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Enumerating Viruses in Coral Mucus

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The distribution of viruses inhabiting the coral mucus remains undetermined, as there is no suitable standardized procedure for their separation from this organic matrix, principally owing to its viscosity and autofluorescence. Seven protocols were tested, and the most efficient separations were obtained from a chemical treatment requiring potassium citrate.

Viruses have been probably the least studied among the biological entities that live in the mucus and form the coral holobiont (dinoflagellates, fungi, bacteria, archaea, cyanobacteria, etc.) (1, 4, 10, 11), until now. Speculation on their presence in coral lacks a scientific basis, and only a few scattered metagenomic studies have provided any new information (6, 12). It is now known that, although viruses can infect any of the microorganisms in the coral holobiont, they are mostly comprised of bacteriophages (6). Moreover, there is no quantitative estimate of their density (per unit of mass of mucus or per unit area) in the literature, and their prevalence in the surface mucus layer is based only on qualitative observations (2, 9). A standard reliable method is thus required for estimating their abundance levels. In this study, seven previously described protocols were tested, and their suitabilities for counting viruses reliably from the coral mucus were compared.

The mucus was collected from six scleractinian species in the Cap d'Agde aquarium (France)—*Fungia* sp., *Turbinaria* sp., *Alveopora* sp., *Favia* sp., *Lobophyllia* sp., and *Platygyra* sp.—and the soft coral *Heteroxenia* sp. Briefly, corals were taken out of the water and exposed to air for 1 to 3 min (7). This stress caused the mucus to be secreted, forming long gel-like threads dripping from the coral surface that were directly collected in cryotubes, fixed with formaldehyde (final concentration of 3%), and stored at −80°C until staining. Mucus samples were processed in triplicates as follows.

Chemical methods. (i) Potassium citrate method. For the potassium citrate method (14), a total of 100 μl of fixed mucus was eluted into 900 μl of 0.02-μm-pore-size-filtered, pH 7 solution of 1% citrate potassium with 10 g potassium citrate, 1.44 g $Na_2HPO_4 \cdot 7H_2O$, and 0.24 g KH_2PO_4 per liter. All tubes were then vortexed at a moderate speed before particles were stained and enumerated (see below).

(ii) Methanol method. For the methanol method (5), a total 160 μl of fixed mucus was incubated with a solution of 0.02-μm-pore-size-filtered methanol (20% final concentration) at 35°C for 15 min and sonicated for 30 s (amplitude of 20).

(iii) Sodium pyrophosphate method. For the sodium pyrophosphate method (1a), a total of 100 μl of fixed mucus was inoculated with tetrasodium pyrophosphate (10 mM, final concentration), vortexed for 1 min, and incubated at ambient temperature for 15 min. The sample was then diluted (1:3) in Milli-Q filtered water and sonicated for 30 s.

(iv) Polysorbate 80 method. For the polysorbate 80 method (15), a total of 100 μl of fixed mucus was diluted in 900 μl of 0.02-μm-pore-size-filtered Milli-Q water and incubated with 20% Tween 80 (polysorbate 80) at 35°C for 15 min and sonicated for 30 s.

Mechanical method. The mechanical method was a homogenization method (6) based on a 1:10 dilution of the mucus after fixing (100 μl) in 0.02-μm-pore-size-filtered Milli-Q water, followed by 1 min of power homogenization (Polytron; PT 10 35 GT) at 5,000 rpm. A combination of this protocol and that based on the potassium citrate protocol was tested by diluting 100 μl of the mucus in 900 μl of 1% potassium citrate solution prior to homogenization.

Enzymatic method. The trypsin method (3) involved 100 μl of fixed mucus eluted into a solution of EDTA (10 mM, final concentration), vortexed for 1 min, and sonicated for 3 min, followed by 1% trypsin incubation for 30 min at 37°C.

Staining procedure. Viral abundances were determined in each mucus sample (treated and untreated) by using the standard technique with nucleic acid dye SYBR Gold and epifluorescence microscopy (8).

Statistical analyses. The treatment effectiveness (TE) was calculated based on the difference in viral abundances between the treated (T) and untreated control (C) mucus samples: $TE\ (\%) = [(T - C)/C] \times 100$. The significance of treatments and coral species on viral abundance was tested using multifactorial analysis of variance (ANOVA) and Tukey-Kramer mean *post hoc* comparison tests (alpha level = 0.05).

The reliability of each protocol was simply inferred from the levels of abundance detected for viruses, which are the net result of parameters such as brightness, contrast, background fluorescence, presence of remaining aggregates, staining quality, even distribution of particles on membrane, etc. Thus, the protocol resulting in the highest values was considered the most effective for separation and enumeration of viruses.

In this study, the 1% potassium citrate solution was the best overall eluent for the mucus samples, regardless of the coral species. Direct counts of viruses following their extraction with this compound ranged from 2.8×10^7 VLP ml^{-1} of mucus for *Fungia* sp. to 11.4×10^7 VLP ml^{-1} for *Alveopora* sp. (Fig. 1). The treatment effectiveness reached up to 252.3% and was significantly higher than that of the other chemical, mechanical, or enzymatic treatments (Tukey-Kramer, $P < 0.05$). Potassium citrate has been successfully used for more than 10 years to desorb viral particles

Received 9 April 2012 Accepted 19 June 2012
Published ahead of print 22 June 2012
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doi:10.1128/AEM.01141-12

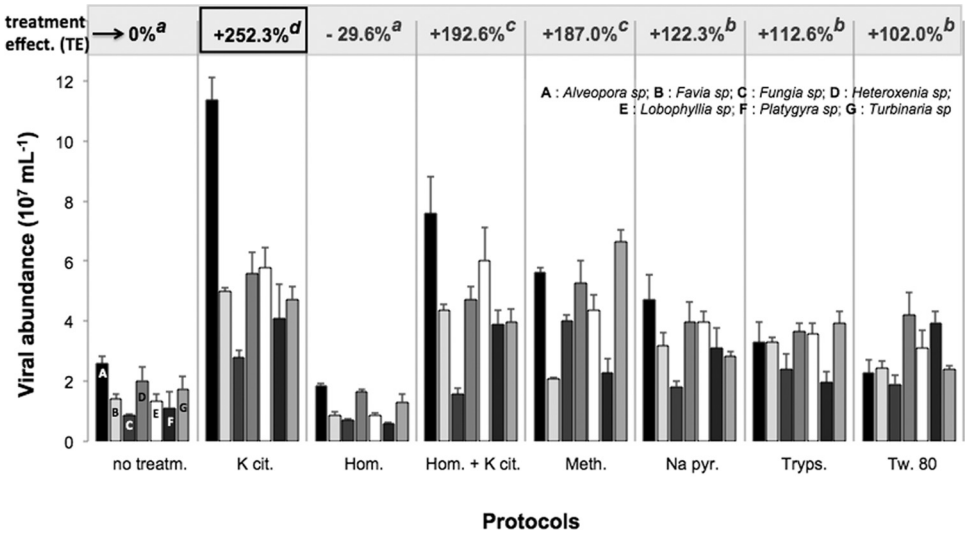

FIG 1 Abundance of viruses as measured after their extraction from coral mucus using the various protocols, shown on the *x* axis. Error bars represent the standard deviations calculated with three values. The treatment effectiveness (TE), expressed as a percentage, represents the ratio between abundances measured for treated and untreated samples. An index (*a*, *b*, *c*, *d*) is assigned to each treatment protocol; two TEs with the same index are not significantly different (multifactorial analysis of variance, and Tukey-Kramer *post hoc* comparison tests, $P < 0.05$). Treatm., treatment; K cit., potassium citrate; Hom., homogenization; Meth., methanol; Na pyr., sodium pyrophosphate; Tryps., trypsin; Tw. 80, Tween 80.

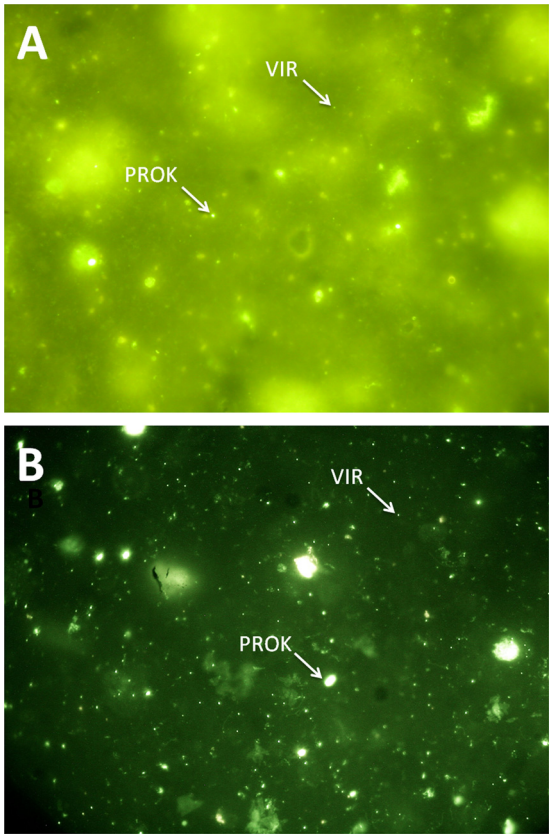

FIG 2 Epifluorescence microscopy image (×100 magnification) of mucus samples stained with SYBR Gold without any preliminary treatment (A) and after being treated with 1% potassium citrate (B). The small dots represent virus-like particles (VIR), the larger dots are prokaryotes (PROK).

from porous substrates, such as sediments and soils (13, 14). By raising the pH of a solution, it increases the electrostatic repulsion between the viruses and the substrate to which they are attached. Furthermore, unlike the other chemical eluents tested in this study, potassium citrate also greatly reduced the autofluorescence of the mucus, which made counting by epifluorescence much more reliable (Fig. 2). Last, the method is very fast, as the effect of the reagent is immediate and does not require any additional incubation. It is also inexpensive, as only a few milligrams are required of a stock at ca. 60 euros per 500 g of potassium citrate (VWR).

Unlike its remarkable efficiency for extracting prokaryotes from mucus (3), trypsin did not give the highest viral counts, possibly because this enzyme might also cause substantial damage to the viral capsid proteins. The homogenization method, previously used for DNA extraction in coral mucus, also resulted in very poor quality observations. This mechanical treatment is a powerful mucus liquefier, but the resulting spreading over the entire membrane surface has seemingly increased the background fluorescence of mucus, thus reducing the contrast and brightness of the stained viruses.

Our results were obtained from cultured corals rather than from *in situ* scleractinian colonies and may not be applicable to natural habitats. Nonetheless, this study shows that the citrate potassium method represents an interesting tool to investigate the dynamic processes of colonization and the spatial and temporal variability of the viral community abundances within the coral holobiont. It also might help to cope with several methodological barriers that prevent single-cell studies of these biological entities, including flow cytometry and electron microscopy.

ACKNOWLEDGMENTS

The research reported here was financially supported by the French EC2CO project CORINE and the Total Foundation.

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