

## Rotavirus VLP2/6: a new tool for tracking rotavirus in the marine environment

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### Abstract

The potential of rotavirus 2/6-virus-like-particles (VLP2/6) for use as tracers in the marine environment was investigated. The stability of bovine rotavirus (strain RF) and VLP2/6 in natural seawater at 25 °C for six days was studied. ELISA and western blot methods were used to quantify the particles. The rates of decline of rotavirus particles and VLP2/6 were similar (approximately 0.5 log<sub>10</sub> per day). Western blot analysis showed that the integrity of capsid proteins VP2 and VP6 was conserved during the incubation time. These results demonstrate that VLP2/6 particles have the same stability in seawater as rotavirus particles. Thus, VLP2/6 can be used as a tracer, which should be of particular value for studying the fate of rotavirus particles in the marine environment.

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### 1. Introduction

Viral gastroenteritis is the leading cause of infectious diarrhea worldwide, and rotavirus is the most common cause of severe diarrhea in infants and young children [21]. Enteric viruses are transmitted primarily by the fecal–oral route and represent a major cause of waterborne gastroenteritis outbreaks [15,18,19]. The clear role of the environment in the transmission of infectious disease highlights the need to monitor the behavior of these viral pathogens.

Co-expression of rotavirus capsid proteins in the baculovirus system results in the assembly of virus-like particles (VLPs) [17]. VLPs maintain the structural and functional characteristics of the native particles: they resemble a real virus but they are non-infectious [6]. The present report describes the potential of rotavirus 2/6 VLP as a tool to study the behavior of rotavirus in the marine environ-

ment. For that, we compared the stability of rotavirus bovine strains RF and VLP2/6, constituted of the proteins from the same virus strain, in natural seawater.

### 2. Materials and methods

#### 2.1. VLPs

VLPs containing full-length VP2 and VP6 were produced as previously described [3,6]. The VLP suspension was quantitated by estimation of the protein concentration by the method of Bradford using bovine serum albumin as a standard [3].

#### 2.2. Rotavirus bovine strain

The bovine rotavirus RF strain was propagated using the fetal rhesus monkey kidney (MA104) cell line as described [20]. Triple-layered particles were purified by two runs of centrifugation in CsCl density gradients. The rotavirus suspension was quantitated using the Bradford method.

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### 2.3. ELISA

Falcon 3915 (Probind assay plate, Becton–Dickinson, France) microtiter plates were coated with 200  $\mu$ l of rabbit anti-rotavirus serum 8148 diluted 1:2500 in carbonate/bicarbonate buffer (pH 9). The plates were incubated overnight at 37 °C and then washed 5 times. All washes were performed with phosphate-buffered saline (PBS) containing 0.1% of Tween 20 (Sigma–Aldrich, France). Then the plates were blocked with 200  $\mu$ l of PBS/4% non-fat dry milk for 1 h 30 min at 37 °C and washed 5 times. Serial dilutions of VLP2/6 and virus particles ranging from  $10^8$  to  $10^3$  particles/ml were made in PBS (100  $\mu$ l) and then incubated in the microtiter plates for 1 h 30 min at 37 °C. The plates were washed 5 times and 100  $\mu$ l of peroxidase-conjugated remi-2 antibody (Argène Biosoft, France) diluted 1:100 in PBS was added and incubated for 1 h at 37 °C. After 5 additional washes, 100  $\mu$ l of 3,3', 5, 5'-tetramethylbenzidine (TMB, Sigma–Aldrich, France) was added and incubated at room temperature in the dark for 15 min. The reaction was stopped by addition of 100  $\mu$ l of H<sub>2</sub>SO<sub>4</sub> 1 M and optical density was read at 450 nm with a spectrophotometer (Safire, Tecan, France). The sensitivity of the ELISA was  $10^4$  particles per ml for both the bovine rotavirus RF strain and the VLPs.

### 2.4. Protein analysis

SDS-PAGE analysis was performed in the Laemmli gel system (12.5% polyacrylamide, 0.1% SDS). Samples were dissociated by boiling for 5 min in sample buffer containing 2% sodium dodecyl sulfate (SDS), 5% 2-mercaptoethanol, 50 mM Tris–HCl, 10% glycerol and bromophenol blue. After blotting on Immobilon-P polyvinylidene difluoride (PVDF) membranes (Sigma–Aldrich, France) by transverse electrophoresis in 10 mM cyclohexylaminopropane sulfonic acid (CAPS), proteins were detected. All washes were performed in TBST buffer (50 mM Tris, 150 mM NaCl, 0.05% Tween 20 (v/v)). The membranes were blocked in 1% western blocking solution (1% (w/v) blocking reagent (Roche Molecular Biochemicals) in TBST) for 1 h at room temperature and then washed 5 times. RV1026 and 164E22 mouse antibodies diluted in western blocking solution were used, respectively, for immunodetection of VP6 and VP2. Membranes were incubated 1 h at room temperature. After 5 washes, peroxidase-conjugated anti-mouse immunoglobulin G (Sigma–Aldrich, France) was added. After 1 h of incubation at room temperature and 5 additional washes, 1 ml of Lumi-Light<sup>PLUS</sup> substrate (Roche Molecular Biochemicals) was added, and the membranes were incubated 10 min in the dark and revealed by chemiluminescence using a Bio-Rad Multi-Imager. The limit of detection for both rotavirus and VLP2/6 was  $10^6$  particles/ml.

### 2.5. Comparison of rotavirus particles and VLP2/6 stability in natural seawater

Natural seawater samples were collected on the French Atlantic coast (Argenton Bay, salinity 36.5 g/l, pH 8.2, turbidity <1 NTU, total coliform  $1.6 \times 10^5$  CFU/ml). Sixty ml of seawater was inoculated with purified virus particles or VLP2/6. The flasks were then incubated at 25 °C in darkness under agitation. Samples (10 ml) were taken at days 0, 1, 2, 4 and 6. The samples were concentrated by filtration using Centriplus YM-100 (Amicon, Millipore, France). Concentrated particles (100  $\mu$ l) were then quantified by ELISA and western blot methods as described above. Positive controls (serial dilutions of virus and VLP2/6 particles) were quantified at the same time to determine the particle concentration in the sample. Four different experiments were performed.

### 2.6. Statistical analysis

Linear regression analysis was performed using the Stat-Graphic software. The probability of a difference between the two slopes of the linear regression curves obtained for VLP2/6 and RV was calculated and tested for significance at the 0.05 confidence level.

## 3. Results

### 3.1. Stability of rotavirus and VLP2/6 as measured by ELISA

Natural seawater was seeded with purified virus particles or VLP2/6 at  $10^8$  particles/ml. For the four experiments, similar concentrations of virus and VLPs were used. As shown by the mean value calculated per day, the initial titer of  $10^8$  particles/ml was reduced by approximately 3 log<sub>10</sub> units after 6 days at 25 °C for both rotavirus and VLP2/6 (Fig. 1). The rates of decline for virus and VLP2/6 as determined by linear regression analysis were similar (–0.47 (95% CI –0.54 to –0.41) and –0.52 (95% CI –0.63 to –0.41), respectively) using a single compartment model (Fig. 1). We cannot exclude the possibility that VLPs have a more complex degradation pattern (with rapid degradation rate during the first two days followed by stabilization of the decay rate). Measured geometric mean levels of the VLP2/6 were lower on all days, including day 0, compared to rotavirus.

### 3.2. Protein analysis

After concentration of seawater samples, the two types of particles were analyzed by SDS-PAGE and western blot to confirm the presence of each protein, VP2 and VP6. Both proteins were detected on the membrane through day 4, with the intensity of the bands decreasing each day. The relative

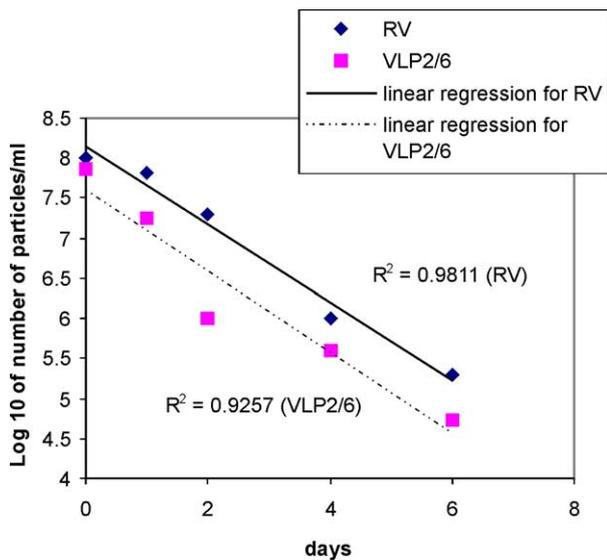


Fig. 1. Comparison of rotavirus particles and VLP2/6 survival in seawater at 25 °C for 6 days.

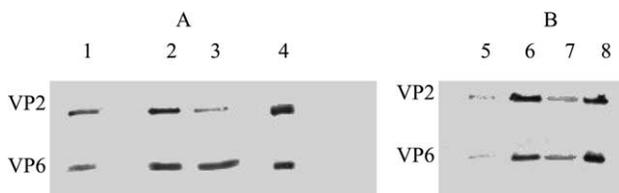


Fig. 2. Analysis of infectious rotavirus and VLP2/6 proteins by western blot. (A) proteins VP2 and VP6 present in rotavirus particles at day 2 (2) and day 4 (1). Positive controls:  $10^6$  (3) and  $10^8$  particles/ml (4). (B) proteins VP2 and VP6 present in VLP2/6 particles at day 2 (6) and day 4 (5). Positive controls:  $10^6$  (7) and  $10^7$  particles/ml (8).

concentrations of rotavirus and VLP2/6 were estimated based on the intensity of the band compared to those of known quantities of positive controls. A concentration of about  $10^8$  particles/ml was found at days 0 and 1 for bovine rotavirus and VLP. Fig. 2 shows results for virus particles and VLP2/6 obtained after days 2 and 4 in seawater. The estimated concentrations of virus particles and VLP2/6 were about  $10^7$  particles/ml at day 2. At day 4, the virus concentration was about  $10^6$  particles/ml, and for VLP2/6, the concentration was estimated at between  $10^6$  and  $10^5$  particles/ml (weak signal detected on the membrane, Fig. 2B). At day 6, concentrations were below the limit of detection using this method. These data are similar to and confirm the results obtained using ELISA.

#### 4. Discussion

Rotavirus transmission via the aquatic environment is a major health problem worldwide and, to date, there have been no tools available to track rotavirus particles in the environment. These viruses are frequently detected in the environment [7,9,11,15,16,18,19,27]. In addition, the low

infectious dose, the high strain diversity, the possibility of cross-species transmission, and the risk of human–animal genetic reassortment leading to the emergence of new rotavirus strains reinforce the need to develop environmental monitoring tools that allow the determination of the fate of viral particles [10,14,22,26].

Field analysis of rotavirus stability to degradation in the environment is difficult, as many strains are difficult to cultivate and viral pathogenicity precludes its use in an open environment. A few studies have been made on rotavirus behavior in a marine environment and all of them have been on a bench scale in a laboratory setting [1,4,13]. The use of an indicator, such as bacteriophage, has been proposed as a surrogate to measure virus persistence in the environment, but differences in phage characteristics compared to rotavirus make interpretation and direct correlation of the results difficult. Our innovative idea was to use rotavirus VLP as a tracer for the native virus. The major interests of rotavirus VLP are the ease of production, the absence of pathogenicity and a structure identical to that of native rotavirus particles. These VLPs have been very helpful in fundamental research to study the relationship between rotavirus and the host cell [2], the immune response [12], the quantification of proteins in living cells [8] and the development of a vaccine [5]. These VLPs are stable for years in sterile water at 4 °C but nothing is known about their stability in other conditions. Furthermore, VLPs made to Norwalk virus have been a useful tool to model filtration of virus particles for this waterborne pathogen [24,25].

Experiments were conducted in natural seawater at 25 °C, as previous studies had demonstrated that the most important parameters for rotavirus inactivation are salts and temperature [1,9,13,23]. The most interesting and innovative observation from our study is the demonstration that VLPs persist in the seawater for 6 days similarly to what is seen with the rotavirus. To concentrate and to select complete particles, ultrafiltration with a cut off of 100 kDa was selected, taking into account the VP6 and VP2 protein molecular weights (44783 and 102431 Da, respectively). Although trimeric VP6 also would be retained with the ultrafiltration conditions used, the observed degradation patterns by ELISA and the identification of both VP6 and VP2 by western blot analysis suggest that these proteins remained associated in particles. Interestingly, similar results were obtained in a single experiment using VLP2/6/7 particles (data not shown).

The concentration of viral particles for study in the seawater was a compromise between the capacity to follow a  $3 \log_{10}$  unit reduction (taking into account the sensitivity threshold of our ELISA method) and to mimic the low level of contamination of natural seawater. For the rotavirus itself, the results obtained in our study are comparable to those obtained by cell-culture titration of infectious virus [4]. A good relationship between decreases in antigenicity and infectivity has also been reported in different types of water [23].

This is the first time that stability of rotavirus VLPs in environmental conditions has been clearly demonstrated. Additional studies will need to confirm the VLP stability in other conditions, but our data showed that VLPs could be a good tool to study rotavirus behavior in seawater, without the risk of using infectious viruses. Such a tracer could also be useful in other field studies where the physical elimination of virus particles needs to be measured (e.g., shellfish depuration, water treatment, and food transformation). Moreover, these studies could be extended to other enteric viruses for which VLPs are available.

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