The role of giant viruses of amoebas in humans.

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ABSTRACT (119 words)

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.

TEXT (2,625 words)

The emergence of giant viruses of amoebas

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

Dozens of additional mimiviruses, which were classified in the family *Mimiviridae*, were isolated in amoebas from environmental water samples collected in various geographical areas worldwide [5;6]. In addition, these studies led to the discovery of the first viruses of viruses, named óvirophagesô, which replicate in the viral factories of mimiviral hosts and can impair their replicative cycle and morphogenesis [7;8]. Moreover, other GVA have been discovered since 2008 [4;9]. Some were classified in the family *Marseilleviridae* and others include pandoraviruses [10;11], *Pithovirus sibericum* [12], faustoviruses [13] and *Mollivirus sibericum* [14], which represent new putative virus families [9]. All these GVA cultured in amoebas display many unique characteristics that put them on the edge of the virus definition, and warrant proposing their reclassification as representatives of a fourth óTRUCô (an acronym for Things Resisting Uncompleted Classifications) of microbes [15] (reviewed by V. Sharma et al. [4]). They have been proposed for classification in a new viral order, *Megavirales*, alongside other double-stranded DNA viruses [16].

GVA appear to be common in our biosphere; they have been isolated from marine water, freshwater and soil samples collected in several countries worldwide (https://www.google.com/maps/d/edit?mid=zA3X4ljlz-uM.kFSrbnCtoBLc) [5;17;18]. This has been corroborated by metagenomic studies that detected sequences matching these viruses in similar environmental samples collected in highly diverse geographical areas [19;20] (reviewed by S. Halary et al. [21]). In addition, their hosts, *Acanthamoeba* spp. (for most of these viruses) or *Vermamoeba vermiformis* (for faustoviruses) are ubiquitous organisms that are common in human environments, very resistant and described as ôTrojan horsesô for their parasitic pathogens [22;23]. Moreover, GVA prevalence was probably underestimated because 'viral' fractions analyzed were most often obtained by filtration through a 0.2 µm-large pore size, which neglects gigantic virions [20]. Taken together, these findings strongly suggest that humans are exposed to GVA. Noteworthy, 12% of 242 samples collected from inanimate surfaces in a Brazilian hospital were positive for Mimivirus DNA by PCR, the incidence being significantly greater in respiratory isolation facilities, and amoebal lysis was obtained from 83% of these samples [24].

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

Evidence for a causative role of giant viruses of amoebas in pathogenicity

Causality criteria

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

Host cells other than phagocytic amoebas for giant viruses of amoebas

All GVA have been isolated on cultures of *A. castellani*, *A. polyphaga*, or *V. vermiformis* [13;36]. Numerous cell lines have been tested for their permissivity to mimiviruses or marseilleviruses. In experimental inoculation tests, Mimivirus was capable of entering professional phagocytes, among which various human myeloid cells including circulating monocytes, monocyte-derived macrophages and myelomonocytic cells, and also mouse myeloid cells [37]. Further experiments conducted with mouse macrophages showed a significant increase in Mimiviral DNA load during a 30-hour period of incubation; in addition, only approximately one quarter of the macrophages were viable after 30 hours, and macrophage extracts led to Mimivirus replication within amoebae and to amoebal lysis. These findings indicated productive infection of macrophage by Mimivirus post-internalization. In addition, Mimivirus was demonstrated to replicate in total human peripheral blood mononuclear cells (PBMC), as measured by the tissue culture infective dose method [38]. Furthermore, Mimivirus was revealed to induce type I IFN production in infected human PBMC and to inhibit interferon stimulated genes expression in these cells. These findings question if amoebae are the exclusive hosts for the giant Mimivirus. Moreover, inoculation of Jurkat cells, which are immortalized human T lymphocyte cells, with a serum sample positive for Giant blood Marseillevirus (GBM) DNA led to detection of this virus by PCR in the culture supernatant, and viral DNA and virions were detected within Jurkat cells 21 days post-infection by PCR, fluorescence *in situ* hybridization, or transmission electron microcopy [39]. Although GBM was not propagated, these results indicated productive infection of these cells. It should be considered that the host barrier may be far more limited for GVA than for other viruses, because GVA infect their hosts by phagocytosis [37]. This was exemplified by the capability of Mimivirus to enter human macrophages through phagocytosis, and this closely resembled Mimivirus entry in amoebas [37]. In addition, mimiviruses, marseilleviruses or faustoviruses have been isolated from different phagocytic protists, including amoebozoa and chromalveolata, and also mammals, including humans, and also insects [26;48;49].

Mimivirus

Serological-only evidence

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

PCR

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

Culture isolation

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

Experimental evidence

Histopathological features of pneumonia, including thickened alveolar walls, inflammatory infiltrates and diffuse alveolar damage were observed in an experimental mouse model following intracardiac Mimivirus inoculation (Table 1) [54]. No other experiment model of inoculation to animal has been conducted to date for another GVA. Such approach is of strong interest but questions on the most appropriate inoculum and inoculation route.

Marseilleviruses

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

Other giant viruses of amoebas

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

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

Metagenomic data

Metagenomics has emerged during the same period as GVA, representing a new technological approach and powerful tool, although it may lack sensitivity and may allow only detecting sequences best matching with GVA [20]. Nevertheless, causing diseases Detection in human metagenomes of sequences related to GVA tends to be correlated with the number of available genomes and time to their release. Mimivirus-like sequences have been detected 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 (Table 1) [20;21;63]. Notably, it has been recently reported that *Mimiviridae* representatives dominated, together with *Poxviridae* representatives, the human gut eukaryotic virome in metagenomic samples of the Human Microbiome Project [63]. Virophage-like sequences have also been found in the human gut [64]. In addition, Marseillevirus-like sequences have been detected in the buccal mucosa, retroauricular crease, vagina and stools from healthy people (Table 1) [20;63]. Recently, metagenome sequences best matching with pandoraviruses, *Pithovirus sibericum*, faustoviruses or virophages have also been detected in human plasma samples from patients with liver diseases [65].

Conclusion

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

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FIGURE LEGENDS

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.

TABLE

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

Technical approaches	Evidence for mimiviruses	Evidence for marseilleviruses	Elements to consider for causality
Serology	Presence of specific IgG and IgM antibodies to Mimivirus in pneumonia patients Greater seroprevalence in pneumonia patients than controls Mimivirus seroconversion in pneumonia patients, including one individual who manipulated the virus (reactivity to 23 Mimivirus proteins) Serological reactivities to the Sputnik virophage in two patients (reactivity to 2 virophages proteins); seroconversion in one case	IgG detection in blood donors, young health adults, multitransfused thalassemia patients, and a lymphadenitis patient	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 Detection of antibodies to Marseillevirus in association with Marseillevirus antigen/DNA detection in a single case-patient
Immunodetection		Detection of Marseillevirus antigens by immunofluorescence and immunochemistry in a lymph node adenitis	Association of Marseillevirus with lymphadenitis
Molecular detection	**Conventional PCR**: Mimivirus DNA found in a broncholalveolar fluid and a serum sample from two pneumonia patients	**Conventional PCR**: Marseillevirus DNA detection in the serum from blood donors, multitransfused thalassemia patients, and a lymphadenitis patient Detection of Marseillevirus DNA by fluorescence *in situ* hybridization in a lymph node adenitis	Association of Mimivirus with unexplained pneumonia and of Marseillevirus with lymphadenitis
	Metagenomics: 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	**Metagenomics:** Marseillevirus-like sequences detection in the buccal mucosa, retroauricular crease, vagina and stools from healthy people	
Culture isolation	Isolation from a broncholalveolar fluid and a faeces sample from two pneumonia patients		Association of Mimivirus with unexplained pneumonia
Experimental models	**Cells:** 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	**Cells:** 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 *in situ* hybridization, or transmission electron microcopy	
	Animal: Pneumonia induction in mice inoculated intracardiacally		Mimivirus causes pneumonia in mice

References for quoted studies are included in the text

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

Henle, Loeffler and Kochôs postulate (1884-1890) [29]

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

Riversô criteria for proof of viral disease causation (1937) [31]

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

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

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

antibodies should regularly result from active infection.

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

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

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

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

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

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

Evansôs criteria for proof of disease causation: a unified concept appropriate for viruses as causative agents of disease based on the Henleï Koch postulates (1976) [33]

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

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

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

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

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

graded response, often from mild to severe.

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

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

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

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

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

Fredricks and Relmanôs molecular guidelines for establishing microbial disease causation (1996) [34]

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

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

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

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

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

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

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

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

Metagenomic Kochôs postulates (2012) [35]

Comparison between a diseased and healthy control animal shows a significant difference between the metagenomic libraries (depicted by the histograms of relative abundance reads). In order to fulfill the metagenomic Kochôs postulates:

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

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

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

Fig. 1

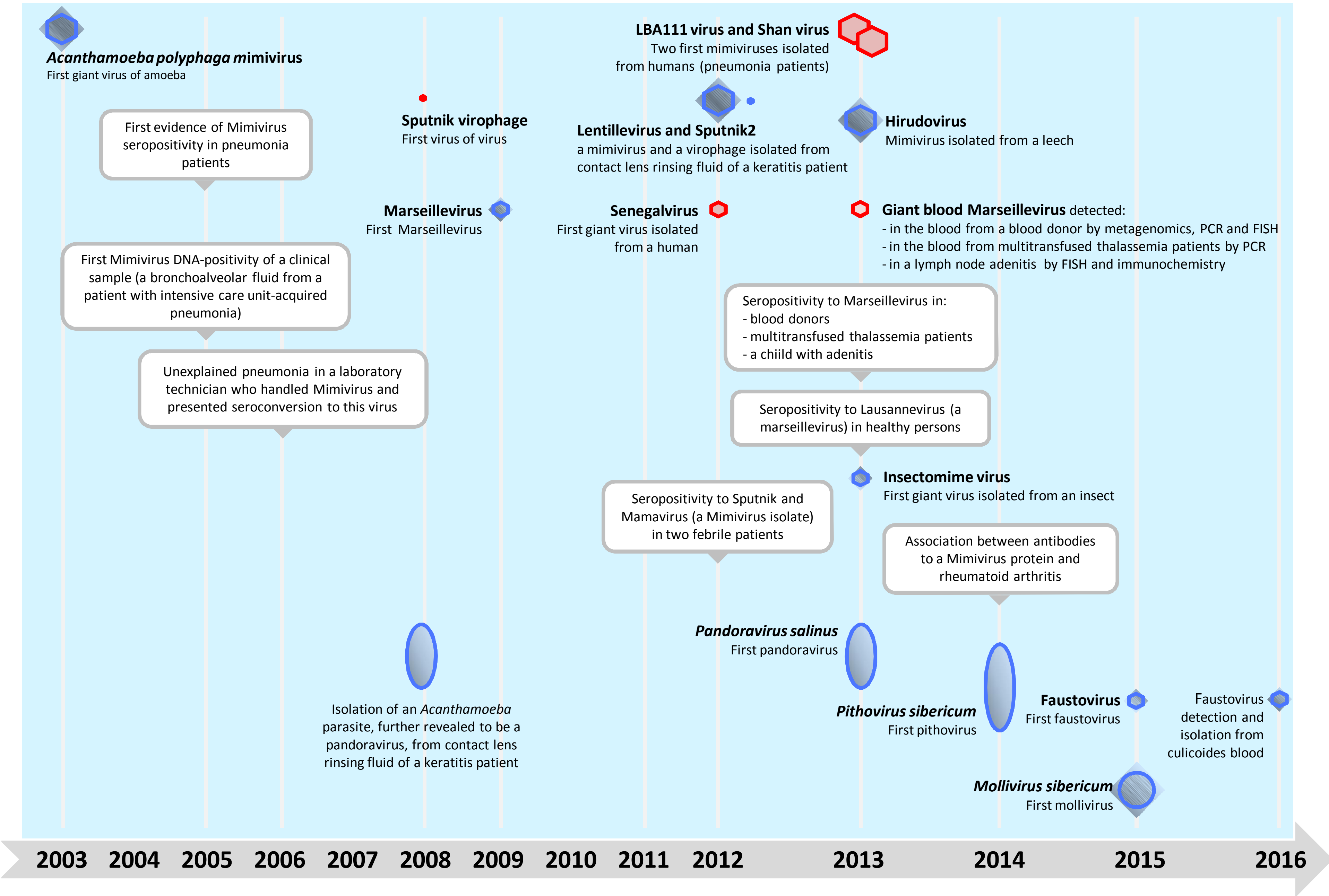
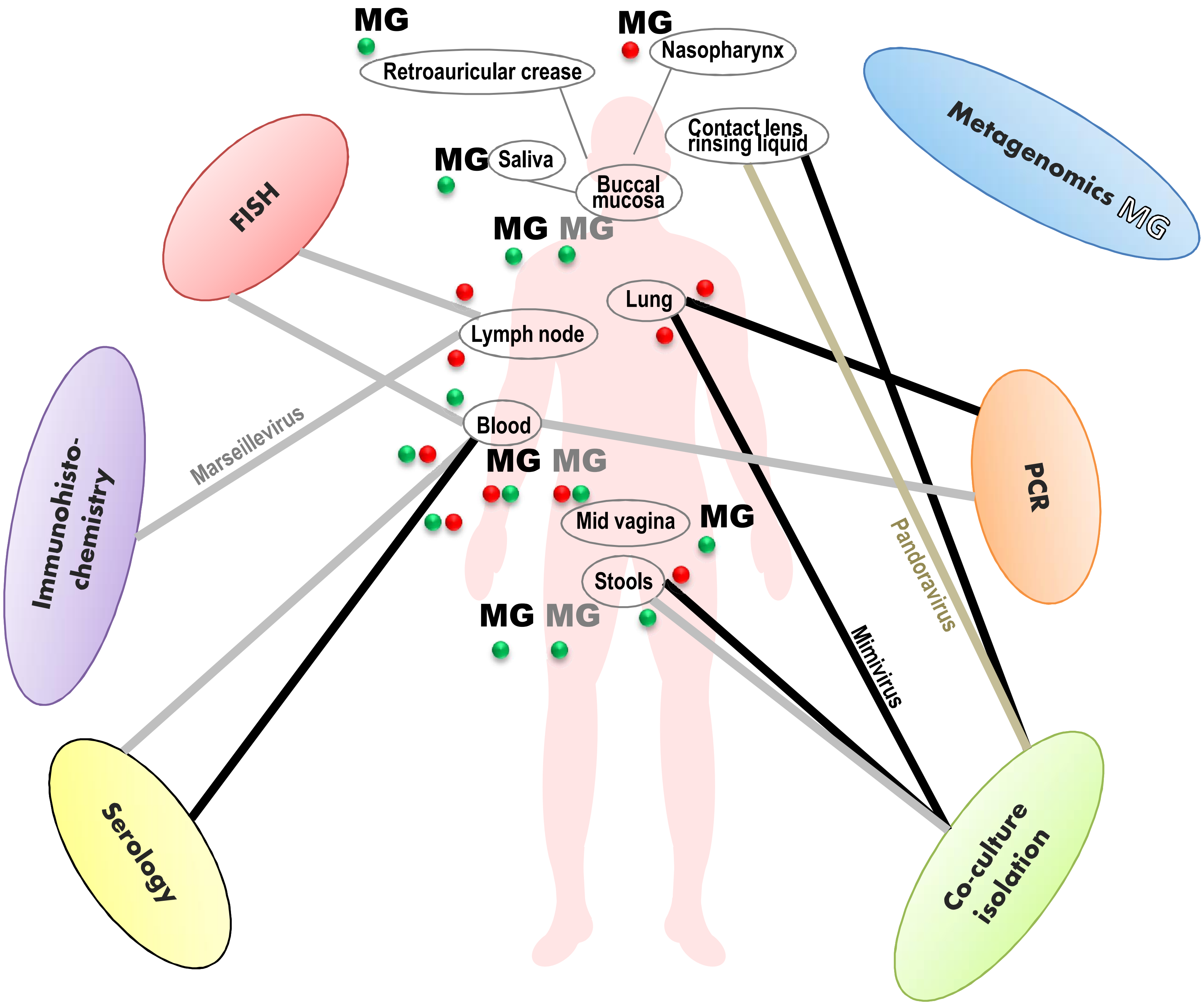

Acanthamoeba polyphaga mimivirus
First giant virus of amoeba
First evidence of Mimivirus seropositivity in pneumonia patients
First Mimivirus DNA-positivity of a clinical sample (a bronchoalveolar fluid from a patient with intensive care unit-acquired pneumonia)
Unexplained pneumonia in a laboratory technician who handled Mimivirus and presented seroconversion to this virus
Sputnik virophage
First virus of virus
Marseillevirus
First Marseillevirus
Isolation of an Acanthamoeba parasite, further revealed to be a pandoravirus, from contact lens rinsing fluid of a keratitis patient
LBA111 virus and Shan virus
Two first mimiviruses isolated from humans (pneumonia patients)
Lentillevirus and Sputnik2
a mimivirus and a virophage isolated from contact lens rinsing fluid of a keratitis patient
Hirudovirus
Mimivirus isolated from a leech
Senegalvirus
First giant virus isolated from a human
Giant blood Marseillevirus detected:
- in the blood from a blood donor by metagenomics, PCR and FISH
- in the blood from multitransfused thalassemia patients by PCR
- in a lymph node adenitis by FISH and immunochemistry
Seropositivity to Marseillevirus in:
- blood donors
- multitransfused thalassemia patients
- a chiild with adenitis
Seropositivity to Lausannevirus (a marseillevirus) in healthy persons
Insectomime virus
First giant virus isolated from an insect
Seropositivity to Sputnik and Mamavirus (a Mimivirus isolate) in two febrile patients
Association between antibodies to a Mimivirus protein and rheumatoid arthritis
Pandoravirus salinus
First pandoravirus
Pithovirus sibericum
First pithovirus
Faustovirus
First faustovirus
Faustovirus detection and isolation from culicoides blood
Mollivirus sibericum
First mollivirus
2003 2004 2005 2006 2007 2008 2009 2010 2011 2012 2013 2014 2015 2016

Fig. 2


MG
MG
Retroauricular crease
Nasopharynx
Metagenomics MG
FISH
MG
Saliva
Contact lens rinsing liquid
Buccal mucosa
MG MG
Lung
Lymph node
Immunohisto-chemistry
Marseillevirus
Blood
PCR
MG MG
Mid vagina
MG
Stools
Pandoravirus
MG MG
Mimivirus
Serology
Co-culture isolation