217 only detecting sequences best matching with GVA [20]. Nevertheless, causing diseases  
218 Detection in human metagenomes of sequences related to GVA tends to be correlated with the  
219 number of available genomes and time to their release. Mimivirus-like sequences have been  
220 detected in metagenomes generated from human coprolites, stools of diarrheal patients and  
221 healthy people, nasopharyngeal aspirates from patients with respiratory tract infections,  
222 buccal mucosa, saliva and retroauricular crease from healthy people, vagina from healthy  
223 women, and blood samples from healthy people or patients with liver diseases of various  
224 etiologies (Table 1) [20;21;63]. Notably, it has been recently reported that *Mimiviridae*  
225 representatives dominated, together with *Poxviridae* representatives, the human gut  
226 eukaryotic virome in metagenomic samples of the Human Microbiome Project [63].  
227 Virophage-like sequences have also been found in the human gut [64]. In addition,  
228 Marseillevirus-like sequences have been detected in the buccal mucosa, retroauricular crease,  
229 vagina and stools from healthy people (Table 1) [20;63]. Recently, metagenome sequences  
230 best matching with pandoraviruses, *Pithovirus sibericum*, faustoviruses or virophages have  
231 also been detected in human plasma samples from patients with liver diseases [65].

232

#### 233 **Conclusion**

234 The presence and impact of GVA and virophages in humans undoubtedly represent an  
235 important field that deserves further investigation in medicine. Such investigations are

236 difficult. However, it has been increasingly demonstrated that GVA can be present in humans.  
237 Evidence is particularly strong for mimiviruses and marseilleviruses, which were isolated  
238 from human feces, bronchoalveolar fluid and blood. Regarding the potential pathogenic role  
239 of these viruses in humans, the link between amoebal mimiviruses and pneumonia has been  
240 the most documented, whereas marseilleviruses have mostly been described in asymptomatic  
241 persons, and in an adenitis patient. Furthermore, for all these GVA, one must consider that  
242 their tremendous gene repertoires confer on them a strong potential for interaction with other  
243 organisms. It is also noteworthy that the closest relatives to faustoviruses are asfarviruses,  
244 which cause a common and severe disease in pigs [13]. Regarding other megaviruses, they  
245 include poxviruses, which are pathogenic in insects and mammals, including humans [66],  
246 and *Acanthocystis turfacea* chlorella virus, a phycodnavirus that was found in human  
247 pharyngeal samples and tentatively associated with cognitive disorders [67]. Until recently,  
248 the belief that all viruses are small entities probably limited the detection of GVA in humans.  
249 As this paradigm has been crumbling for a decade, future research should clarify the  
250 prevalence and consequence of their presence in humans. It appears particularly relevant to  
251 continue searching for mimiviruses in respiratory samples and stools, and for marseilleviruses  
252 in blood and in lymph nodes. Nevertheless, a broader panel of human samples from healthy  
253 and sick people should be tested; for instance, urine samples might be studied. In addition,  
254 investigations should involve a broad range of technological approaches, including serology,  
255 immunohistochemistry, immunofluorescence, FISH, targeted and random nucleic acid  
256 amplification, Sanger and next-generation sequencing, cytometry, microscopy, and high  
257 throughput culture isolation. Particularly, metagenomes currently extensively generated from  
258 human samples should be more exhaustively, thoroughly and recurrently screened for the  
259 presence of sequences best matching these GVA. Finally, experimental models on cells or  
260 animals would be helpful to gain a better understanding of the consequences of GVA

261 presence in humans.

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## REFERENCES AND RECOMMENDED READING

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## FIGURE LEGENDS

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**Figure 1.** Schematic of the chronology of major findings that support the presence and possible pathogenic role in humans of giant viruses of amoebas

A majority of the findings are for mimiviruses and marseilleviruses, which were the oldest giant viruses described, in 2003 and 2009, respectively. Other giant viruses of amoebas have been described over the three last years.

**Figure 2.** Schematic of findings that support the presence and possible pathogenic role in humans of giant viruses of amoebas.

Supportive arguments involve a broad range of technological approaches including serology, immunohistochemistry, immunofluorescence, culture isolation, electron microscopy, fluorescence *in situ* hybridization (FISH), targeted and random nucleic acid amplification, qPCR, or Sanger and next-generation sequencing. Green and red circles indicate human body sites for which GVA evidence were obtained in healthy people and in diseased people, respectively.

497 **Table 1.** Summary of evidence of associations of mimiviruses or marseilleviruses with humans and of a possible pathogenic role

498

Technical approaches	Evidence for mimiviruses	Evidence for marseilleviruses	Elements to consider for causality
<b>Serology</b>	<p>Presence of specific IgG and IgM antibodies to Mimivirus in pneumonia patients</p> <p>Greater seroprevalence in pneumonia patients than controls</p> <p>Mimivirus seroconversion in pneumonia patients, including one individual who manipulated the virus (reactivity to 23 Mimivirus proteins)</p> <p>Serological reactivities to the Sputnik virophage in two patients (reactivity to 2 virophages proteins); seroconversion in one case</p>	<p>IgG detection in blood donors, young health adults, multitransfused thalassemia patients, and a lymphadenitis patient</p>	<p>Recurrent evidence of serological reactivities, including in association with Mimivirus isolation in one case; seroconversion to the Mimivirus and the Sputnik virophage in patients; association with Mimivirus handling in a patient with unexplained pneumonia, and with hospital-acquired pneumonia</p> <p>Detection of antibodies to Marseillevirus in association with Marseillevirus antigen/DNA detection in a single case-patient</p>
<b>Immunodetection</b>		<p>Detection of Marseillevirus antigens by immunofluorescence and immunochemistry in a lymph node adenitis</p>	<p>Association of Marseillevirus with lymphadenitis</p>
<b>Molecular detection</b>	<p><b>Conventional PCR:</b> Mimivirus DNA found in a bronchialveolar fluid and a serum sample from two pneumonia patients</p> <p><b>Metagenomics:</b> Detection in metagenomes generated from human coprolites, stools of diarrheal patients and healthy people, nasopharyngeal aspirates from patients with respiratory tract infections, buccal mucosa, saliva and retroauricular crease from healthy people, vagina from healthy women, and blood samples from healthy people or patients with liver diseases of various etiologies; detection of virophage-like sequences in the human gut</p>	<p><b>Conventional PCR:</b> Marseillevirus DNA detection in the serum from blood donors, multitransfused thalassemia patients, and a lymphadenitis patient</p> <p>Detection of Marseillevirus DNA by fluorescence <i>in situ</i> hybridization in a lymph node adenitis</p> <p><b>Metagenomics:</b> Marseillevirus-like sequences detection in the buccal mucosa, retroauricular crease, vagina and stools from healthy people</p>	<p>Association of Mimivirus with unexplained pneumonia and of Marseillevirus with lymphadenitis</p>
<b>Culture isolation</b>	<p>Isolation from a bronchialveolar fluid and a faeces sample from two pneumonia patients</p>		<p>Association of Mimivirus with unexplained pneumonia</p>
<b>Experimental models</b>	<p><b>Cells:</b> Entry in various human myeloid cells including circulating monocytes, monocyte-derived macrophages and myelomonocytic cells; entry in of mouse myeloid cells; productive infection of macrophage by Mimivirus post-internalization; replication in total human peripheral blood mononuclear cells; interaction with type I IFN production in these cells</p> <p><b>Animal:</b> Pneumonia induction in mice inoculated intracardially</p>	<p><b>Cells:</b> Inoculation of immortalized human T lymphocyte cells with a serum sample positive for Giant blood Marseillevirus (GBM) DNA led to virus DNA detection in the culture supernatant, and viral DNA and virions detection within these cells 21 days post-infection by PCR, fluorescence <i>in situ</i> hybridization, or transmission electron microscopy</p>	<p>Mimivirus causes pneumonia in mice</p>

499 References for quoted studies are included in the text

500 **Box 1. Evolving criteria for proof of disease causation that can be applied to viruses**

501

502 **Henle, Loeffler and Koch's postulate (1884-1890) [29]**

- 503 1. The microorganism must be found in abundance in all organisms suffering from the  
504 disease, but should not be found in healthy animals.
- 505 2. The microorganism must be isolated from a diseased organism and grown in pure culture.
- 506 3. The cultured microorganism should cause disease when introduced into a healthy organism.
- 507 4. The microorganism must be re-isolated from the inoculated, diseased experimental host and  
508 identified as being identical to the original specific causative agent.

509

510 **Rivers's criteria for proof of viral disease causation (1937) [31]**

- 511 1. A specific virus must be found associated with a disease with a degree of regularity.
- 512 2. The virus must be shown to occur in the sick individual not as an incidental or accidental  
513 finding but as the cause of the disease under investigation.
- 514 3. Information concerning the presence of antibodies against the agent and the time of their  
515 appearance in the serum of patients is equally important as evidence of etiological  
516 significance of the virus.

517

518 **Huebner's prescription for the virologist's dilemma: conditions necessary for  
519 establishing a virus as cause of a specific human disease (1957) [32]**

- 520 1. Virus must be a "real" entity: A new virus must be well established by passage in the  
521 laboratory in animal or tissue cultures.
- 522 2. Origin of virus: the virus must be repeatedly isolated from human specimens and shown not  
523 to be a viral contaminant of the experimental animals, cells, or media employed to grow it.
- 524 3. Antibody response: An increase in neutralizing or other serologically demonstrable

525 antibodies should regularly result from active infection.

526 4. Characterization and comparison with known agents: A new virus should be fully  
527 characterized and compared with other agents including host and host-cell ranges, pathologic  
528 lesions, types of cytopathogenic effects, size, susceptibility to physical agents, etc.

529 5. Constant association with specific illness: The virus must be constantly associated with any  
530 well-defined clinical entity and isolated from diseased tissue, if available.

531 6. Studies with human volunteers: Human beings inoculated with a newly recognized agent in  
532 "double blind" studies should reproduce the clinical syndrome.

533 7. Epidemiologic studies: Both "cross-sectional" and "longitudinal" studies of community or  
534 institutional groups to identify patterns of infection and disease.

535 8. Prevention by a specific vaccine: One of the best ways to establish an agent as the cause.

536 9. Financial support: A consideration so absolutely necessary that it deserves to be called a  
537 postulate.

538

539 **Evans's criteria for proof of disease causation: a unified concept appropriate for viruses**  
540 **as causative agents of disease based on the Henle-Koch postulates (1976) [33]**

541 1. Prevalence of the disease is significantly higher in subjects exposed to the putative virus  
542 than in those not so exposed.

543 2. Incidence of the disease is significantly higher in subjects exposed to the putative virus than  
544 in those not so exposed (prospective studies).

545 3. Evidence of exposure to the putative virus is present more commonly in subjects with the  
546 disease than in those without the disease.

547 4. Temporally, the onset of disease follows exposure to the putative virus, always following  
548 an incubation period.

549 5. A regular pattern of clinical signs follows exposure to the putative virus, presenting a

550 graded response, often from mild to severe.

551 6. A measurable host immune response, such as an antibody response and/or a cell-mediated  
552 response, follows exposure to the putative virus. In those individuals lacking prior experience,  
553 the response appears regularly, and in those individuals with prior experience, the response is  
554 anamnestic.

555 7. Experimental reproduction of the disease follows deliberate exposure of animals to the  
556 putative virus, but nonexposed control animals remain disease free. Deliberate exposure may  
557 be in the laboratory or in the field, as with sentinel animals.

558 8. Elimination of the putative virus and/or its vector decreases the incidence of the disease.

559 9. Prevention or modification of infection, via immunization or drugs, decreases the incidence  
560 of the disease.

561 10. The whole thing should make biologic and epidemiologic sense.

562

563 **Fredricks and Relman's molecular guidelines for establishing microbial disease**

564 **causation (1996) [34]**

565 1. A nucleic acid sequence belonging to a putative pathogen should be present in most cases  
566 of an infectious disease. Microbial nucleic acids should be found preferentially in those  
567 organs or gross anatomic sites known to be diseased (i.e., with anatomic, histologic, chemical,  
568 or clinical evidence of pathology) and not in those organs that lack pathology.

569 2. Fewer, or no, copy numbers of pathogen associated nucleic acid sequences should occur in  
570 hosts or tissues without disease.

571 3. With resolution of disease (for example, with clinically effective treatment), the copy  
572 number of pathogen-associated nucleic acid sequences should decrease or become  
573 undetectable. With clinical relapse, the opposite should occur.

574 4. When sequence detection predates disease, or sequence copy number correlates with



575 severity of disease or pathology, the sequence-disease association is more likely to be a causal  
576 relationship.

577 5. The nature of the microorganism inferred from the available sequence should be consistent  
578 with the known biological characteristics of that group of organisms. When phenotypes (e.g.,  
579 pathology, microbial morphology, and clinical features) are predicted by sequence-based  
580 phylogenetic relationships, the meaningfulness of the sequence is enhanced.

581 6. Tissue-sequence correlates should be sought at the cellular level: efforts should be made to  
582 demonstrate specific in situ hybridization of microbial sequence to areas of tissue pathology  
583 and to visible microorganisms or to areas where microorganisms are presumed to be located.

584 7. These sequence-based forms of evidence for microbial causation should be reproducible.

585

#### 586 **Metagenomic Koch's postulates (2012) [35]**

587 Comparison between a diseased and healthy control animal shows a significant difference  
588 between the metagenomic libraries (depicted by the histograms of relative abundance reads).

589 In order to fulfill the metagenomic Koch's postulates:

590 1. The metagenomic traits in diseased subject must be significantly different from healthy  
591 subject.

592 2. Inoculation of samples from the disease animal into the healthy control must lead to the  
593 induction of the disease state. Comparison of the metagenomes before and after inoculation  
594 should suggest the acquisition or increase of new metagenomic traits. New traits can be  
595 purified by methods such as serial dilution or time-point sampling of specimens from a  
596 disease animal.

597 3. Inoculation of the suspected purified traits into a healthy animal will induce disease if the  
598 traits form the etiology of the disease.

599

**Fig. 1**

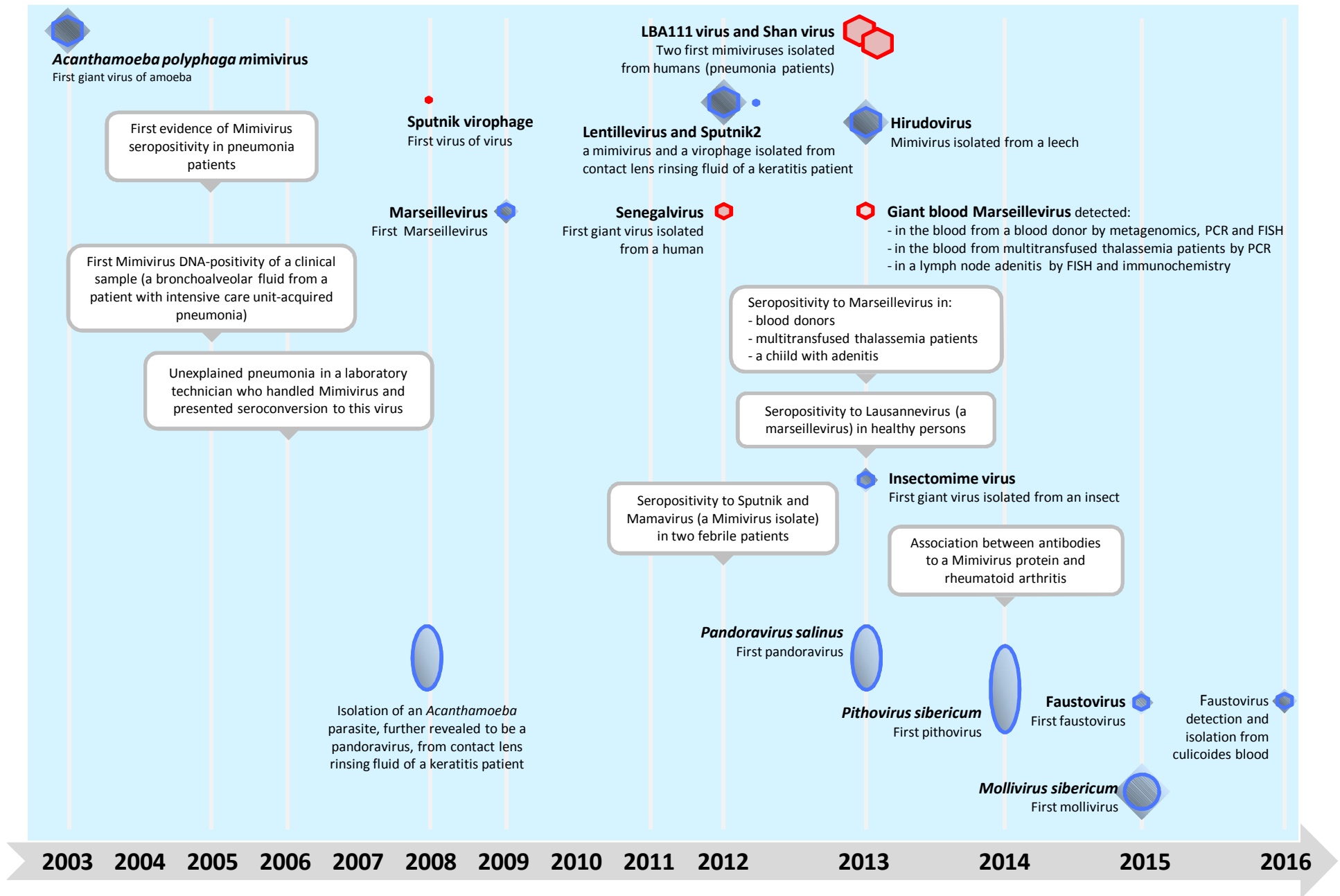


Fig. 2

