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Looking at protists as a source of pathogenic viruses

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ABSTRACT

In the environment, protozoa are predators of bacteria and feed on them. The possibility that some protozoa could be a source of human pathogens is consistent with the discovery that free-living amoebae were the reservoir of *Legionella pneumophila*, the agent of Legionnaires' disease. Later, while searching for *Legionella* in the environment using amoeba co-culture, the first giant virus, *Acanthamoeba polyphaga mimivirus,* was discovered. Since then, many other giant viruses have been isolated, including *Marseilleviridae*, *Pithovirus sibericum, Cafeteria roenbergensis virus* and *Pandoravirus* spp. The methods used to isolate all of these viruses are herein reviewed. By analogy to *Legionella*, it was originally suspected that these viruses could be human pathogens. After showing by indirect evidence, such as sero-epidemiologic studies, that it was possible for these viruses to be human pathogens, the recent isolation of some of these viruses (belonging to the *Mimiviridae* and *Marseilleviridae* families) in humans in the context of pathologic conditions shows that they are opportunistic human pathogens in some instances.

1. Introduction

In the environment, protozoa are predators of bacteria and feed on them. The most studied of these protists as potential vectors of pathogens are free-living amoebae. Free-living amoebae (FLA) are ubiquitous unicellular eukaryotes that have been isolated worldwide from soil, water, air and even vertebrates. In the environment, they colonize natural biofilms, grazing on them to search for bacteria, their major sources of nutrients. In fact, free-living amoebae are natural phagocytes that feed on large particles (>0.5 μm) in the extracellular environment, independently of a recognition system and based only on particle size [1]. They supposedly do not feed on smaller particles that are able to pass through a 0.20 μm filter [2]; however, as some may phagocytose small viruses (<0.2 μm), it is possible that this phagocytosis may be mediated by specific recognition in some instances.

Historically, the search for pathogens in protozoa followed the discovery of *Legionella pneumophila*, the agent of Legionnaires' disease in humans. After that, TJ Rowbotham used FLA as a support to isolate *Legionella* spp. from the environment, especially cooling towers, the most common environmental reservoir of *Legionella* at the origin of epidemics. Using the procedure developed for this purpose, TJ Rowbotham and others could isolate *Legionella* from infected human samples, including sputum and stool [3,4]. In addition to *Legionella*, several bacterial species were also isolated and named amoeba-resisting microorganisms (ARMs). These ARMs consist mainly of bacteria that belong to various phylogenetic clades dispersed throughout the prokaryotic tree [5], and among these facultative and obligate intracellular species, some are human pathogens. However, while searching for ARMs, a giant viruses was discovered, resembling bacteria in its size. This first one was *Acanthamoeba polyphaga mimivirus*. After an inaugural work on the survival of *Coxiella burnetii* in *Acanthamoeba castellanii* [6], our interest in amoeba-associated organisms continued when Richard J. Birtles took up a post-doctoral fellowship in the laboratory, bringing along a collection of obligate intra-amoebal bacterial parasites. The parasites were recovered by Dr Tim Rowbotham over a period of nearly twenty years from environmental water samples collected as part of the Legionnaires' disease outbreak investigations by his employer, the Public Health Laboratory Service of England and Wales. Most of these bacteria were referred to as "*Legionella*-like amoebal pathogens" (LLAPs) [7]. In addition to cultures of LLAPs were two cultures of apparently Gram-positive coccoid bacteria, referred to as "the Bradford coccus" and "Hall's coccus", the latter of which had been sent to Dr Rowbotham by a

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colleague in the USA and later identified as a new genus, *Parachlamydia acanthamoeba* [8].

However, all of the attempts to amplify 16S rDNA from the Bradford coccus failed despite the use of PCR assays that incorporated different sets of "universal" pan-bacteria primers. To search for a particular cell wall structure that could explain the inefficiency of our DNA extraction protocols, we decided to examine the ultrastructure of the Bradford coccus by electron microscopy [9]. To our great surprise, we observed icosahedral particles resembling giant Iridoviruses within infected amoebae. The suspicion of the viral origin of the Bradford coccus was confirmed by further preliminary work, in which we showed that it contained a large double-stranded DNA chromosome coding for typical viral genes and underwent an eclipse-phase replication typical of viruses. Furthermore, we now know that the assembly of virus particles takes place in specific intracellular locations, which have been termed "virus factories" when previously observed for viruses, including Iridoviruses [10]. A particle diameter of 600 nm and a genome size of 1.2 Mb made this virus the largest known ever and the first member of the giant viruses, later called giruses. Phylogeny indicated that it groups with other nucleocytoplasmic large DNA viruses (NCLDVs) including the Iridoviridae, Baculoviridae, Phycodnaviridae and Poxviridae. After this seminal work and the incidental isolation of Mimivirus, the search for viruses of protists began, a strategy developed by our laboratory that was then followed by several other laboratories across the world.

In all of those cases, protist-associated viruses have been isolated according to two different strategies. The most commonly used strategy is to reproduce the isolation of Mimivirus by inoculating samples on axenic FLA using amoebae as a support for culture, exactly as is done with the culture of intracellular bacteria with cell lines. In that case, samples (human or environmental) are inoculated on an amoebal monolayer cultivated in buffer with antibiotics that prevent the multiplication of bacteria, and the cultures are examined to detect amoeba lysis, indicative of an amoeba pathogen that can potentially be a giant virus. In such cases, amoebae from the genus *Acanthamoeba*, *A. polyphaga* and *A. castellanii* have been primarily used, but recently, other protozoa such as *Harmanella vermiformis* have been tentatively used (unpublished data). The second strategy consists of isolating protists and then searching for the presence of giant viruses developing in them. This strategy allows the isolation of unique species of giant viruses although it is less commonly used. These different strategies are analyzed here with a specific view on isolates from humans.

2. Isolation of giant viruses using amoeba co-cultures

The first isolate was *Acanthmoeba polyphaga mimivirus,* and it was isolated by the original method described by TJ Rowbotham to isolate Legionella sp [4]. In this method, isolation was performed on *A. polyphaga* strain AP L1501/3A. This strain was chosen because it could be produced axenically in PYG medium and because it is a slow-growing amoeba. The speed of growth is important because the relative number of *Legionella* and amoebae is critical to observe *Legionella* growth. If there are too many amoebae, they can encyst before their lysis due to *Legionella* growth is detected, and if there are too few amoebae, the growth of *Legionella* may be missed. The strain *A. polyphaga* (Linc-AP1) we used for propagating Mimivirus and for isolating giant viruses was provided by TJ Rowbotham to us twenty years ago, and it is thought to be comparable to strain AP L1501/3A. We replaced our co-culture on shell vials with a 6- to 12-well microplate system, allowing us to test more samples at the same time [11–13]. Due to the massive multiplication of Mimiviruses and Marseilleviruses, we do not believe that this relative amount of viruses to amoeba is critical to isolate Mimiviruses. However, we showed that the multiplication of APMV is dependent on the virus/amoeba ratio [14]. Our study suggested that a low virus/amoeba ratio was more efficient for the production of infectious particles; at a virus/amoeba ratio (virus in TCID50) of a factor of 0.01, Mimivirus was more productive than at any other ratio. However, we believe that in natural conditions, this ratio is smaller. Therefore, in our recent procedures, we performed two blind subcultures on the amoebae before searching for amoeba lysis [13]. In our first studies searching for amoeba pathogens, after inoculating hundreds of environmental samples without a mix of antibiotics containing vancomycin and colistin, we isolated almost exclusively bacteria [15,16]. We isolated few giant viruses, including the second Mimivirus isolate shown to be infected by a virophage, which we named Mamavirus, and the first Marseillevirus isolate [17,18]. Our colleague G. Greub, who was searching mostly for Parachlamydia, had the same experience and isolated a unique giant virus, which represents of a new lineage of Marseillevirus he named Lausanne virus [19].

In order to increase our chances of isolating viruses, we modified our culture strategy by isolating and testing the antibiotic susceptibility of the contaminating bacteria [20]. This allowed us to isolate 19 giant viruses from 105 environmental samples, including Marseilleviruses and Mimiviruses, for an isolates/samples ratio never obtained at such a level since then. This strategy allowed us, for example, to identify the presence of different lineages within Mimiviruses, A, B and C, with one of these lineages corresponding to a Mimivirus later named Megavirus [21]. This strategy also allowed us to isolate a second virophage, which was later demonstrated to be integrated in the Mimivirus genome and which would be named later as a provirophage [22]. The major difficulty in obtaining this high isolates/samples ratio was clearly the fine detail of the procedure. To avoid the meticulous observation of microplates searching for amoeba lysis, we added a blind enrichment and searched for lysis on agar plates [23]. After 3 days of culture in amoeba without antibiotics, the supernatant was subcultured to a microplate with antibiotics. Then, after 2 days, the supernatant was inoculated as a drop on an agar plate seeded with a monolayer of *A. polyphaga.* The virus was detected by observing a clear area corresponding to amoeba lysis. This procedure allowed us to isolate 11 new *Marseilleviridae* strains and four new *Mimiviridae* strains. This procedure was thus shown to be highly efficient, as it allowed the isolation of the first Mimiviruses of human origin [24,25], but it has some limitations, the major being that it cannot be used with highly motile protozoa. Other research groups used this procedure to discover additional isolates of Marseilleviruses and Mimiviruses, the last being isolated in Brazil [26]. With now more than 7000 inoculated samples, we have isolated 43 strains of *Mimiviridae* and 17 strains of *Marseilleviridae* [13], whereas at the same time, another team isolated and described two new virus families, *Pandoravirus* and *Pithovirus,* using *A. castellanii* as a support for isolating amoeba-associated viruses [27,28]. Although these viruses seem to be mostly environmental, a recent work suggests that humans are in contact with them through contact lens-associated keratitis [29]. The team of JM Claverie first isolated *Megavirus chilensis,* which corresponds to the Mimiviridae we identified as lineage C. It was isolated from sea water in Chile [21]. In their work, they supplemented 1 L of sea water with 4% rice. The mixture was then incubated at room temperature in the dark to allow the heterotrophic bacteria to grow and to be fed on by protozoa, allowing the protozoa to expand and thus increase the viral population. After filtration, the membrane was mixed with antimicrobial agents (penicillin, gentamicin, streptomycin, and fungizone) for 3 days then inoculated on several *Acanthamoeba* species in microplates. It is not clear if the membrane was inoculated on *A. castellanii*, but the

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