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Alcohol Disinfection Procedure for Isolating Giant Viruses from Contaminated Samples

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Key Words
Giant viruses · Megavirales detection · Disinfection protocols

Abstract
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. ***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. ***Results:*** Of all the treatments, a brief ethanol treatment did not significantly reduce the titer of viable viral particles of *Acanthamoeba polyphaga mimivirus* or *Marseillevirus*, whereas it efficiently killed *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. ***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 *Acanthamoeba* spp.

Introduction

The discovery of the ability of free-living amoebae to be infected by a large variety of microorganisms has led to the use of amoebae for the culture of uncommon bacteria [1–3]. The interest in amoebae for the culture of intracellular microorganisms was stimulated by the discovery of *Acanthamoeba polyphaga mimivirus* (APM), the first known giant virus, which has a complex genome (more than 1,000 genes) [4] and is the same size as a small bacterium (400 nm) [5]. APM forms a large virus factory for multiplication within the amoeba [6]. The first isolation of a giant virus was inadvertently achieved during the investigation of a pneumonia epidemic via amoebal coculture in search of *Legionella* spp., and it prompted a prospective search that led to the isolation of a new giant

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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 pretreatment 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 amoebal 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 5×10^5 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 10^7 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-

ed with 30, 40, 50, 60 and 70% ethanol for 5 and 10 min, respectively. For exposure to ultraviolet light, 1 ml of each virus or bacterium was uniformly deposited in a Petri dish and exposed to UV light under aseptic conditions at 253,7 nm wavelength 15 W intensity and a distance of 12 cm. Each microorganism received 5, 10, 15, 20 and 30 min of UV exposure. For the desiccation treatment, 1 ml of viral or bacterial suspension was deposited in Petri dishes and allowed to dry under aseptic conditions for 48–96 h until complete desiccation was observed. The dried pellets were resuspended in 1 ml of PAS. For the thermal treatment, 3 temperatures were tested: 55, 65 and 75°. For each temperature, 1 ml of microbial suspension was placed in a water bath at the given temperature and incubated for 20 and 30 min. The exposure to glutaraldehyde was performed by adding 80 µl of a glutaraldehyde solution to 1 ml of microbial suspension for a final concentration of 2%. The suspensions were incubated for 5 min in the dark and centrifuged for 30 min at 1,984 *g*. The supernatants were discarded and the pellets were resuspended in 1 ml of PAS. After the treatments, we obtained 1 ml of each viral or bacterial suspension for each disinfection trial. The suspensions 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].

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 Marseillevirus-like giant virus (Seb1Sol), and the other was positive for a Mimivirus-like giant virus (Goulette1) [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 for 30 min at 15,000 rpm, and the pellets were resuspended in 250 µl of PAS. A sterility control was performed by inoculating 50 µl of this suspension on Columbia agar medium with 5% sheep blood (Biomerieux) and on BCYE agar (Oxoïd, Dardilly, France). A volume of 100 µl of each sample was inoculated onto an amoebal monolayer at a concentration of 5×10^5 amoebae/ml seeded in 24-well microplates. After 3–5 days of incubation at 32°, 10 µl of each coculture was inoculated on a non-nutritive agar plate coated with amoebae at a concentration of 2×10^6 amoebae/ml. This method allowed for the observation of a lysis plaque on the agar after 12 h of incubation at 32° in the presence of giant viruses, as previously described [19]. An identical experiment was performed for both samples using sterile water substituted for 30, 40, 50, 60 and 70% ethanol to serve as a control for the efficiency of the treatment. The control for the ethanol treatment was performed on Columbia agar medium with 5% sheep blood (Biomerieux) and on BCYE agar (Oxoïd).

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° (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 viruses decreased 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° led to the complete inactivation of all tested viruses. At 65°, 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°. At 55°, all of the viruses were

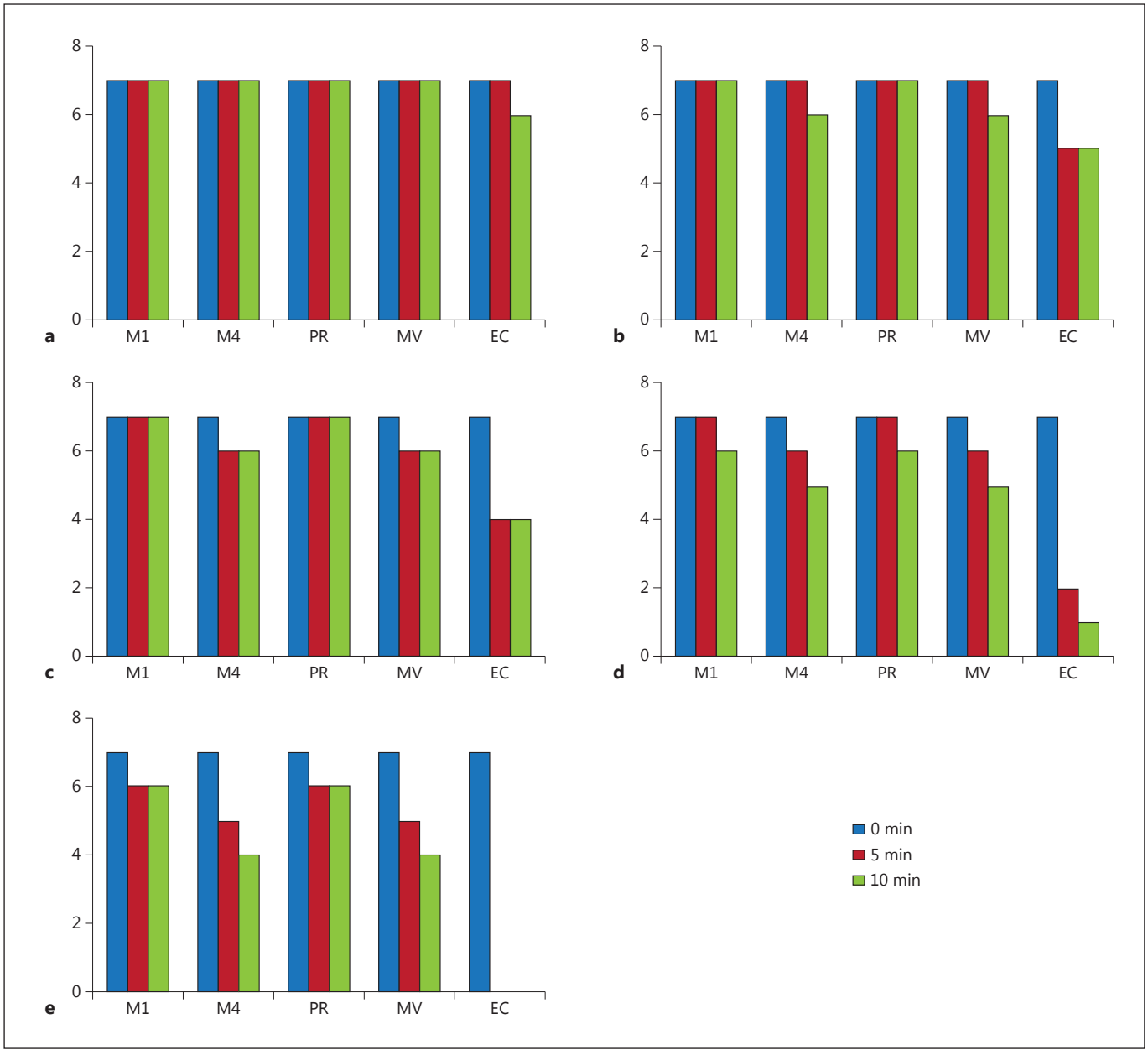

Fig. 1. The effect of ethanol on the giant viruses M1, M4, PR and MV. A strain of the bacterium *E. coli* (EC) was used as a control for treatment efficiency. The infectivity of the microorganisms was measured using an end-point dilution method and expressed as the log of the microbial concentration.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

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).

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

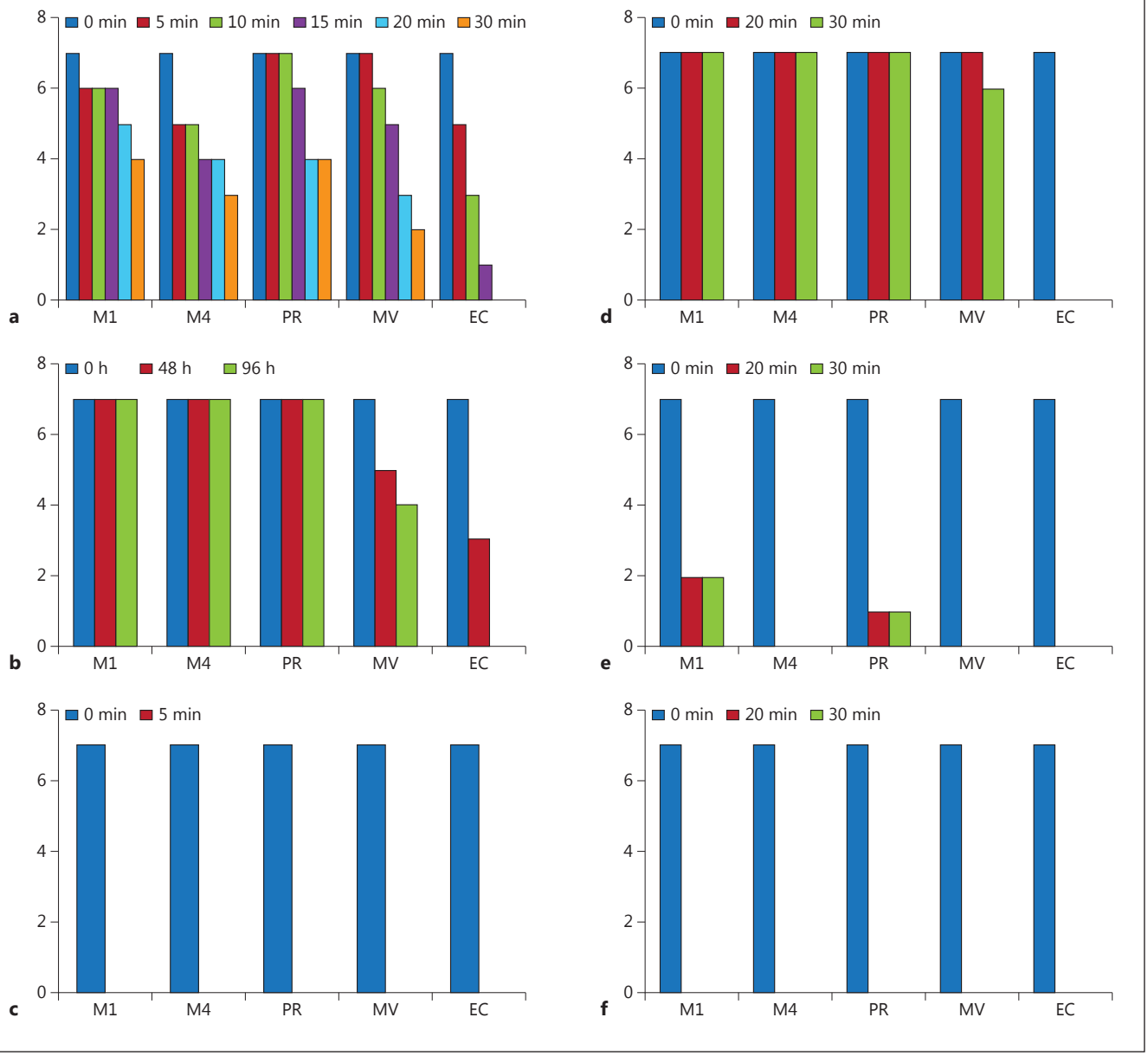

Fig. 2. The effect of several inactivation treatments on the giant viruses M1, M4, PR and MV. A strain of the bacterium *E. coli* (EC) was used as a control for treatment efficiency. The infectivity of the microorganisms was measured using an end-point dilution method and expressed as the log of the 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 (**d**), 65 (**e**) and 75° (**f**), under several incubation times.

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°. After treatment with 70% ethanol, the agar plates remained sterile after 48 h of incubation at 32°, 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

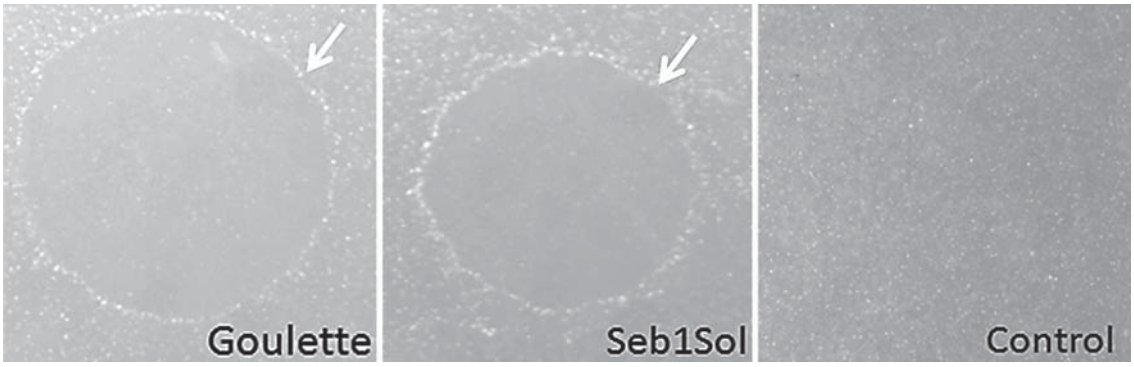

Fig. 3. Non-nutritive agar plate coated with a concentrated amoebal suspension. For the Goulette1 and Seb1Sol samples, a lysis plaque is observable; for the negative control, no lysis plaque can be observed. The lysis plaques are indicated by arrows.

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

coculture 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.

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