Acta virologica **54**: 85 – 90, 2010 doi:10:4149/av_2010_02_85

Minireview

Viruses, virophages, and their living nature

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Received September 4, 2009; accepted March 16, 2010

Summary. – Over 100 years viruses have fascinated scientists around the world. Although biologists, chemists, physicians, veterinarians, and even physicists attempted to elucidate the nature of viruses, the question still remains "Are viruses alive?" Different theories have aimed at unifying our views of virology to provide an answer. However, the discovery of a mimivirus, its genome organization and replication cycle, in addition to the recently found virophage challenged the established frontier between viruses and parasitic cellular organisms. Consequently, the old controversy whether viruses are inert agents at the threshold of life or a different form of life was reignited. This review reopens the debate about the living nature of viruses from the classical concepts to the recent discoveries in order to rationally discuss our beliefs about the living or non-living character of viruses.

Keywords: filterable agent; mimivirus; virophage

Contents:

1. First steps in virus history

The scientific community has disputed about the living status of viruses for over a century. Early in 19th century, the viruses were considered as a poison and later on, as a microscopic form of life or biochemical agents. Today, 110 years after their original description as "filterable agents" was coined, the viruses still linger in an enigmatic and controversial position between living and non-living microbes. Although viruses cannot replicate by themselves, they are able to hijack the host cellular machinery for their replication and at the same time they deeply affect life cycle of the host.

When we want to support the living nature of viruses, we have to go back to the mid-19th century, the period of time before their discovery. Two scientists, Louis Pasteur and Robert Koch, took part in the history of microbiology by challenging the prevailing dogmas in biology. Through fermentation studies, Pasteur (1860) showed that "microscopic life" was not spontaneously generated, but it was a result of contamination with living germs. On the other hand, Koch (1876) provided the dogmas for the microbiology through the formation of "Koch's postulates" that apparently could be used for classification of the nature of infectious diseases. According to these postulates, a disease was the result of infection with

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Abbreviations: APMV = Acanthamoeba polyphaga mimivirus; ICTV = International Committee on Taxonomy of Viruses; TMV = Tobacco mosaic virus

a microorganism that was able to grow on an artificial medium and could be re-inoculated in a healthy host developing a specific disease (Koch, 1876). In principle, Koch´s postulates worked as a very useful guide for the methodical study of the origin of infectious diseases. At the same time, they presented a limitation for the characterization of causal agents that did not fit in those rules. Later, their validity was confronted and questioned more than once and their limitations stimulated the creation of new concepts and causality criteria. Hill (1965) postulated a wider set of conditions to assign the causality of a disease as the strength of association, consistency, specificity, temporality, dose-response relationship, biological plausibility, coherence, and experimental evidence.

Although Pasteur (1890) was one of the first scientists working empirically with viruses, at that time the term "virus" was almost exclusively used to describe a poison. Later on, viruses were accepted as the infectious agents of the microbial world. In the late 19th century, after Pasteur and Koch had largely forged the bacteriological era, the first reports about the existence of agents that challenged these dominant dogmas made their first appearance. Mayer (1882, 1886) proposed the existence of an "infectious soluble agent, possibly of an enzymatic-type", although scientific support for such an analogy was missing. Ivanovsky (1892) published a short paper about the tobacco mosaic disease, where he stated that "the sap of leaves attacked by the mosaic disease retains its infectious qualities even after filtration through Chamberland filter candles". During the Congress of the Academy of Sciences in Amsterdam, Beijerinck (1898) presented one of the most important discoveries of biology, postulating that a *contagium vivum fluidum* was the cause of tobacco mosaic disease, now known to be caused by Tobacco mosaic virus (TMV) (Kluyver, 1940). This discovery in a conjunction with the use of porcelain filters developed by Chamberland allowed first characterization of the viruses as filterable agents.

A few years after demonstration that the disease on the tobacco leaves was caused by a microorganism or a toxin that passed through the bacteria-proof filters, Ivanovsky (1903) reported that this *contagium vivum fluidum* extracted from the diseased tobacco leaves was able to grow in culture medium. In parallel with these experiments, Loeffler and Frosch (1898) working with foot-and-mouth disease in cattle (today known to be caused by Foot-and-mouth disease virus) came to similar conclusions about the nature of the causative organism of this disease. Then, they attributed many diseases of animals and humans to these filterable agents (Loeffler, 1898).

Some years after studying contagious bovine pleuropneumonia, a disease that fitted Koch's postulates, Roux (1903) published the first review of "Viruses" including the description of *contagium vivum fluidum* of tobacco mosaic disease and a causative agent of pleuropneumonia describing them as "invisible" microbes.

2. Discoveries of the 20th century

During the first 3 decades of 20th century, viruses had been studied only from the perspective of their transmissibility and effects on their host. The discovery and rapid increase of viral diseases, particularly during the 1920s increased the need to recognize the intrinsic and biological properties of such agents (Rutherford *et al.*, 1929; McKinley, 1932). One of the first descriptions of viruses appeared, when Stanley (1935) succeeded in the isolation of a crystalline protein possessing the properties of TMV from the diseased plants. Although TMV was erroneously described as an autocatalytic enzyme, this achievement along with the subsequent characterization of TMV as a "nucleic acid/protein complex" by Bawden and Pirie (1937) provided the most significant advance in the understanding of viruses as real physico-chemical entities and their subsequent genetic characterization. However, it was not until 1938 that the first definition of viruses appeared as "non-cellular, small packages of non-host genetic information; obligate parasites lacking any physiological machinery of their own", in other words according to Laidlaw (1938), as microorganisms that live "a borrowed life".

In this short summary, using the words of biochemist Fraenkel-Conrat (1962) "In design and function, viruses really are at the threshold of life" and in agreement with other authors that a reproductive ability is one of the most fundamental features related to life, viruses are the agents that best define the threshold between the living and non-living systems (Villarreal, 2004).

Currently, the International Committee on Taxonomy of Viruses (ICTV) defines viruses as "elementary biosystems that possess some of the properties of living systems such as having a genome and being able to adapt to a changing environment" (van Regenmortel, 2000). In contrast to this definition, traditional concepts argue that the viruses as an issue belong to the biology, since they possess genes, are able to replicate, to evolve, and are adapted to the particular host, biotic habitat, and ecological niches. However, viruses cannot capture and store free energy and they are not functionally active outside their host cells. Although they are pathogens, viruses should not be considered as pathogenic microorganisms, since they are not alive. For many traditional biologists the simplest system that can be considered as living is a cell. The cells acquire an autonomy that is characteristic of living systems thanks to the integrated complex of metabolic activities. However, none of the constituent systems of cells, such as organelles or macromolecules, can be said to be alive. A virus becomes part of a living system only after it has infected a host cell and its genome has become integrated with the cell genome, what is a specific case of retroviruses. Viruses replicate only through the metabolic activities of infected cells and therefore occupy a unique position in biology (van Regenmortel and Mahy, 2004; Büchen-Osmond, 2006).

Table 1. Selected biggest viruses, smallest bacteria, and virophage (adapted from Claverie *et al.*, 2006)

Virus/Bacteria/Virophage	Genome size (bp)	Family	GenBank Acc. No.
Acanthamoeba polyphaga mimivirus	1,181,404	*Mimiviridae*	NC_006450
Acanthamoeba castellanii mamavirus	1,200,000	*Mimiviridae*	Not reported
Bacillus phage G	497,513	*Myoviridae*	02.043.0.00.014[a]
Emiliania huxleyi virus 86	407,339	*Phycodnaviridae*	NC_007346
Marseillevirus	368,454	*Not classified*	NC_013756
Canarypox virus	359,853	*Poxviridae*	NC_005309
Fowlpox virus	288,539	*Poxviridae*	NC_002188
Pongine herpesvirus 4	241,087	*Herpesviridae*	NC_003521
Human herpesvirus 5, strain Merlin	235,646	*Herpesviridae*	NC_006273
Human herpesvirus 5, strain AD169	230,290	*Herpesviridae*	NC_001347
Mycoplasma hyopneumoniae 232	892,758	*Mycoplasmataceae*	NC_006360
Chlamydia trachomatis D/UW-3/CX	1,042,519	*Chlamydiaceae*	NC_000117
Rickettsia prowazekii	1,111,523	*Rickettsiaceae*	NC_000963
Sputnik virophage	18,343	*Not classified*	NC_011132

Bacteria (dark grey shade) and sputnik virophage (light grey shade). [a]Identity number in ICTV database available at URL: pbi.bio.pitt.edu.

Based on the premises formulated by Koshland (2002), a living organism is an organized unit, which carries out metabolic reactions, defends itself against injury, responds to stimuli and contains the ability to reproduce himself, at least with the help of a partner. Viruses could almost completely fulfill all of the above conditions including their ability to produce viral progeny, when they interact with their "partner", a host cell. Consequently, this premise would raise them again to the status of "a living organism".

Recently Koshland (2002) also introduced "The Seven Pillars of Life" from a different perspective that could be considered in this discussion. The requirements of living organisms are: 1) capacity to respond to self-program, 2) capability to improvise, 3) ability to involve physical compartmentalization, 4) ability of energy production, 5) potential to regenerate, 6) adaptability to environment in order to survive, and 7) seclusion, e.g. the skill to isolate oneself. According to these conditions the viruses lack ability to produce their own energy that is identified as a movement of chemicals from the body or its components. In other words, viruses do not pose an open system that exchanges molecules and carries out metabolic and chemical reactions inside and outside the organism. The absence of this exchange would inevitably lead to a lack of living essence in any existing being without independent metabolism.

3. Mimivirus, a giant virus

Recent discovery in the virology brings out the validity of above requirements. The discovery of Acanthamoeba polyphaga mimivirus (APMV) and the analysis of its full genome caused an astonishment in the international community of virologists (La Scola *et al.*, 2003; Raoult *et al.*, 2004), not to mention evolutionists, whose debates seem to be increasingly more intense (Moreira and López-García, 2005; Iyer *et al.*, 2006). This new microorganism attracts attention because of its size, gene content, and phylogenetic characterization. The information encoded in the viral genome challenges many orthodox concepts about viruses, particularly in terms of those biochemical components that are not supposed to be encoded by a virus genome. Nevertheless, they make us wonder what could have been the possible origin of such viral agents.

APMV (the genus *Mimivirus*, the family *Mimiviridae*) contains a double-stranded DNA enclosed in an icosahedral capsid. The virus replicates in the amoeba (*Acanthamoeba polyphaga*) and constitutes part of the group known as nucleo-cytoplasmic large DNA viruses. To regard APMV as a giant virus is no exaggeration, since its genome contains 1,181,404 base pairs (bp), what moves APMV at the top of large viruses. In the second position appears Bacillus phage G, previously considered as the biggest virus with 497,513 bp, the third position is filled with Emiliania huxleyi virus 86 with 407,339 bp belonging to the genus *Coccolithovirus* and the sixth place is took by the recently discovered and yet unclassified Marseillevirus with 368,454 bp (Boyer *et al.,* 2009). It should be noted that within the scale of the largest viruses, animal viruses make their appearance only at the 5th and 6th position with the poxviruses (Canarypox and Fowlpox viruses) of the subfamily *Chordopoxvirinae* with 359,853 and 288,539 bp, respectively. Down the list at the 6th position is Orangutan herpesvirus known as Pongine herpesvirus 4 with 241,087 bp followed by the human cytomegaloviruses, Human herpesvirus 5, strain Merlin and strain AD169 with 235,646 and 230,290 bp, respectively (Table 1). Genomically

speaking, a mimivirus is one of the giants of the viral world and is even bigger than many bacteria such as Mycoplasma, Chlamydia, and Rickettsia. Considering the large diameter of its icosahedral capsid (400 nm), the size of the virion is comparable to a mycoplasma cell (Claverie *et al*., 2006).

Describing the size of APMV, we are not necessarily referring to its most amazing feature. Also, the study of the viral genome showed that it consists of 1,262 putative ORFs, of which 10% exhibit a high homology with proteins of known functions. Additionally, APMV has other characteristics that differentiate it from other large nucleo-cytoplasmic DNA viruses, including the presence of genes encoding central components of the protein translation system, 6 transfer RNAs (tRNAs), 4 aminoacyl-tRNA synthetases, eukaryotic release factor-1 (eRF1), elongation factor EF-Tu, and translation initiator factor 1. APMV also encodes topoisomerase class I and II that are important components of DNA repair machinery, enzymes for polysaccharide synthesis and intein (protein intron) capable of self-splicing and re-joining to its own protein (Ogata *et al*., 2005a).

Reductionist biology divided life on Earth into three domains: Eukaryotes, Eubacteria and Archaea. Eubacteria are the simplest organisms lacking a nucleus to keep their genetic material together. Eubacteria are followed by Archaea that are supposed to have evolved in different ways due to the different phospholipids compositions in Archea membrane. Finally, the Eukaryotes contain a true nucleus inside the cells, where genetic material is assembled. Amazingly, the mimivirus has 7 genes that are common in the mentioned three domains. For some researchers, this phenomenon puts APMV at the same definition of life as is attributed to those domains, or at least APMV deserves its own definition of life (Peplow, 2004; Ogata *et al*., 2005b).

A feature common to almost all existing viruses is their total dependence on the host translational machinery for protein synthesis. However, it is surprising that APMV encodes genes for almost all steps of messenger RNA translation, but lacks ribosomes (Ogata *et al*., 2005a). If we return to the Koshland's "seven pillars of Life", the mimivirus completely fulfills these postulates that would qualify it as a living organism, being an exception in the viral world. Although this exception does not represent the wide diversity of viruses, it is a proof that the current characterization is not sufficient enough to define a virus as a living or non-living organism.

According to these recent findings in virology, we should probably redefine a virus according to the Raoult and Forterre (2008), who suggest that virus is "a capsid-encoding organism composed of proteins and nucleic acids that self-assembles in a nucleocapsid and uses a ribosome-encoding organism for the completion of its life cycle". Alternatively, Wolkowicz and Schaechter (2008) recommend that a better definition of a virus should be based on the knowledge that it presents a microorganism that breaks up and loses its bodily integrity with its progeny becoming reconstituted after replication from newly synthesized parts independent of the time when this phenomenon occurs.

4. Virophage, a new agent in the virology era

However, when we thought we had reached the highest possible level of surprise with the discovery of APMV, the global community of virologists witnessed a new surprise. La Scola *et al*. (2008) discovered a new strain of APMV that was named a mamavirus. Using electron microscopy they found a new APMV strain even larger than the previously described mimivirus and in addition, they demonstrated the presence of small icosahedral particles of approximately 50 nm inside the APMV viral factories. Given its association with the mimivirus, authors called this new agent "sputnik" (a Russian word meaning satellite). Studies of this "unknown virus" provided two major findings to the current virology: (1) sputnik acts as a "parasite" within the APMV viral factories producing its progeny faster than APMV and generating an abnormal formation of APMV viral particles characterized by partial thickening of the viral capsid, production of defective viral particles and loss of the morphology, (2) in some cases, it was possible to observe encapsidation of sputnik´s virions into APMV. However, it was not possible to infect *Acanthamoeba castellanii* (the host a mamavirus was isolated from) with sputnik only. When APMV and sputnik were co-inoculated, there was a 70% reduction in the infectivity of APMV and a threefold decrease of its lytic ability, what demonstrated its detrimental potential on the infectivity of APMV. Additionally, the analysis of the sputnik genome showed that it is a circular double-stranded DNA with 18,343 bp that contains genes phylogenetically related to the Eukaryotes, Eubacteria, and Archaea (La Scola *et al*., 2008).

Given the similarity between bacteriophages that infect and cause disease in bacteria by subverting their host for their own replication, the name "virophage" was coined for sputnik. Additionally, since the virophage carries genes closely related to APMV genes, it is possible to assume that this virus can perform a certain level of horizontal transfer of genes between viruses as is known for bacteriophages that infect bacteria and share the genetic material with them, a mechanism commonly described as transfection that could even affect bacterial virulence (La Scola *et al*., 2008).

It is necessary to state that the sputnik virophage has as yet not been classified by ICTV due to the fact that "the decisions about creating new species is based on the description of more than one unique isolate; therefore we should look

forward to the identification of more such isolates in the future so that a clear taxonomic description can be made" (Eric B. Carstens, President of ICTV, personal communication). Additionally, the sputnik should not be confused with satellite viruses that are sub-viral agents relying on a "helper" virus to produce co-infection and to generate new viral progeny, but which do not appear to affect replication and production of helper virus progeny.

5. Open debate

In light of the new scientific evidence, it seems appropriate and necessary to restore the old controversy about the living nature of viruses. Rational discussion should go on the new findings based on the scientific literature and leaving out our beliefs and judgments about what should be considered "alive" or inert in terms of the nature of viruses. It is important to develop this controversy and try to reach a scientific consensus or at least to show a broader perspective based on these new paradigms and also recognize the frequent surprises of microbiological research. Recently, Moreira and Lopez-García (2009) published a controversial paper with "ten reasons to exclude viruses from the tree of life" including the statement that a virus is not alive. Although the subsequent discussion has turned properly evolutionistic, we agree with some authors who emphasize that a proper definition of life using all established biological data should be integrated (Raoult, 2009). Viruses similar to all living organisms follow Darwin's theory of "the survival of the fittest", acquire mutations and evolve to sustain in a new environment (Hegde *et al.*, 2009). On the basis of the genomic and phylogenetic analyses, viruses could be defined as the fourth domain of life (Raoult *et al.*, 2004; Claverie and Ogata, 2009).

For now, we prefer to employ the classic, but not out-of-date definition of "non-cellular life" coined by the botanist Novak (1930), who stated that the viruses represented a phylogenetic kingdom formed by the organisms living without their own cells. In addition, he supported the phylogenetic classification of viruses as life forms. However, this definition is not the recent one, but it fits to the majority of features required to confer "life" to viruses without contradicting all existing classifications and denominations that will be most likely discussed in forthcoming meeting of ICTV.

Acknowledgment. The authors thank Anne-Lise Haenni for revision of the English and critical reading of the manuscript.

Conflict of interest. The authors declare that there is no conflict of interest with the ideas put forward in the final version of manuscript.

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