Alcohol Disinfection Procedure for Isolating Giant Viruses from Contaminated Samples

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Our results demonstrate that ethanol treatment can be used to evaluate large collections of environmental samples for the presence of giant viruses and to provide insight into understanding their ecology. This study should also facilitate the isolation of giant viruses using other species of protozoa in addition to \textit{Acanthamoeba} spp.

**Key Words**
Giant viruses · Megavirales detection · Disinfection protocols

**Abstract**

\textbf{Objective:} Giant viruses of the Megavirales order have been neglected in the literature because they are removed from samples during viral purification for viral metagenomic studies. Isolation via amoeba coculture has low efficiency and is extremely time-consuming. Thus, our objective was to improve Megavirales detection and recovery by using a new protocol that will eliminate most bacteria present in environmental samples while preserving giant virus viability.

\textbf{Methods:} In this study, we tested the ability of a number of disinfection protocols to kill contaminating bacteria. These treatments were ethanol, UV irradiation, desiccation, glutaraldehyde and thermal shock.

\textbf{Results:} Of all the treatments, a brief ethanol treatment did not significantly reduce the titer of viable viral particles of \textit{Acanthamoeba polyphaga mimivirus} or \textit{Marseillevirus}, whereas it efficiently killed \textit{Escherichia coli}. This treatment was applied to environmental samples that previously tested positive for giant viruses and was shown to eliminate contaminating bacteria, whereas it allowed for the isolation of the giant viruses.

\textbf{Conclusion:} Our results demonstrate that ethanol treatment can be used to evaluate large collections of environmental samples for the presence of giant viruses and to provide insight into understanding their ecology. This study should also facilitate the isolation of giant viruses using other species of protozoa in addition to \textit{Acanthamoeba} spp.
virus resembling Mimivirus, named Mamavirus [7]. This isolation was followed by the isolation of an additional giant virus, Marseillevirus, which was the first member of a new family of amoeba-associated viruses [8]. Sputnik, a small virus that infects the viral factory of Mamavirus, was concomitantly identified, which led to the novel concept of a virophage [7]. Subsequently, two additional viruses of the Marseilleviridae family were isolated using the same coculture method: Lausannevirus, which has novel genomic features, was isolated from the Seine (Paris) [9], and Senegalvirus was isolated from the stool of a healthy patient from Senegal [10]. These early studies had a low rate of virus isolation and were characterized by laborious methods using amoeba cocultures. Subsequent to the initial studies, the use of a modified approach of coculture with the addition of a number of antibiotics on 105 environmental samples led to the isolation of 19 new giant viruses [11]. Three of these viruses belonged to the Marseillevirus family, and 16 were assigned to the APM family. One of the latter giant viruses, Lentille virus, had genome virophage integrated in its genome [12]. Several isolates have been obtained using the original techniques, including Megavirus chilensis [13], which is larger than Mimivirus, leading to the designation of the proposed new Megavirales order [14]. These authors used a novel enrichment procedure, although the isolation procedure was roughly identical to our approach [13]. M. chilensis is a member of the genomic group C of APM and corresponds to Courdo11, which was previously isolated using our multiantibiotic approach [11]. The antibiotic-based approach improved the rate of isolation in environmental samples by inhibiting the growth of bacteria within and outside of the amoebae. Up to eight antibiotics (i.e. colimycin, vancomycin, gentamicin, cotrimoxazole, erythromycin, rifampin, doxycycline and ciprofloxacin) could be combined to inhibit the bacterial overgrowth that inhibits the growth of viruses [11]. A number of antibiotics or combinations of antibiotics negatively affected the growth of the amoebae, which is one of the likely reasons that so few viruses have been effectively isolated despite their high abundance in the environment, as predicted by numerous metagenomic studies [15, 16]. In a previous work, we briefly investigated the potential resistance of APM to different treatments and demonstrated that APM continued to be infectious after 1 year at 4, 25 or 32° in Page’s amoebal saline (PAS) buffer and was not affected by 48 h of desiccation [6]. In a recent work, the resistance of APM to a number of chemical biocides, especially alcohol solutions, was demonstrated [17]. In this work, to simplify and expedite the isolation of viruses by avoiding the use of antibiotics that have deleterious effects on amoebae, we investigated the possibility of using diverse types of pre-treatment to kill contaminating overgrowth of bacteria without killing the APM or Marseilleviridae.

Materials and Methods

Coculture

The giant viruses used in this study were the original strain of APM (M1) [5]; a bald form of Mimivirus that was obtained after 150 culture passages of the original strain (M4) [18]; an additional viral strain of the Mimiviridae family, Pointerouge1 (PR) [11], and the original strain of Marseillevirus (MV) [8]. The four viruses were grown in an A. polyphaga suspension (strain Linc AP-1) in the nutritive medium PYG (proteose peptone yeast extract). The amoebae were lysed after 3 days of incubation at 32°, and the culture supernatant containing the viruses was distributed into 50-ml Falcon® tubes (BD Bioscience, USA). After centrifugation for 10 min at 318 g, the pellets containing the amoebal cysts and debris were removed, and the supernatants were collected and filtered through a 0.8-μm syringe filters (Sartorius, France) to remove the remaining small amoeba debris. The filtered viral supernatant was distributed into 50-ml tubes and frozen at –80° until use. To assess the efficiency of the antimicrobial treatments, we used a strain of Escherichia coli bacterium (ATCC 25922) as a control. The bacteria were grown on Columbia agar medium with 5% sheep blood (Biomerieux, Marcy l’Etoile, France); after 24 h of incubation at 37°, the cells were harvested and resuspended in PAS, vortexed, distributed into 50-ml tubes and frozen at –80° until use. The viruses were titrated using an end-point dilution method. The suspensions were serially diluted (1/10) in PAS buffer, and 50 μl of each dilution was inoculated onto 450 μl of an amoebal suspension calibrated at 5 × 105 amoebae/ml aliquoted into a 24-well microplate. We inoculated 4 wells per dilution to a final volume of 500 μl per well. The plates were incubated for 4–6 days at 32° to determine the highest dilution that led to amoebal lysis in one or two of the four replicate wells. We considered this dilution to correspond to the inoculation of 1 microorganism per well. The bacteria were titrated using an end-point dilution method, and the presence of bacteria was verified by growth on the agar plates. The samples were serially diluted (1/10) in PAS buffer, and 100 μl of each suspension was inoculated onto Columbia agar medium with 5% sheep blood (Biomerieux) and incubated at 37°. After 24 h of incubation, the colony-forming units (CFU) were counted, and the highest dilution leading to the growth of less than 10 CFU/plate was used to quantify the number of viable bacteria in each suspension. After titration, the viral and bacterial suspensions were adjusted to a concentration of 105 viruses or bacteria/ml for further experimentation.

Disinfecting Treatments

A number of disinfection methods were identified based on previously published research [5, 17] and the practicality of each treatment application in the context of routine laboratory procedures. We selected ethanol disinfection, ultraviolet light exposure, desiccation, thermal inactivation and glutaraldehyde exposure. All of the treatments were tested for several exposure durations. All viruses and bacteria were pelleted via centrifugation at 1,984 g for 30 min, the supernatants were removed, and the pellets were treat-
Isolation of Viruses from Environmental Samples

From our collection of environmental samples, we selected 2 environmental water samples that were previously shown to be positive for giant viruses. One sample was positive for a Marseille-virus-like giant virus (Seb1Sol), and the other was positive for a Mimivirus-like giant virus (Gouletet) [19]. The 1-ml samples were centrifuged at 15,000 rpm for 30 min, and the pellets were treated with 250 μl of 30, 40, 50, 60 and 70% ethanol for 5 and 10 min, respectively. The samples were centrifuged again, and the pellets were resuspended in 1 ml of PAS buffer. The final suspensions were used for the titration of microorganisms. For the viruses and bacteria, the titrations were performed using an end-point dilution method, as described above. All of the concentration results are expressed in log [C].

Virus Characterization

After the appearance of a lysis plaque on the agar coated with the amoebae, the plaque was cut around the perimeter and shaken in PAS buffer to resuspend the viruses in the buffer. This suspension was inoculated onto a fresh amoebal monolayer and, after lysing the amoebae, the supernatant was observed using electron microscopy. Negative staining was performed, as previously described, to prepare these samples for electron microscopy [6].

Results

Disinfection Treatments

For the ethanol treatment, we observed that bacterial contamination persisted when concentrations ranging from 30 to 60% ethanol and exposure times of 5 and 10 min were used (fig. 1a–d). The application of 70% ethanol with an exposure time of 5 and 10 min led to the elimination of bacterial contamination (fig. 1e). For UV exposure at 0–15 min, a number of bacteria remained viable, whereas the bacteria were eliminated after exposure for 20 and 30 min (fig. 2a). After 96 h of desiccation, no bacteria were detected on the agar plate (fig. 2b). After exposure to a solution of glutaraldehyde, the bacteria were completely inactivated after 5 min of incubation in the dark (fig 2c). For thermal inactivation, the bacteria were inactivated after 20 and 30 min of incubation at all three temperatures: 55, 65 and 75°C (fig. 2d–f). Regarding the viruses, 30% ethanol did not lower the number of viable virus particles for all of the viruses. The ethanol treatment from 40 to 60% had a small effect on the viability of viral particles in a number of cases. We could observe a small decrease of 1 log [C] after 5 min for M4 and MV with 40% ethanol and after 10 and 5 min for M4 and MV with 50% ethanol. Using a 60% ethanol titer, a small loss of 1 log [C] of the viable virus particles was observed for M1 and PR after 10 min of incubation. For M4 and MV, the amount of viable virus decreased by 2 log [C] after 10 min of incubation. For a 70% ethanol titer, we observed a partial loss of viable viral particles, the effect being less important for M1 and PR: a 1 log [C] decrease was observed after 5–10 min of incubation. For M4 and MV, the amount of viable virus particles was reduced by 2 log [C] and 3 log [C] after 5 and 10 min of incubation, respectively (fig. 1). Irradiation with UV light led to a loss of viable viral particles for all virus types. The decrease in viability with increasing UV exposure was approximately identical for all of the viruses, with a relatively greater resistance observed for PR and M1 (fig. 2a). Desiccation had no effect on the viability of the giant viruses, with the exception of MV, in which case the viability was decreased by 2 log [C] and 3 log [C] after 48 and 96 h of desiccation, respectively (fig. 2b). The thermal treatments at 75°C led to the complete inactivation of all tested viruses. At 65°C, we observed a small residual level of viable viruses for the original strain of Mimivirus, M1, and for PR, the second Mimiviridae used in this study. The other viruses, M4 and MV, were completely inactivated after 20 min of incubation at 65°C. At 55°C, all of the viruses were...
resistant to the treatment, and a small reduction of the number of MV particles was observed after 30 min of incubation (fig. 2d–f). The treatment by exposure to glutaraldehyde led to the inactivation of all the viruses, Mimivirus M1 and M4, PR and MV, after 5 min of incubation (fig. 2c).

**Fig. 1.** The effect of ethanol on the giant viruses M1, M4, PR and MV. A strain of the bacterium *E. coli* (EC) was used as a control for treatment efficiency. The infectivity of the microorganisms was measured using an end-point dilution method and expressed as the log of the microbial concentration. The assay was performed using various exposure times. **a** Ethanol 30%, **b** Ethanol 40%, **c** Ethanol 50%, **d** Ethanol 60%, **e** Ethanol 70%.

**Treatment of Environmental Water Samples with Ethanol**

After treating the samples with 30, 40, 50, 60 and 70% ethanol for 5 min, using sterile water as a control, the efficiency of the treatment was assessed via growth on Columbia agar with 5% sheep blood and on BCYE agar. For
the samples treated with sterile water, we observed the growth of bacterial strains in the samples. For the samples treated with 30–60% ethanol, the control agar plates were contaminated after 48 h of incubation at 32°C. After treatment with 70% ethanol, the agar plates remained sterile after 48 h of incubation at 32°C, demonstrating the efficiency of the treatment for all bacterial species present in the samples. After the different ethanol treatments, the samples were washed in PAS buffer and inoculated in the coculture onto an amoebal monolayer. After an enrichment stage of 3 days, the cocultures were inoculated on non-nutritive agar plates coated with a confluent amoebal monolayer.

Fig. 2. The effect of several inactivation treatments on the giant viruses M1, M4, PR and MV. A strain of the bacterium *E. coli* (EC) was used as a control for treatment efficiency. The infectivity of the microorganisms was measured using an end-point dilution method and expressed as the log of the microbial concentration. **a** Effect of UV irradiation using various exposure times. **b** Effect of desiccation using various desiccation times. **c** Effect of glutaraldehyde at 2% over a 5-min duration. Effect of thermal inactivation at 55°C (d), 65°C (e) and 75°C (f), under several incubation times.
monolayer and incubated at 32°. After 1 day, the presence of giant viruses was apparent, as indicated by the formation of a lysis plaque on the agar surface (fig. 3). This effect was observed for the sample containing Goulette1 and the sample containing Seb1Sol. The analysis of the lysis plaques via electron microscopy after the subculture confirmed the presence of giant viruses. In the Goulette1 sample, we observed a giant Mimivirus-like virus with a capsid size of 450 nm, and in the Seb1Sol sample, we detected a giant virus resembling Marseillevirus with a capsid size of 220 nm.

Discussion

The efficiency of all the tested treatment methods was assessed using the effective inactivation of the bacterium *E. coli*. The treatments had variable effects on the viruses. We noted that PR and M1 showed a greater resistance to the treatments than the other two strains. The degenerated bald form of M4 Mimivirus and MV were more fragile, especially regarding the thermal and desiccation treatments. It is possible that the presence of fibrils on M1 and PR confers a protective effect to the virus [18]. The purpose of our study was to find an efficient method to isolate giant amoeba-associated viruses from environmental samples. The isolation of Mimiviridae and Marseilleviridae was dependent on the use of a number of antibiotic treatments to avoid bacterial contamination [5, 7–11, 13]. The classic technique of coculture with an amoeba, which involves labor-intensive and time-consuming procedures such as numerous Gimenez stainings [20] and the use of several antibiotic treatments, was the only available method for isolating amoeba-associated viruses. This method allowed for the isolation of many giant viruses, but bacterial contamination was a consistent problem, and we presume that viral culturing often failed. Based on the results from several metagenomic studies, we expected a higher proportion of giant viruses in the environment to have been cultured [15, 16, 21, 22]. We aimed to alleviate this constraint by developing a pretreatment process that could eliminate bacterial contamination without altering giant viruses. We could disregard glutaraldehyde and high-temperature treatment because bacteria and viruses are effectively eliminated by those treatments. Low-temperature treatment allowed for the elimination of bacteria while maintaining the viability of Mimiviridae and Marseillevirus; this step required a minimum of 20 min. Desiccation for 24 h did not effectively eliminate bacteria, and a longer desiccation duration was effective regarding bacteria and Marseillevirus. The compromise between obtaining efficacy against bacteria (requiring a short time of manipulation and having no effect on the viability of viruses) was observed using the ethanol treatment. After 5 min of contact with a 70% ethanol solution, the bacteria were completely inactivated; a loss of 1 log [C] of M1 and PR was observed and, for the more fragile M4 and MV, we observed a loss of 2 log [C]. We employed the 70% ethanol method for the treatment of samples known to be positive for giant viruses. These 2 positive samples were used in the previous isolation via a high-throughput method of Seb1Sol and Goulette1 [19]. We determined that after an enrichment stage in coculture with amoebae, the viruses were viably present on the nutritive agar plate when using the identical high-throughput method. A lysis plaque was observed at the inoculation point of the

Fig. 3. Non-nutritive agar plate coated with a concentrated amoebal suspension. For the Goulette1 and Seb1Sol samples, a lysis plaque is observable; for the negative control, no lysis plaque can be observed. The lysis plaques are indicated by arrows.

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coclure using the positive samples, and this lysis plaque contained viruses that could be observed in the coculture after re-inoculation via electron microscopy with negative staining. We demonstrated that a direct treatment of the samples with a 70% ethanol solution could facilitate the elimination of bacteria and could, more specifically, target the isolation of giant viruses. In the future, a combination of ethanol pretreatment of the sample and the use of a recently described high-throughput system of isolation [19] could lead to the isolation of many more viral strains and facilitate understanding of the ecology and environmental presence of amoeba-associated viruses. Ethanol pretreatment could also be adapted to the classic saline buffer coculture with other protozoa in addition to Acanthamoeba spp.

Disclosure Statement

The authors declare that there is no potential conflict of interest or financial disclosure.

References

1. Birtles RJ, Rowbotham T, Raoult D, Harrison TG: Phylogeographic diversity of intra-amoebal legionellae as revealed by 16S rRNA gene sequence comparison. Microbiology 1996;142:3525–3530.
2. Adeleke A, Pruckler J, Benson R, Rowbotham T, Halablab M: Fields B: Legionella-like ame-
bal pathogens – phylogenetic status and possible role in respiratory disease. Emerg Infect Dis 1996;2:225–230.
3. Greub G, Raoult D: Microorganisms resistant to free-living amoebae. Clin Microbiol Rev 2004;17:413–433.
4. Raoult D, Audic S, Robert C, Abergel C, Reste

poto, Opatia H, La Scola B, Suzan M, Clave-

erie JM: The 1.2-megabase genome sequence of Mimivirus. Science 2004;306:1344–1350.
5. La Scola B, Audic S, Robert C, Jungang L, deLamballerie X, Drancourt M, Birtles R, Clave-

erie JM, Raoult D: A giant virus in amoebae. Science 2003;299:2033.
6. Suzan-Monti M, La Scola B, Barrassi L, Espi-
nosa L, Raoult D: Ultrastuctural characterization of the giant volcano-like virus factory of Acanthamoeba polyphaga Mimivirus. PLoS One 2007;2:e328.
7. La Scola B, Desnues C, Pagnier I, Robert C, Barrassi L, Four-
nous G, Merchant M, Suzan-Monti M, Forterre P, Koonin E, Raoult D: The virophage as a unique parasite of the giant Mimivirus. Nature 2004;455:100–104.
8. Boyer M, Yutin N, Pagnier I, Barrassi L, Four-
nous G, Espinosa L, Robert C, Azza S, Sun S, Rossmann MG, Suzan-Monti M, La Scola B, Koonin EV, Raoult D: Giant Marseillevirus highlights the role of amoebae as a melting pot in emergence of chimeric microorganisms. Proc Natl Acad Sci USA 2009;106:21848–21853.
9. Thomas V, Bertelli C, Colly F, Casson N, Tele-
teni A, Goesmann A, Crozatto A, Greun G: Lausannevirus, a giant amoeba virus encoding histone doublets. Env Microbiol 2011;13:1454–1466.
10. Lagier JC, Arnaqugom F, Million M, Hugon P, Pagnier I, Robert C, Bittar F, Fournous G, Gimenez G, MaraninchI T, Trappe JF, Koonin EV, La Scola B, Raoult D: Microbial cul-
turomics: paradigm shift in the human gut microbiome study. Clin Microbiol Infect 2012;18:1185–1193.
11. La Scola B, Campocasso A, N’Dong R, Four-
nous G, Barrassi L, Flandrops C, Raoult D: Tentative characterization of new environmental giant viruses by MALDI-TOF mass spectrometry. Intervirology 2010;53:344–353.
12. Desnues C, La Scola B, Yutin N, Fournous G, Robert C, Azza S, Jardot P, Monteil S, Cam-
pocasso A, Koonin EV, Raoult D: Proviro-

phages and transpovirons as the diverse mob-

ilome of giant viruses. Proc Natl Acad Sci USA 2012;109:18078–18083.
13. Arslan D, Legendre M, Seltzer V, Abergel C, Claverie JM: Distant Mimivirus relative with a larger genome highlights the fundamental features of Megaviridae. Proc Natl Acad Sci USA 2011;108:17486–17491.
14. Colson P, de Lamballerie X, Fournous G, Ro-
thod: Reclassification of giant viruses composing a fourth domain of life in the new order Megavirales. Intervirology 2012;55:321–332.
15. Ghedin E, Claverie JM: Mimivirus relatives in the Sargasso sea. Virol J 2005;2:62.
16. Yau S, Lauro FM, DeMaere MZ, Brown MV, Thomas T, Raftrey MJ, Andrews-Franko C, Lewis M, Hoffman JM, Gibson JA, Cavic-

chioli R: Virophage control of antarctic algal host-virus dynamics. Proc Natl Acad Sci USA 2011;108:6163–6168.
17. Campos RK, Andrade K, Ferreira PC, Bon-
jardin CA, La Scola B, Kroon E, Abrahao J: Viricidal activity of chemical biocides against Mimivirus, a putative pneumonia agent. J Clin Virol 2012;55:323–328.
18. Boyer M, Azza S, Barrassi L, Close T, Campo-
casso A, Pagnier I, Fournous G, Borg A, Rob-
cert C, Zhang X, Desnues C, HenriSSat B, Ross-

mann MG, La Scola B, Raoult D: Mimivirus shows dramatic genome reduction after intra-
amoebal culture. Proc Natl Acad Sci USA 2011;108:10296–10301.
19. Boughalmi M, Saadi H, Pagnier I, Colson P, Fournous G, Raoult D, La Scola B: High-
throughput isolation of giant viruses of the Mimiviridae and Marseilleviridae families in the Tunisian environment. Environ Micro-
bioi 2013;15:2000–2007.
20. Gimenez DF: Staining Rickettsiae in yolk-sac cultures. Stain Technol 1964;39:135–140.
21. Yamada T: Giant viruses in the environment: their origins and evolution. Curr Opin Virol 2011;1:58–62.
22. Claverie JM, Grzela R, Lartigue A, Bernadac A, Nitsche S, Vacelet J, Ogata H, Abergel C: Mimivirus and Mimiviridae: giant viruses with an increasing number of potential hosts, including corals and sponges. J Invertebr Pathol 2009;101:172–180.