

Serendipitous Discovery in a Marine Invertebrate (Phylum Chaetognatha) of the Longest Giant Viruses Reported till Date

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Abstract

The recent discovery of a nuclear giant virus that infects chaetognaths (small marine invertebrates) led us to reanalyze the surprising structures previously observed in this taxon. These elements, initially thought to be bristles and, later, bacteria, have been observed in two species and are in fact viral particles that likely correspond to two host-specific species. All of these viral particles have a spindle (fusiform) shape, an outer envelope and a tegument-like structure surrounding one internal membrane delimiting a compartment containing the genome and proteins. Electron photographs have provided a view of the sequential viral assembly and egress processes, which are concomitant and occur through the cytoplasmic membrane. During viral budding, the tegument-like wall self-assembles from a ring structure. Moreover, in the cell cytoplasm, the viral nucleoid is surrounded by two membranes. The virions that infect *Paraspadella gotoi* have a length range of ~2.5-3.1 μm and are not completely covered by the envelope revealing a kind of small "paintbrush" that is probably protein in nature. This structure does not appear in the viral particles infecting *Spadella cephaloptera*, who's the size of a virus exceeds 4 μm , which is approximately twice the length of the bacterium *E. coli* and represents the longest known length of a virus. Moreover, they are perhaps the first known photographs of giant viruses (1967). Future genomic studies and further ultra-structural investigations are needed to improve knowledge of these viruses, which may represent a novel virus family for we provisionally propose the name *Klothoviridae* and the type species *Klothovirus casanovai*.

Keywords: Giant virus; *Klothoviridae*; Chaetognatha; Internal membrane; Budding

Introduction

Chaetognaths are a small phylum of marine invertebrates with a size range of 2-120 mm [1]. Despite their soft bodies, early Cambrian (~540-520 million years ago) chaetognath fossils with morphologies almost identical to recent forms have been discovered in China, suggesting a Precambrian origin [2]. Chaetognaths exhibit numerous peculiarities including with respect to their nuclear and mitochondrial genomes [3-5]. Little is known regarding the interaction of viruses with this taxon; only a recent study reported the discovery of a novel giant virus that infects the species *Adhisagitta hispida* [6]. The virions, named Meelsvirus, have a mean size of 1.25 μm that includes an ovoid nucleocapsid joined to a conical tail, both of which are surrounded by a thin envelope. Giant viruses are potentially comparable to some bacteria with respect to their particle size and genomic length [7]. Although variable thresholds are currently used, there appears to be a consensus that giant viruses must have a minimum particle size and genome length of approximately 350 nm (making them visible by light microscopy) and 300 kbp, respectively [8]. Apart from the recent isolation of a retro-giant virus from human leukemia T cells [9], all of the other known giant viruses have double-stranded DNA (dsDNA) genomes. After the discovery of the first giant virus (Mimivirus) in 2003 [10], numerous species of giant viruses have been identified from both terrestrial and aquatic samples [8]. Almost all of these viruses have unicellular eukaryotes as hosts, but because numerous "classical" viruses infecting animals are the causative agents of devastating

infectious diseases, it would be very useful to identify giant viruses with animals as hosts.

In light of Shinn and Bullard's study [6], the goal of this article is to reanalyze different chaetognath-associated viral structures, including the following: 1) those described in 2003 as bacteria-like organisms that appear to push back the membrane of the apex of epithelial cells of the chaetognath *Paraspadella gotoi* [11]; 2) Those from our unpublished photo libraries that infect *P. gotoi* and *Spadella cephaloptera*; and 3) Those said to be "bristles" reported in 1967 that infect *S. cephaloptera* [12]. These studies resulted in the discovery of a new family of giant virus that infects the epidermal cells of these chaetognath species. In ancient Greek, one of the names for spindle was κλωστήρ (transliterated as "kloster" in Latin characters), a name that originating from the verb κλώθω (klotho) which means "to spin wool". Moreover, in Greek mythology, the Moirai were the three goddesses of fate who personified the inescapable destiny of man. The names of these goddesses were Klotho, "the Spinner", who spun the thread of life using generally a spindle (hence his name); Lakhesis, who measured the thread and Atropos, who cut it short. Because of the spindle shape of these viruses, and since viral infections can seriously jeopardize the survival of hosts, the proposed family name *Klothoviridae* appears highly appropriate.

Methods

This paper does refer to electron photographs and a photocopy issuing from two old publications [11,12] and our photo libraries, so the methods have been previously published and only details

concerning electron microscopy preparation procedures will be mentioned here.

Published and unpublished studies from our former laboratories

Paraspadella gotoi was collected in the vicinity of the Amakusa Marine Biological Station (Kumamoto Pref., Japan), while *Spadella cephaloptera* was collected off Villefranche-sur-Mer (Mediterranean French Coast). All of the specimens assayed by electron microscopy were prepared as follows: fixation for 4-8 h at 4°C in a mixture of 3% glutaraldehyde, 1% paraformaldehyde and 30% sea water in a 0.2 M sodium cacodylate buffer (pH 7.3; final osmolarity of ~1.200 mOsm); rinsing for 8-12 h in the same buffer supplemented with 9% glucose; post-fixation for 1-2 h at 4°C in 1% osmium tetroxide in the same buffer; and after routine dehydration and embedding in Araldite, thin sections were stained with uranyl acetate and lead citrate and observed using a Philips EM300 electron microscope [11].

Study by Horridge et al. [12]

For electron microscopy investigations, whole live specimens of *S. cephaloptera* obtained from Plymouth (southern part of the Channel), United Kingdom were fixed in a solution of equal volumes 2% osmic acid and sea water at 0°C for 2-4 h. The material was embedded in Araldite, sectioned, and stained in uranyl acetate for 5 min, followed by lead citrate for 5 min.

Results

Giant viruses of *Paraspadella gotoi*

Paraspadella gotoi species belong to a lineage of benthic chaetognaths (*Spadellidae*) that has developed limb-like appendages on the caudal part of the body. The most complex appendages are those of *P. gotoi*, which are used as props with the tip of the tail to allow for a highly elaborate courtship mating behavior [13], justifying a detailed study conducted by Casanova et al. [11]. According to these last authors that analyzed Figures 1A and 1B: « In all the specimens studied, the more distal cells contain prokaryote-like structures. Their aspect evokes bacteria; indeed, there is a central clear nucleoid-like structure surrounded by cytoplasm filled with ribosomes. They are seen sometimes in great number and gathered together, more or less elongated, probably circulating in the cells. Numerous elongated bacteria-like organisms push aside the cell apex membrane, where they are fixed by the mean of a peculiar collar resulting in a thickening of their sheath which is constituted of three membranes. As for the presence of numerous bacteria, not seen on other parts of the body but very dense at the secretory cells level, it is more difficult to explain. An infestation during breeding cannot be ruled out, but no cell reaction against the bacteria observed in the cells is visible and the prokaryotes are regularly found at the tip of the appendages. A symbiosis seems more probable, perhaps to supply the cells with organic molecules which remain to be identified and which can circulate between cells *via* gap junctions. The 'bristles' observed on the epidermis of *S. cephaloptera* maintained under laboratory conditions by Horridge et al. [12] are probably also infesting prokaryotes. Such cells are unknown in the epidermis of the species of the planktonic genus *Sagitta* ».

Casanova et al. [11] suggested that the elements leaving the cell apical poles had three membrane-like structures, leading them to suggest that they could be Gram-negative bacteria, which are

characterized by cell envelopes composed of a peptidoglycan cell wall sandwiched between an inner cytoplasmic cell membrane and a bacterial outer membrane. The first report of a giant virus identified as such was published in 2003 [10] and was named Mimivirus for being a microbe-mimicking virus, since it was initially misidentified as a Gram-positive bacterium for eleven years. Indeed, during a pneumonia outbreak in 1992 in England, a microorganism growing in amoebae was isolated and referred to as "Bradford coccus" due to its large particle size that was easily visible with a light microscope and its mild Gram-coloration (this virus is non-enveloped) [10]. Moreover, other giant viruses have also been misidentified as bacteria [14,15].

Because the elements egressing from the cells do have not the same structure inside and outside the cells (principally due to the absence of the very electron-dense shell surrounding the part of the particle presents in the cytoplasm), and because the external portion appears to grow from the cell apical poles (see, particles n°1 and n°3 in Figures 1A and 1B, respectively), the hypothesis that these elements are bacterial in nature can be rejected in favor of their having a viral nature. However, there may be some doubt as to the interpretation of the photographs of Figure 1. Do they represent the first stages of viral infection (attachment and cytoplasmic entry of the viral nucleoid) or do they show the release of viral particles? The particles n°1 and n°3 in Figures 1A and 1B, respectively, show a viral release step, i.e., the viral "wall" is not present inside cell. In addition, cells exhibit protuberances from their cytoplasmic membranes (n°4 and n°5 in both Figures 1A and 1B, and n°2 in Figure 1D) in front of viral particles that are not in contact with the cells. The most logical hypothesis is that these cellular protuberances are generated at the site of where the viral particles broke away from the cell membrane. As there is no viral nucleoid surrounded by a double membrane in the cytoplasm in contact with these protuberances, this observation cannot correspond to the very beginning of the self-assembly step.

One of the primary characteristics of the viral particles is their spindle shape and their number of enveloping structures. When the thin cuts were longitudinal, the particles being formed and those that are free exhibit a "weaving shuttle" shape. The particle sizes have been measured for the two virions cut longitudinally (n°2 in both Figures 1A and 1B) and for the unique particle being formed whose length exceeds that of the virions n°2 (n°1 in Figure 1D). The maximum lengths were ~2.5, ~2.5 and ~3.1 µm, respectively. Moreover, several transverse cuts showed that the section is circular, the maximal diameter of the three measured particles were ~0.6, ~0.7 and ~0.7 µm, respectively. It should also be noted that the measurements of the maximum particle sizes must often be underestimated because it is not known if the section were made at the longest lengths and widths. However, the authors made serial tissue sections and chose the photographs in which the "bacteria-like" structures appeared to have their maximal sizes [11].

Casanova et al. [11] mentioned that the "bacterial-like" structures have three membranes. In actually, the various "layers" surrounding the inner area that must contain the viral genome are not always visible. However, it frequently appears that these structures could be interpreted as an internal membrane resembling the cytoplasmic membrane surrounded by a tegument-like structure composed of at least two layers. Moreover, an outer layer that resembles the cytoplasmic membrane appears in some areas of particles, suggesting that the viral particles are enveloped. In addition, the apical ends of the particles exhibit a very electron dense area that is likely composed of

proteins and has the appearance of the tuft of a paintbrush, which will hereafter be referred to as the "paintbrush".

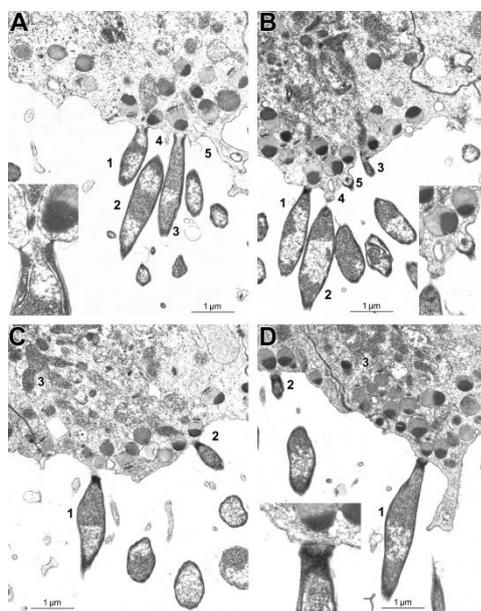


Figure 1: Electron micrographs of the secretory epithelial cells on the distal part of the limb-like appendages of *Paraspadella gotoi* showing the successive stages of virions release from the cells. Panels (A) and (B) were reproduced with permission from [11]; panels (C) and (D) are original photographs. Several oval secretion granules are concentrated in the cytoplasm and are clearly visible in the photographs. The numbers were assigned to identify some viral particles (1-3 on (A) and (B), 1 and 2 on (C) and 1 on (D)), and protuberances on cell apical surfaces (4, 5 on (A) and (B), and 2 on (D)). Ring-like structure of the particles n°1 is shown enlarged in inserts in both (A) and (D), protuberances n°4 and 5 are shown enlarged in insert in (B).

The connection between this structure and the tegument-like structure is indiscernible but it is never covered by the envelope. In contrast, the basal pointed end probably exhibits the three types of enveloping structures. Moreover, in the basal part of all the egressing virions, there is a very electron dense ring-like structure from which the tegument-like wall seems to be self-assembled (Figure 1 and inserts enlarged in Figures 1A and 1D).

Furthermore, in all of the photographs of Figure 1, vesicles of various forms surrounded by two membranes (although not always completely observable) are visible and contain material that appearing similar to that of the intracytoplasmic portion of the nucleoids being encapsidated. Moreover, vesicles appear to bud as the n°3 in both Figures 1C and 1D.

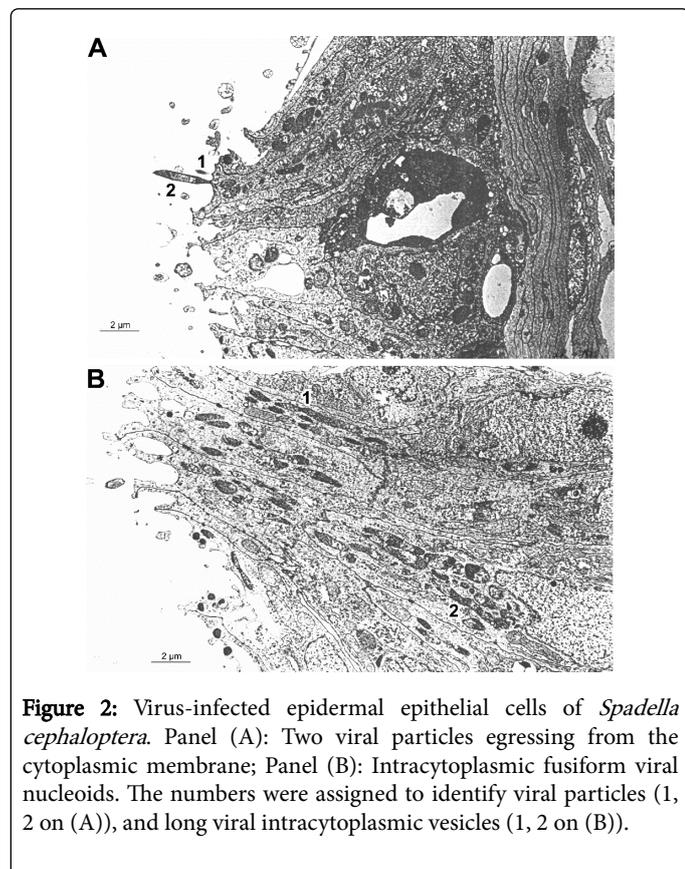
Giant viruses of *Spadella cephaloptera*

Only two photographs been taken by our former laboratories of a *Spadella cephaloptera* specimen collected off the French Mediterranean Coast exhibiting infected cells. Unfortunately, the negatives and the original photographs have been lost, and only an annotated photocopy has been preserved. However, despite the

mediocre quality of this image, this document is still of great interest and is represented in Figure 2. *Spadella cephaloptera* adults can attach themselves to substrates with adhesive papillae that are localized in the mid-ventral surface of the body [16], and the photographs were taken in this area. In Figure 2A, two forming viral particles are observable, for one particle (n°1), the orientation of the cross-section is not relevant, whereas for the second (n°2), the entirety or almost of the particle is shown. No "paintbrush" structure can see at the apical end of the latter particle and it is impossible to determine the characteristics of the layers of the enveloping structures or even their number. The length of the longest viral particle is ~2.9 μm . In Figure 2B, several cells contain intracytoplasmic viral nucleoids, and unlike those present in *P. gotoi* cells, they have a spindle shape. Duvert [11], who had taken these photographs, considered that the intracytoplasmic elements were of the same nature as the large one observed emerging from the cell in Figure 2A. Given the poor quality of the picture, it is difficult to determine if these structures are surrounded by a double membrane or not, even if something appearing to be a double membrane is visible on small portions of the periphery of certain particles. Moreover, the intramembranous viral material with electron-dense and other clear zones has an appearance similar to that observed in the particle n°2 in Figure 2A and in the free or forming particles observed in *P. gotoi* photographs (Figure 1). Very long nucleoids, i.e., $\geq 5 \mu\text{m}$ in length (numbered 1 and 2 in Figure 2B), which exhibit elsewhere other than at the ends a narrowing diameter that may suggest a form of binary fission, even if a bias of the section cannot be excluded. Unlike *P. gotoi*, infected cells do not contain oval secretion granules, with the exception of one cell exhibiting one viral nucleoid (bottom left in Figure 2B). The infected cells might have lost their secretory activity; however, apparently uninfected cells also do not contain secretion granules.

In 1967, Horridge et al. [12], who were studying the organs involved in vibration sensing in *S. cephaloptera*, observed two types of structures, bristles and ciliary sensory organs. However, the bristles described by these authors *via* electron microscopy (Figures 3B-3D and Figure 4) are actually giant viral particles analogous to those observed in *P. gotoi*. According to these last authors: « The bristles project from the epithelium in small tufts along both sides of the body, usually alternating with ciliary sense organs. They are clearly visible in life (Figure 3A), whereas to see the ciliary organs a phase-contrast microscope is required. Each bristle is approximately 0.1 mm long and 0.7 μm in diameter, narrowing to 0.2 to 0.3 μm at the base where it enters the cell body. Each bristle (virus particle) is a long straight extension of the epithelial cell (Figure 3B), and its outermost membrane is continuous with that of the cell. The (virus particle) has two inner membranes, presumably of endoplasmic reticulum, which run along its whole length. These inner two membranes join together at the base to form a tube which fits tightly within the outer wall of the (virus particle) (Figure 3C). The cytoplasm of the (virus particle) is separated from that of the cell by a peculiar collar of membranes (Figures 3C, 3B and 4) and the interior of the (virus particle) is composed of a granular material which is distinct from the cytoplasm of the cell. Below the junction of (virus particle) and cell there is frequently a cluster of circular bodies with dense granular contents enclosed in a single membrane (Figure 4). These bodies appear to contain material similar to that within the (virus particles). The triple membrane and dense contents of the (virus particles) must account for their stiffness because they have no internal fibrils or skeleton. The (virus particles) show no trace of a basal body and cannot be modified

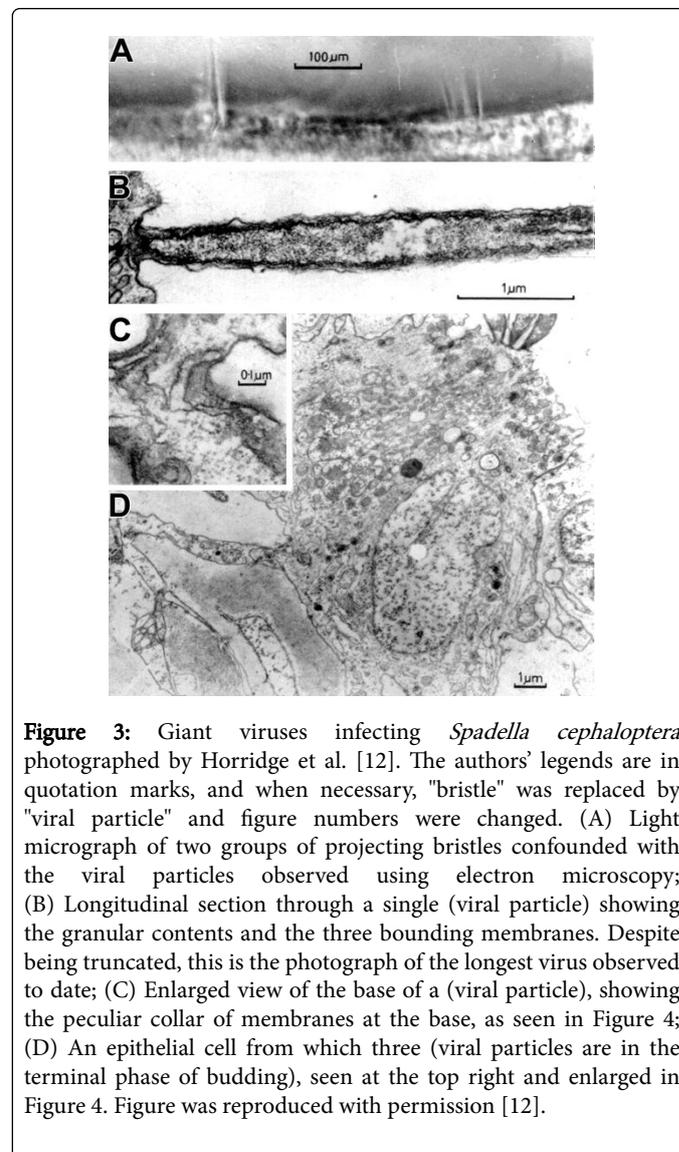
cilia. They break off very easily and in fixed material are commonly lost ».



As the true bristles are very fragile, using electron microscopy, the authors [12] took the only structures outside of the cytoplasm other than the ciliary sensory organs for bristles (Figures 3B-3D and 4 vs. Figure 3A). This led to the confusion regarding the sizes, the length (0.1 mm) corresponds to the bristles while all other values smaller than a micrometer (diameter and base sizes) must be related to the viruses. However, numerous comments made by these authors are highly appropriate. There is continuity between the cytoplasmic membrane and those surrounding the particle (i.e., the envelope) (Figures 3B and 3C). For particle n°1 in Figure 4, a small portion of the viral material is still in the cytoplasm, and the innermost membrane is partly visible. This latter membrane surrounds the internal viral material as has already been observed for *P. gotoi*. At this point, Horridge et al. [12] also mentioned three membranes that they considered to be of cytoplasmic origin; however, there is some confusion regarding their description, indeed, the "outer wall" likely corresponds to the tegument-like structure. Moreover, the envelopes in Figure 4 have detached themselves from the viral particles from which they are located several hundred nanometers away. Similarly, to those infecting *P. gotoi*, the virions have three enveloping structures and the ring-like structure is connected to the layers of the tegument-like material (Figures 3B and 4).

The viral particle shown in Figure 3B appears rigid and has probably a spindle shape similar to those observed in photographs of *P. gotoi* (Figure 1) and especially in the photograph of a particle egressing from a *S. cephaloptera* cell (n°2 in Figure 2A). In contrast, the three viral particles shown in Figure 4 appear rather flaccid and fewer spindles

shaped. Moreover, the envelope is located very far from the rest of the particles and even has partly disappeared (particle n°3), and the shell structure is also strongly degraded.



Although this could be due to an artifact related to the fixation method, the damages underwent by these particles allow the laminated structure of the tegument-like material to be observed; this wall is characterized by parallel layers stacking successively on each other. In Horridge et al. electron study [12], no "paintbrush" structure is visible on the apex of the two viral particles, which are not truncated (Figure 4). The lack of these structures could be due to the preparation of the sections, but the envelope totally surrounds the apical end of particle n°2 suggesting that viruses infecting *S. cephaloptera* are different from those having *P. gotoi* has a host. Moreover, the difference in the shape of the intracytoplasmic nucleoids (Figure 2B vs. Figure 1) reinforces this hypothesis.

It is very difficult to determine what types of cells are represented in the photographs taken by Horridge et al. [12], as these authors mentioned that the bristles project from the epithelium in small tufts along both sides of the body, which could not correspond to the

adhesive papillae on the ventral surface of the body. However, the description is too imprecise on this point.

In Figure 3B, despite being truncated, this is the photograph of the longest virus observed to date. Its size in the photograph is 3.9 μm suggesting that the whole length of this viral particle greatly exceeds of 4 μm whereas its maximal diameter is $\sim 0.5 \mu\text{m}$. The sizes of the viral particles in Figure 4 are inconclusive due to their degradation.

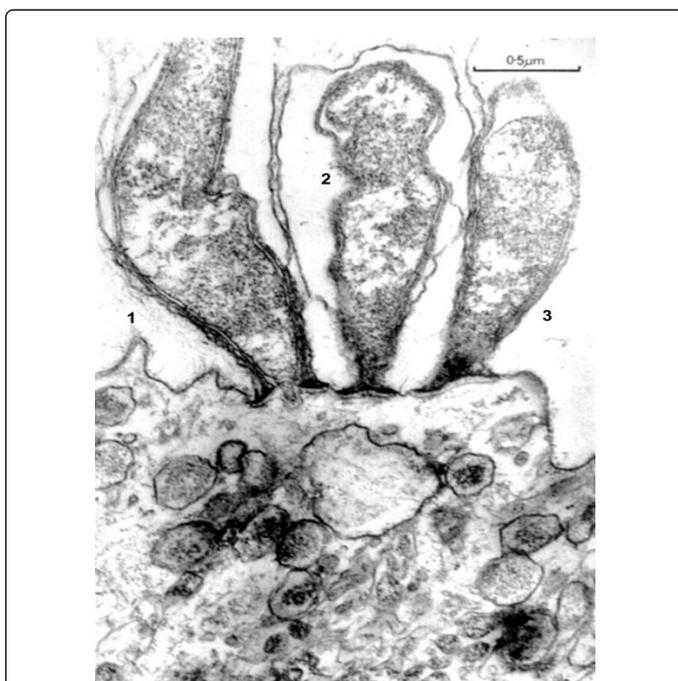


Figure 4: Enlarged view of the three viral particles budding from the apical extremity of the epithelial cell shown in Figure 3D. Numbers have been added to identify the viral particles. Figure was reproduced with permission [12].

Discussion

Morphological characteristics of *Klothoviridae* particles

Based on their unique morphology, the viral particles infecting *P. gotoi* and *S. cephaloptera* certainly belong to a new family with the provisional name *Klothoviridae*. Moreover, the differences exhibited by the virions infecting the two chaetognath species suggest that there are two viral species belonging to two different genera provisionally named *Klothovirus* and *Megaklothovirus*. The rules of the ancient Greek language dictate that the adjective Μεγάλη (Megale), meaning "great", should be used; however, as is often the use in nomenclature, the prefix "Mega" will be employed. For the name of the type species infecting *P. gotoi*, *Klothovirus casanovai* has been proposed, as Professor Casanova has studied at length the chaetognaths and notably described *P. gotoi*. Concerning the viral species infecting *S. cephaloptera*, the name *Megaklothovirus horridgei* is proposed.

All of these viruses have a spindle shape and their cross section appears to be circular. Concerning *K. casanovai* particles, the range in their total length is $\sim 2.5\text{-}3.1 \mu\text{m}$. For *M. horridgei*, despite measurements having only been taken for two specimens, the total length is from 2.9 μm to more than 4 μm making it the longest viral

particle observed to date, with the latter size exceeding the average length of an *E. coli* cell by approximately two-fold (Figure 5). Figure 5 also shows comparison of the size of two giant viruses (Mimivirus and a medium-sized *Pithovirus sibericum*) with *K. casanovai* and *M. horridgei*. Moreover, *M. horridgei* is slightly longer than an exceptional specimen of the *Pithoviridae* giant virus family (a prolate spheroid up to 2.5 μm in length and 0.9 μm in diameter) that was previously the longest viral particle known [17]. In addition, pithoviruses range in width from ~ 0.6 to $\sim 0.9 \mu\text{m}$ showing that *Megaklothovirus* have not the largest volume known for a viral particle.

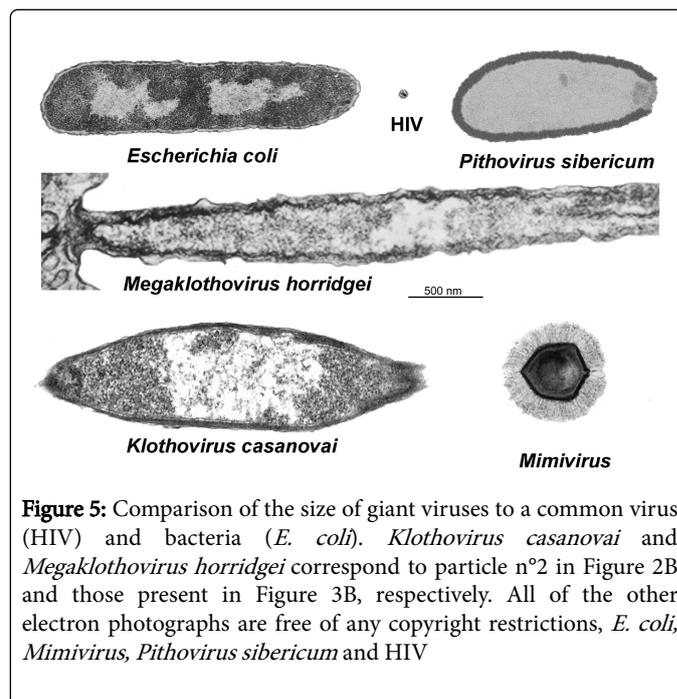


Figure 5: Comparison of the size of giant viruses to a common virus (HIV) and bacteria (*E. coli*). *Klothovirus casanovai* and *Megaklothovirus horridgei* correspond to particle n°2 in Figure 2B and those present in Figure 3B, respectively. All of the other electron photographs are free of any copyright restrictions, *E. coli*, *Mimivirus*, *Pithovirus sibericum* and HIV

Klothoviridae particles appear to consist of three enveloping structures surrounding the nucleoid. The first one forms a discrete layer that resembles the cytoplasmic membrane and it is covered by a tegument-like structure. Moreover, it is possible to observe another membrane similar to the cytoplasmic one surrounding parts of the surface of the particles, demonstrating that they are enveloped (e.g., see the extracellular part of the particle n°1 in Figures 1A-1C, 3B, 3C and 4). Generally, the internal membrane differs from the envelope with respect to content of structural proteins [18]; however, no difference is visible in the photographs. In *K. casanovai* particles, the envelope does not surround the entirety of the particle; at the apical pole a type of protein "paintbrush" is not covered. In the photographs, the basal end of the "paintbrush" cannot be differentiated from the shell. The "paintbrush" structure has never been observed in *M. horridgei*, but the apical part is only visible on three particles; two of which are probably degraded, and there is no guarantee that the cut was made at the end of the particles. The "paintbrush" or an analogous element was unknown to us in the world of giant viruses. However, some of giant viruses (e.g., Mollivirus, Pandoravirus and Pithovirus) do not have a total closure of their capsid-like structure [8]. *Klothoviridae* particles exhibit a very electron-dense tegument-like structure composed of at least two layers, but its thickness is difficult to measure due to the resolution of the photographs. The laminated aspect of the shell is easily visible in some parts of both *K. casanovai* and *M. horridgei* particles. Due to the substructure of the shell of

Klothoviridae particles, the word capsid will not be used and will be replaced by tegument-like structure or shell. However, the terms encapsidation and encapsidate will always be used because there is no synonym so precise. All known viruses have a capsid or a shell structure playing an equivalent role as for some giant viruses which is a protein coat that surrounds and protects their nucleic acid in the central core. A typical virus possesses a capsid composed of multiple copies of coat proteins arranged in a symmetrical fashion which shares one of two possible basic structures: icosahedral or helical, giving a pseudo-spheric or polyhedre, or rod-shape particle, respectively. All the non-icosahedral giant viruses isolated up to now appear to have tegument-like structure [19] composed of two or more layers [8]. Moreover, these enveloping structures do not enclose totally the nucleoid as for pandoraviruses and pithoviruses which have oval wall with an opening at one end or as *Mollivirus sibericum* which is spherical with an aperture [8]. Moreover, conventional transmission electron microscopy reveals that wall thickness is achieved in different ways in different lineages of giant viruses suggesting that they have evolved independently [20]. In addition, a large internal volume probably necessitates a particularly thick wall to maintain the structural integrity of particles [6,8].

In *Klothoviridae*, the internal membrane enclosing the nucleoid closely follows the internal volume of the shell, except for particles n°2 and n°3 in Figure 4; but this discrepancy could be due to an artefact generated during the fixation process. Furthermore, the internal membrane delimits a compartment that is mostly devoid of discernible substructures but does not appear homogeneous. Whereas some areas of the internal material are electron-dense, others are electron-lucid, and their distribution varies among the particles. Intracytoplasmic nucleoids of *M. horridgei* have a similar appearance, and this distribution is also observed in the bacterial cytoplasm (e.g., Figure 5). Electron-lucid regions may correspond to areas containing the genome. It is unclear how the genomes are packaged and organized within the diverse dsDNA giant virions [17]. However, the genome of *A. castellanii* lausannevirus, a giant virus of the *Marseilleviridae* family, encodes histone-like proteins, suggesting the presence of a DNA packaging mechanism that is similar to those observed in eukaryotes [21]. Similarly, the only retro-giant virus identified to date also encodes histone-like proteins [9]. Therefore, it cannot be ruled out that the nucleic material may be condensed in some areas of *Klothoviridae* particles. Moreover, the encapsidation process strongly suggests that associated proteins are packed in the core with the genome.

For *Klothoviridae* particles in the process of being formed, the part of the nucleoid still in the cytoplasm appears to be rich in granules that were previously interpreted as ribosomes [11], suggesting a very high rate of protein synthesis (particles n°1 and n°3 in Figures 1A and 1B, respectively). Moreover, "sandy" ribosome-like structures are also encapsidated in the particles, even though they are present at much lower numbers. This is an area of study worth investigating in the future, as only the genomes of organisms from the three domains of life (Archaea, Bacteria and Eukarya) are known to encode the constituents of ribosomes. Although ribosomal proteins of the host are packaged into *Mollivirus sibericum* virions [22] and some enveloped RNA viruses (e.g., Rift Valley fever virus (Phenuiviridae), mouse mammary tumor virus (Retroviridae) and arenaviruses) contain host ribosomes, but they cannot function within the virion [23-25]. All viruses identified to date lack the genes that encode ribosomal proteins and this criterion is currently used to exclude them from the living world [26].

Klothoviridae multiplication cycle

Most productive animal viral infections follow similar stages in their multiplication cycle: attachment to the cell surface, penetration, uncoating, replication, synthesis of transcripts and translation, assembly, and the release of progeny virions [27]. However, regarding *Klothoviridae*, electron microscopy analyses of host cells only revealed data concerning the intracytoplasmic nucleoids, particle assembly and budding from the cytoplasmic membrane. Thus, the description of the viral cycle will begin with these latter processes.

Self-assembly of viral particles and their release: Both in Figures 1A and 1B, there is one particle (n°1 and n°3, respectively) of which a part of the nucleoid that has not yet been encapsidated is in the cytoplasm. Inside the cells, these nucleoids are surrounded by two membranes that appear to be similar to the cytoplasmic or intracellular membranes. In addition, in the cytoplasm of cells of Figure 1, there are vesicles that have a similar appearance to the intracellular material of the particle n°1 and n°3 (Figures 1A and 1B, respectively), suggesting that it may be material of viral origin, especially as these vesicles were not observed in cells considered to be uninfected. Moreover, parts of the surrounding double membrane are visible at least for *K. casanovai* vesicles. In addition, electron photographs suggest a fusion between the cytoplasmic and external viral membranes, which would occur at the very beginning of the budding process. Thus, the morphogenesis of *Klothoviridae* particles appears to always be spatially separated from the cytoplasm by a "septum". This could help to maintain the integrity of the cell membrane when the mature virions separate completely from the cell. Furthermore, the presence of the "septum" at the base of virions egressing from the *S. cephaloptera* cells strongly indirectly suggests that the intracytoplasmic nucleoids of *M. horridgei* are also surrounded by two membranes. In the space separating the viral particle from cytoplasm, a very electron-dense ring-like structure is formed, that is very clearly visible in several photographs (Figures 1, 3B, 3C and 4). The cytoplasmic viral material enters in the particle through the lumen of the ring, from which the neo-synthesis of the layers of the shell would begin (particle n°1 in Figure 1A, and Figures 3B and 4). The tegument-like structure appears to grow by accretion of materials from the ring structure, but the origin of the constituent elements remains unknown. The latter could either originate from the cytoplasmic viral nucleoid or from the cytoplasm. Horridge and Boulton [12] mentioned that « below the junction of (viral particle) and cell there is frequently a cluster of circular bodies with dense granular contents enclosed in a single membrane (and) these bodies appear to contain material similar to that within the (viral particles) ». Even if we do not agree with the latter part of this comment, it could be possible that some of the material necessary for the syntheses of new particle layers is transported by this type of structure. However, these "vesicles" are only present in two photographs of *S. cephaloptera* (Figures 3B and 3D enlarged in 4). Their presence could be artifactual, since they are not present in photographs of *P. gotoi*. However, clusters of vesicles in close proximity to the "viral factory" have been observed at the onset of capsid assembly for Mimivirus [28]. Viral factories are perinuclear or cytoplasmic structures where viral replication and assembly take place and are functional dynamic structures that occupy large areas of infected cells [29].

In *Klothoviridae*, during encapsidation, the intracytoplasmic viral material appears to replenish the available space as the internal volume of the shell expands and always remains surrounded by the most internal membrane (particles n°1 and n°3 in Figures 1A and 1B, respectively) whereas the second membrane remaining in the cell

forms the "septum". The volume of viral particles may depend on the amount of material to be packaged suggesting that the internal volume could be adaptable. The apical portion of *K. casanovai* viral particles exhibit a "paintbrush-like" structure that does not appear on *M. horridgei*, suggesting that for these latter viruses, envelope surrounds the entirety of the particle. Moreover, for this last species, the assembly of shell proteins would be around a pre-existing spindle shape structure. For two membranes, there is continuity between the part belonging to the extracellular particle and the one that is not, which concerns the internal membrane (surrounding the nucleoid) and the outermost layer, i.e., the cytoplasmic membrane becoming the envelope during budding (Figure 3C).

The photographs of *P. gotoi* infection make it possible to follow the sequence of events concerning the release of the particles, starting with particles n°1 and n°3 (Figures 1A and 1B, respectively), followed by n°3 and n°1 (Figures 1A and 1B, respectively) and finally particle n°2, which is newly separated from the protuberance n°4 (Figure 1B).

The becoming of the ring after the release of the particles remains an unanswered question. In the photographs, the ring is never visible where the basal part of the free particles is relatively clean. Thus, it is certainly not properly packaged and therefore cannot be used as a type of pore that allows the release of the nucleoid into the cytoplasm after infection. Very electron-dense material is present at the apical end of the protuberances in Figure 1B (n°4 and especially in n°5) and in Figure 1D (n°2), which could be viral remains after the release of viral particles. The various photographs allow the following sequence to be proposed. When the viral particle is detached, the ring remains bound to the cell but is separated from the cytosol by the "septum" (n°2 for Figure 1D). Moreover, because the apical pole end of the protuberance is not surrounded by a membrane, this part is open to the external environment (n°2 of Figure 1D). As a result, the material of viral origin which is external to the cell spreads in the sea water (n°4 and 5 in Figure 2B). Subsequently, electron-dense material is no longer observed at the apical pole of the protuberances where the septum, which is fused with the cytoplasmic membrane, ensures the continuity of the membrane (n°4 of Figure 1A).

In summary, because the particles are formed by self-assembly, they are viral entities [30]. Members of *Klothoviridae* family have the following distinctive combination of morphological attributes:

1. The particles are very long.
2. They have a spindle shape.
3. Three "enveloping structures" surround the nucleoid in the particles: a likely bilayer lipid membrane, a tegument-like structure certainly comprised of proteins and an outer envelope.
4. For the electron-dense ring occurs the self-assembly of the tegument-like structure.
5. In the cytoplasm, viral nucleic acids and other material are surrounded by a double membrane forming large vesicles.

Some hypotheses concerning the first steps of Klothoviridae multiplication cycle: The cytoplasm and surfaces of apparently normal and infected cells have been examined for the possible presence of early stages of infection but without success. Therefore, for these steps of the multiplication cycle that generally corresponds for viruses infecting animals to attachment, penetration, uncoating, genome replication, syntheses of transcripts and translation, only some hypotheses can be issued from the physical properties of *Klothoviridae*

and from prior knowledge concerning other viruses. After binding of viral anti-receptors to cell-surface receptors, i.e., viral attachment, both naked and enveloped animal viruses are internalized through a variety of endocytic processes, including macropinocytosis [31]. However, for numerous enveloped viruses, another mechanism of penetration is used, where the external viral membrane fuses with the host cell membrane (viral fusion), allowing the nucleocapsid to be delivered directly into the cytoplasm [32]. As Meelsvirus is an enveloped virus, it can use this last penetration mechanism [6]; and this mechanism could also be the most likely hypothesis for *Klothoviridae*, even if an endocytic process cannot be rejected.

Nonetheless, because a part of *K. casanovai* particles are not totally enveloped (due to the "paintbrush"), and similar to *M. horridgei*, they have one internal membrane, alternative hypotheses can be proposed. Even if the discovery of giant viruses has changed our view on the viral world, viruses have long been considered "optimized" selfish biological objects that hijack the host machinery to make more particles. Nevertheless, without being Panglossian, it can be assumed that the presence of a cytoplasmic- or intracellular-like membrane in the viral particles, and possibly that of the "paintbrush", must play essential roles in *K. casanovai* multiplication cycle.

One or more internal membranes are present in virions, that infect archaea, bacteria or eukaryotes, including several giant virus species [8,33]. For example, some bacteriophages exhibit, under the capsid, an internal lipid membrane surrounding the viral genome [34]. Among these bacteriophages, the internal membrane of PRD1 and Bam35 (belonging to the *Tectiviridae* family) allows them to infect Gram-negative or Gram-positive bacteria, respectively, through a fusion process involving their outer or inner membrane [35]. Moreover, Plasmavirus bacteriophages can induce non-cytocidal infection because of their internal membrane, where progeny phages are released by a budding process from the host mycoplasma membrane, with the host surviving as a lysogen [36].

Nucleo-Cytoplasmic Large DNA Viruses (NCLDV) are a group of virus families exhibiting a core containing a large dsDNA genome and that have at least a cytoplasmic stage in their replication cycle. To date, NCLDV include members of the taxa *Ascoviridae*, *Asfarviridae*, *Iridoviridae*, *Phycodnaviridae*, *Poxviridae*, *Marseilleviridae*, *Mimiviridae*, *Mollivirus*, *Pandoraviridae* and *Pithovirus* [37]. Apparently, all the members of NCLDV have a core surrounded by at least one lipid membrane that underlies a capsid or shell structure [8,19,38]. Interestingly, *Paramecium bursaria chlorella virus* (PBCV-1), a member of the *Phycodnaviridae* family known to infect eukaryotic algae, has a bacteriophage-like replication cycle; indeed, in its early infection stages PBCV-1 first creates a hole in the host cell wall, then a tunnel is generated by the fusion of the viral internal membrane with the host membrane, and the viral genome is subsequently ejected into the cytoplasm of the host cell, leaving an empty capsid on the cell surface [39].

Apparently, all of the largest viruses with dsDNA genomes have at least one internal lipid membrane (although, the number of membranes inside some giant viral particles is still debated and under investigation) that surrounds the genomic material and interacts with the capsid (or shell) proteins [40]. For example, this is the case for *Mimivirus*, *Mollivirus*, *Pandoravirus*, *Pithovirus*, and *Cedratvirus* (a possible new genus in the putative *Pithoviridae* family), all of which infect amoebae. The origin of the internal membrane(s) is unknown; however, it was hypothesized, at least for mimiviruses that it would originate from host-derived membrane vesicles [28]. After penetrating

amoebae by phagocytosis, Mimivirus, Mollivirus, Pandoravirus and Pithovirus use the same mechanism to reach the cytoplasm. Following the opening of their delivery portal, the internal membrane unfolds, protrudes and then fuses with that of the vacuole membrane. The nucleo-core containing the viral dsDNA and associated proteins is released through the created channel into the cytoplasm, and the replication cycle can begin [8,41]. For most of these viruses, a single portal is used for genome packaging and delivery but for mimiviruses genome, delivery and packaging occur through two distinct portals [8,38]. In addition, a portal-like structure may exist at the end of the capsid bordering the tail in Meelsvirus virions [6].

Viral particles infecting eukaryotes with shapes resembling those of *Klothoviridae* have been unsuccessfully investigated. With respect to viruses having prokaryotes as hosts, spindle-shaped virion morphology is very common among archaeal viruses that infect a wide range of hosts frequently living in extreme aquatic environments [42]. Among them, Fuselloviridae are the most abundant archaea-specific group of viruses [43]. These viruses are enveloped, pleomorphic in shape, assuming shapes ranging from thin-cigar-like to pear- or lemon-like, with short tail fibers attached to one pole [44]. Among them, Sulfolobus spindle-shaped virus 6 (SSV6), whose length range is 100-250 μm [45], has a morphology very similar to viruses of *Klothoviridae* family. Moreover, a study of the morphogenesis of SSV1 showed that assembly and egress are concomitant and occur at the cellular cytoplasmic membrane *via* a process that is highly reminiscent of the budding of enveloped viruses infecting animals [43]. Electron photographs show extracellular particles still attached to the cell, similar to what is observed for *Klothoviridae* but on a 20 or more-fold smaller. Moreover, SSV1 virions contain a single or two electron-dense ring-like structures resembling the budding necks observed prior to the membrane scission during the egress of several enveloped viruses of eukaryotes [43]. In addition, due to the budding process, which is unusual for a prokaryote virus, the host cells are not killed, with their growth only being slowed down [43]. Otherwise, archaeal viruses with a tail have been speculated to possibly directly inject their dsDNA into host cells, similarly to head-tailed bacteriophages [46].

For conventional viral capsids (i.e., with helical or icosahedral symmetry), this is the part already built that determines the area where the future self-assembly will take place, and this type of structure is generally very rigid and constrained. For giant viruses that have non-icosahedral shell as and Mollivirus, Pandoravirus and Pithovirus, the assembly is somewhat similar to the model previously cited [8]. The capsid-like structures of Meelsvirus are oval rather than pseudo-icosahedral shaped and this structure appears to be rigid, without conformational changes occurring in the parts already built [6]. In contrast, for *Klothoviridae*, the diameter of the ring from which the tegument-like structure is assembled does not appear to vary much and would most often be much smaller than those of the associated zone in the mature viral particle. This could suggest that the tegument-like structure of *Klothoviridae* would be flexible, at least during the first stages of particle synthesis. For example, particle n°1 in Figure 1A has only approximately half the expected length for a mature particle. This indicates that the assembled material has a diameter that is approximately three-fold smaller than that observed in the middle of a mature particle. This scenario could be an argument against the synthesis of a true capsid, because protein sub-units could not generally change shape after their assembly. This flexibility could be a characteristic of an unconventional outer shell as suggested by studies of other giant viruses. Indeed, *Cedratvirus getuliensis* and *Pithovirus sibericum* particles, which have non-icosahedral capsid-like structures,

exhibit significant variations in size due to the plasticity of their outer shell [17,41]. Moreover, during the formation of the Pithovirus virions, which also exhibit a tegument-like shell, the particles are first built as cylinders, with their shape subsequently modified into their mature form before their release after amoeba cell lysis [20]. Similarly, mollivirus particles can lose their spherical morphologies when in vacuoles [22]. However, it would be premature to conclude that the outer shell of *Klothoviridae* does not consist of true capsid proteins. Indeed, herpesvirus procapsids undergo maturation, and similar to phage capsids, their shape changes from round to polyhedral, although the procapsids stay essentially the same size [47]. However, it is interesting to note that these enveloped viruses have an icosahedral capsid surrounded by a tegument-like structure [48]. Nevertheless, influenza viral particles, which do not have this latter structure, exhibit a high degree of structural variability [49]. In addition, SSV1 archeal virions undergo a possible shape transition [43] and unconventional capsid protein assembly [44].

Klothoviridae could combine the two advantages of possessing an internal membrane and an envelope, although it should also be noted that other viruses have both of these attributes but are not giant viruses (e.g., poxviruses). As shown for several giant viruses, the internal membrane allows fusion with various types of intracellular membranes to always isolate the nucleoid or viral factories from the cytosol [8]; although similar to the envelope, this membrane can also fuse with cytoplasmic membrane (as for PBCV-1, even if it rarely does so [39]). In the cytoplasm, the second membrane could be acquired when the nucleoid is integrated into a vesicle. Otherwise, naked viruses are more resistant to desiccation, heat, and chemical agents than enveloped viruses, but these disadvantages for the latter group become most of the time lapsed in marine environment. Although the release of newly formed naked viral particles frequently requires the lysis of the cell membrane, for *Cedratvirus getuliensis*, some particles are also released by exocytosis [41]. Viral envelope confers many advantages to viral survival, among others, many enveloped viruses, can be produced during an extended period by budding without host cell dying. It has been assumed that viral envelope may be an adaptation to animal cells [35], whereas, the internal lipid membranes could be likely primarily be an adaptation to prokaryotic and unicellular eukaryotic infections.

In *Klothoviridae*, the "nucleoid vesicle" is loaded at the basal level of the viral particle being formed, whereas only in *K. casanovai*, the "paintbrush" forms at the opposite side. In the first steps of the infection cycle, the "paintbrush" could act as a genome-delivery portal. Moreover, enveloped viruses infecting archaea (Fusellovirus) [46] and an unenveloped phycodnavirus [39] eject their genome into their host through the cell wall, with the former virions exhibit a small tail that is not covered by the envelope and the latter virions belong to NCLDV, suggesting that the physiological role of the protein-like "paintbrush" of *K. casanovai* deserves to be studied.

Genome replication, synthesis of transcripts and translation: To the best of our knowledge, only one retro-giant virus has been discovered to date [9], and more likely members of *Klothoviridae* family have a DNA rather than an RNA genome; however, this requires confirmation. Among giant viruses, there is no strict correlation between their particle sizes and genome sizes [17], but the very large particle size of *Klothoviridae* could be associated with a large genome size. Future studies of *Klothoviridae* genomes are expected with great interest.

The replication scheme of most common giant viruses has begun to be well understood. Some of them are purely cytoplasmic viruses,

whereas others are nucleocytoplasmic, with their virions undergoing self-assembly in the host cell cytoplasm [8,41]. Proteomic analyses generally allow for discrimination between the two types [50]. For example, the transcription of Mimivirus and Pithovirus genomes occurs exclusively in the cytoplasm of amoebae, whereas in Mollivirus and Pandoravirus transcription begins with a nuclear phase in which early genes are transcribed by the cellular machinery [8]. Moreover, some very large viruses are classified as nuclear viruses because almost all of the viral events occur in the nucleus, e.g., the chaetognath-associated Meelsvirus [6] or Whispovirus, which infects aquatic crustaceans [51]. Concerning *Klothoviridae*, because the appearance and size of the nuclei of the infected epithelial cells are similar to those apparently devoid of viruses, and "nucleoid vesicles" are only observed in the cytoplasm, these observations strongly suggest that replication is cytoplasmically located. In cells infected by *Klothoviridae*, several vesicles that appear to be of viral origin can be observed, but there are differences according to the virus species. In cells infected by *K. casanovai*, vesicles with characteristics similar to the cytoplasmic part of the nucleoids being encapsidated can be observed. These vesicles have no precise shape and are surrounded by two membranes. In addition, some of these vesicles appear to bud to yield intracytoplasmic nucleoids ready to be encapsidated. Similarly, viral particles of the retro-giant virus isolated from human T cells are "budding" around a much larger giant particle [9]. *Klothovirus* vesicles of large sizes may correspond to viral factories that must enclose one or more viral genome (s), many proteins, transcripts and ribosomes. The mechanism is in part different for *M. horridgei*, the intracytoplasmic viral nucleoids have a spindle-shape suggesting that they have some rigidity and the internal material has an appearance similar to that observed in viral particles with electron-dense and electron-lucid areas. Moreover, new spindle vesicles could appear by a binary fission process (scissiparity), although this requires confirmation. Intracytoplasmic spindle shaped structures would constitute viral factories that could produce the nucleoids to be encapsidated.

Infection route, cell tropism and pathogenicity

Klothoviridae infectious route: Similar to Shinn and Bullard's assumptions concerning Meelsvirus [6], two different (non-exclusive) modes of *Klothoviridae* transmission can be proposed. First, the virions may be scattered in the environment and have a relatively low probability of encountering chaetognaths, with this mode principally depending on the concentration and "half-life" of free viral particles in seawater and of the number of potential hosts. Alternatively, the transmission may preferentially occur during mating. Although chaetognaths are hermaphrodites, cross-fertilization is typical and obligatory [52]. Therefore, the comparison of infection rates between immature and adult chaetognaths would be very informative. Moreover, *Klothoviridae* virions infect adhesive cells of the distal part of the limb-like appendages of adult *P. gotoi* and of the mid ventral region of, at least, Mediterranean *S. cephaloptera*. Young specimens of these two species are fixed to the substrate by cephalic adhesive papillae [16,53]. However, "bacteria-like" or "virus-like" structures have never been reported in this latter region to date.

Cell tropism and cytopathology: The meelsviruses infect surface gland cells of the host's stratified squamous epidermis of the host *Adhesisagitta hispida* [6]. Similarly, the host cells of *Klothoviridae* belong to the epithelium of the body of animals. However, differences between species and with respect to their origin have been observed. In Horridge and Boulton's photographs [12], only some cells of the outermost layer of epithelium of *S. cephaloptera* collected in the

southern part of the Channel are shown and these cells apparently have no secretory activity, although this could be due to the infection. According to one photograph of Mediterranean *S. cephaloptera*, *M. horridgei* can infect the cells of the first two outer layers of epithelium of the body. The thin sections were made at the level of the adhesive papillae, but seemingly uninfected cells have no secretory activity. In Figure 2A, a single cell is apparently infected and those around it do not contain secretion granules, which are only visible in the cell located at the bottom right of the photograph. In Figure 2B, except for one cell, those exhibiting intracytoplasmic nucleoids do not contain secretion granules, while two neighboring cells exhibit them, but one also contains an intracytoplasmic nucleoid. *Klothoviruses* infect *P. gotoi*, but only the distal cells of the epidermis of the limb-like appendages, and only the part in contact with the substrate is affected. In *P. gotoi*, the host cells are highly polarized and have an intense secretory activity probably associated with adhesion [11].

Because viral budding only occurs at the apex of the cells, there could be a link between the secretory capacities of cells and cell tropism. However, the specificities of the apical plasma membrane could also play an important role as suggested by observations of *M. horridgei*. In the three laboratories of the authors of the 2003 article [11], studies have been carried out for decades on both the external and internal structures of various species of chaetognaths, and in all the other tissues analyzed (e.g., muscles, gut, nervous system and gonads), no recognizable infected cells have been observed.

The results of the study by Horridge et al. [12] suggest that the level of contaminated cells in *S. cephaloptera* would be significant. In the same species but in specimens collected from the Mediterranean Sea, despite the large number of photographs taken, only one individual exhibited recognizably infected cells. With respect to viruses infecting *P. gotoi*, even if it concerns only a much localized area, all the secretory cells are apparently contaminated. Furthermore, meelsviruses are likely very cytotoxic [6], which is probably not the case for *Klothoviridae* virions released by budding from the cytoplasmic membrane. The infected cells of Mediterranean *S. cephaloptera* maintain an appearance that is similar to that of uninfected cells, and the size and shape of the nucleus remains similar. Moreover, the infected cells of *P. gotoi* continue to produce large quantities of secretory granules, suggesting that at least this function is not disrupted. Especially for *P. gotoi*, the cells appear to tolerate the infection well. The viral and cellular genomes would share the potential for cell synthesis, and the two metabolisms, cellular and viral, coexist according to an acceptable "compromise". Presumably, the host and the virus have been associated for a long time and have evolved a *modus vivendi*.

Concerning Meelsvirus, Shinn et al. [6] mentioned that only two of the several dozen apparently healthy adult chaetognaths observed were determined to be infected (one was collected in 1986 and the other in 1988). However, they noted that individuals that appeared unhealthy were discarded. Moreover, in the wild, predation may also be preferential on infected individuals. As Horridge et al. [12] have confused bristles and viruses, their study would suggest that all of the *S. cephaloptera* species specimens were infected. However, the lack of precise information in this article should make be approached with cautious. Concerning the Mediterranean *S. cephaloptera*, only one infected individual has been identified. In contrast, in all the *P. gotoi* specimens studied (a dozen); all of the more distal cells of the limb-like appendages in contact with the substrate visible in the photographs are infected.

Pathogenicity of giant viruses: Viruses are obligatory parasites that can only multiply within appropriate living cells and can likely infect all types of "true living organisms". In the oceans, viral lytic infection leads to the lysis of a very large number of bacteria and phytoplankton, converting them into newly produced viruses and cellular debris. Giant viruses have also been shown to be associated with important ecological processes, including the collapse of algal blooms [54]. Moreover, released dissolved and particulate organic matter from lysed cells could constitute the major source of food for chaetognaths explaining part of their success in both number and biomass [55].

Metagenomic approaches have allowed the global distribution and diversity of giant viruses in environments and organisms to be demonstrated [56,57], but few giant viruses are known to infect metazoans to date, as they primarily have unicellular eukaryotes for hosts. However, these viruses have often been isolated through strategies involving the use of cultures of protists, which introduces considerable bias. Giant viruses have been isolated from multicellular organisms such as humans and leeches [58,59] but there is no evidence that cells of these metazoans can be infected. Moreover, the link between dsDNA giant viruses and human specific diseases remains very elusive [60-62]. However, *Acanthocystis turfacea* chlorella virus 1 (*Phycodnaviridae*), which is known to naturally infect green algae, has been identified in human oropharyngeal samples, can replicate in human macrophages and is associated with *impaired cognitive* capabilities in humans and mice, probably *via* the induction of inflammatory factors [63,64]. Because the notion of large viruses

remains rather vague, in Table 1 it was chosen to list only viruses whose particle size is at least approximately 350 nm (see for this criterion [8] regardless of their genome sizes especially since they are not always known. Moreover, it does not take into account the giant viruses infecting unicellular eukaryotes and the filamentous viruses. The only retro-giant virus known to date has a very large genome (~1861 ORFs) which contains viral oncogenes and therefore, it could induce diseases in humans [9]. Despite their particle sizes, sturgeon nucleocytoplasmic large DNA viruses have been included in Table 1 because phylogenetical analyses strongly suggest that they belong to the *Mimiviridae* family [65]. The list of viruses in Table 1 is not meant to be exhaustive, but it should be noted that within the large viruses, known to date, infecting metazoans, a great disparity has been observed regarding the viral characteristics (particle shapes, replication sites and structures of the genome) and range of host animals and cell types [6,9,51,65-70]. Except for the viruses infecting chaetognaths, the particle sizes are quite small; generally well below 500 nm and only *Klothoviridae* far exceed the micrometer size. Genome sizes, when they are known, are relatively low (always less than 380 kbp). Almost all the virions mentioned in Table 1 have a viral envelope which, as already mentioned, may be an adaptation to animal cells [35]. Moreover, it must be noted that the hosts are principally invertebrates, and viruses infecting organisms living in aquatic environments are well represented despite there being less data on these ecosystems than on terrestrial environments.

Virus	N.A.	C/L	Gen. size range	Particle shape	Particle size range in nm	Env.	Replication site	Hosts	Cell and tissue tropism	Reference
Retro-giant virus	R	n.d.	n.d.	Spherical	~400 Ø	+	n.d.	Humans	T cells	[9]
<i>Ascoviridae</i>	D	C	150-190	Ovoidal, bacilliform or allantoid	~130 Ø 200-400 L	+	NC	Lepidoptera	Most host tissues or fat body	[68,70]
<i>Hytrosaviridae</i>	D	C	120-190	Rod-shaped	50-100 Ø 500-1000 L	+	N	Diptera	Secretory epithelial cells of the salivary glands	[66]
<i>Iridoviridae</i>	D	L	150-303	Icosahedral	120-350 Ø	+ or -	NC	Invertebrates, amphibians, reptiles, fishes	Most host tissues	[67]
<i>Poxviridae</i>	D	L	130-375	Brickshaped or ovoidal	220-450 L 140-260 W* 160-190 Ø 250-300 L	+	C	Vertebrata, insects	Diverse cell types and tissues	[67,70]
Sturgeon NCLDVs	D	n.d.	n.d.	Icosahedral	242-262 Ø	n.d.	NC	Acipenseridae	Integumentary system	[65,69]
<i>Whispovirus</i>	D	C	~300	Ovoidal to bacilliform	65-70 Ø 300-350 L	+	N	Aquatic crustaceans	Tissues of ectodermal and mesodermal embryonic origin	[51,67]
<i>Klothoviridae</i>	n.d.	n.d.	n.d.	Fusiform	2500-	+	C	Chaetognatha	Secretory or not epithelial cells of the body	This study

<i>Meelsvirus</i>	n.d.	n.d.	n.d.	Persian meels	>4000 L	+	N	Chaetognatha	Secretory epithelial cells of the body	[6]
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Table 1: Characteristics of large viruses infecting metazoans. Abbreviations and symbols: N.A. for nucleic acid; R or D for nature of the nucleic acid, RNA or DNA, respectively; C/L for structure of the nucleic acid, C or L for circular or linear, respectively; Gen. size range for genome size range in kbp; Particle size range Ø, L or W for diameter, length or width; * sizes only for brick-shaped particles; Env. for envelope; Replication site NC, N or C for nucleocytoplasmic, nuclear and cytoplasmic, respectively; n.d. for not determined; Ref. for references.

Conclusion

Serendipity, an unplanned and fortunate discovery has often played a crucial role in science. It was assumed that the majority of the most important and revolutionary discoveries in biology and medicine have a serendipitous element in them [71]. Moreover, the notion of serendipity is intrinsically related to the history of giant viruses from the identification of Mimivirus to those of chaetognath viruses [6]. Among all the many other examples, it could be quoted that a Pithovirus-like virus was first spotted in *Acanthamoeba* more than 15 years ago, but misinterpreted as an archaeal endocytobiont [14,15]. However, from the early 1970's through the 1990's, "large particles" found in or around various types of supposed hosts have been photographed and it was assumed that they could be viruses [72,73].

This study also highlights the importance to publish works with amazing results or difficult interpretations. In 1967, Horridge et al. [12], despite their misinterpretation, probably took the first known photographs of a giant virus. In addition, the first photographs of viruses infecting *P. gatoi* date from 1994 but some of them were only published in 2003 [11]. This also shows the interest of keeping archives of the scientific works of our "elders" who often unfortunately end up in garbage cans.

The discovery of giant virus has generated stimulating debates and thoughts in the scientific, and even philosophical, community. Authors have hypothesized that giant viruses might represent a distinct fourth domain of life [74,75], but the latter may be artifactual, due to compositional heterogeneity and homoplasmy, and horizontal gene transfers with their eukaryotic hosts; so, this proposition is very criticized [76]. Moreover, large acellular entities, with genome and physical size comparable to those of cellular organisms strengthen some arguments to redefine the life concept [77]. Indeed, this opens new paths of philosophical reflections where "life" would not necessarily be the antonym of "death" and where "life" and "living" would designate concepts that overlap only partially. The viruses *sensu lato*, do not have independent life; they express their "life", the controlled reproduction of oneself, solely by the use of a foreign cellular apparatus belonging to the living organisms (Archaea, Bacteria or Eukarya). However, this characteristic is also shared by strict intracellular parasitic bacteria, but unlike the latter, the viruses do not have their own metabolism. Moreover, virions (i.e., free virus particles) are inert in all respects unless they encounter a suitable host cell. Furthermore, even if they are only analogies, giant virions with an internal membrane and a tegument-like structure and those having in addition an envelope exhibit enveloping structures which have similarities with those of Gram-positive and Gram-negative bacteria, respectively.

Main directions for future research can be proposed, this includes, sequencing of viral genomes and evolutionary relationship of *Klothoviridae* to other viruses, determination of the detailed structure of viral particles and understanding of the mechanism of infection of

new hosts. Moreover, several lines of evidence suggest that *Klothoviridae* could have an unusual mode of multiplication. Besides, the determination of the nature of ribosome-like structures observed in particles constitutes also very an exciting challenge. To date, members of *Klothoviridae* family have been found in two species belonging to two closely related genera (*Spadella* Langerhans, 1880 and *Paraspadella* von Salvini-Plawen, 1986). The search of this type of viruses in members of various families of the two current orders of chaetognaths (*Phragmophora* and *Aphragmophora*) could possibly provide information on the antiquity of these viruses; moreover, it should be kept in mind that the phylum Chaetognatha appeared in the Precambrian; so, this could provide useful information to explore the origin of giant viruses. Studies of these latter infecting metazoans will surely bring new exciting challenges for the whole scientific community and will give answers concerning their degree of pathogenicity.

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Potential Conflicts of Interest

The authors have declared that no competing interests exist.

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Contribution

E. Faure was responsible for the study conception. E. Faure wrote the original draft. R. Barthélémy contributed to the design of figures. All the authors critically reviewed and edited drafts. All the authors have read and approved the final manuscript.

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