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NOTE

A population of giant tailed virus-like particles associated with heterotrophic flagellates in a lake-type reservoir

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ABSTRACT: Using transmission electron microscopy (TEM), a population of giant virus-like particles (VLPs) with a head diameter of ca. 405 nm and a flexible ca. 1100 nm long tail was detected in a lake-type reservoir. These giant VLPs were abundant *in situ* at the start of a survey period (3.3×10^4 particles ml^{-1}) and increased by 7-fold within 96 h. This VLP population vanished in dialysis bag incubations of 0.8 µm-filtered reservoir water (free of bacterivorous flagellates) but increased markedly in the enhanced bacterivory treatment, i.e. 5 µm filtered water. In the latter, incubation, heterotrophic nanoflagellate (HNF) abundance increased approximately 15-fold during the study. A multiple regression analysis using microbial abundances and grazing rates as parameters indicated that 78% of the variability in the abundance of giant VLPs was explained by HNF abundance and grazing rates. Our data support the hypothesis that this virus population infects flagellates. Observation of a presumptive lysing flagellate cell suggests a viral burst size of 15. Estimations of decay and net production rates from dialysis bag incubations indicate that lysis due to giant viruses could cause between 10 to 60% of the mortality of the total flagellate community and, thus, viruses are potentially a significant factor shaping the population dynamics of flagellates in freshwater.

KEY WORDS: Viral infection · Mortality · Burst size

INTRODUCTION

Flagellates are usually the major consumers of auto- and heterotrophic bacterioplankton thus, linking bacteria with the grazing food chain, and they are significant remineralizers of inorganic nutrients (Hahn & Höfle 2001, Montagnes et al. 2008). Consequently, heterotrophic nanoflagellates (HNF) are a key component of pelagic ecosystems (e.g. Azam et al. 1983). In limnetic systems, population densities of flagellates are thought to be controlled by grazers, specifically larger protists such as ciliates, and larger zooplankton such as rotifers, copepods, and cladocerans (Jürgens et al. 1997, Jürgens & Jeppesen 2000, Sommer et al. 2012, Šimek et al. 2014). During the last two and a half decades, a plethora of information has been accumulated on viruses controlling bacterioplankton (e.g. Weinbauer 2004, Winter et al. 2010) and phytoplankton (e.g. Brussaard 2004, Brussaard et al. 2008, Sharoni et al. 2015). However, our knowledge of the viruses of non-photosynthetic protists (such as HNF and ciliates) is still sparse and mainly

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restricted to investigations of a few isolated virus–host systems (e.g. Nagasaki et al. 1993, Garza & Suttle 1995, Massana et al. 2007).

During an *in situ* survey and an experiment performed in the mesotrophic lake-type Římov Reservoir (South Bohemia, Czech Republic) to assess the role of protistan grazing on bacterial community dynamics and composition and on viral infection of bacterioplankton (Šimek et al. 2001), we detected a morphologically distinct giant virus-like particle (VLP) population (potentially infecting flagellates) and followed its development *in situ* and estimated decay and potential production rates.

MATERIALS AND METHODS

The *in situ* study and the experiment were conducted in the meso-eutrophic Římov Reservoir (for details, see Šimek et al. 2001). The sampling site is located above the former river valley (water depth 30 m), about 250 m from the reservoir dam. On 28 May 1999, water samples were collected with a 2 l Friedinger sampler from a depth of 0.5 m and a final volume of 30 l was pooled in a 50 l plastic container. The water temperature was ca. 19°C.

The experiment was run during the late clear-water phase (28 May to 1 June 1999), i.e. when phytoplankton populations are relatively sparse due to increased predation pressure of large filter-feeding zooplankton (Sommer et al. 2012). For the experiment water samples were sequentially size fractionated through 5 and 0.8 µm poresize Poretics filters (diameter 47 mm; OSMONIC.) producing 2 fractions: <5 µm (viruses, bacteria and HNF only) and <0.8 µm (viruses and bacteria only). These 2 fractions represent (1) a treatment with enhanced bacterivory due to removal of predators of bacterivorous flagellates and (2) a bacterivore-free treatment (Šimek et al. 2001). The 0.8 µm filtration step was conducted in sterilized glass Poretics filter holders to avoid contamination by small flagellates. The water fractions were placed in 2 l pre-treated (distilled water rinsed and boiled) dialysis tubes (diameter 75 mm, molecular mass cutoff 12 000 to 16 000 Da; Poly Labo). The dialysis bags were incubated in the reservoir at a depth of 0.5 m, oriented horizontally in open Plexiglas holders. Dialysis bags were used to allow exchange of nutrients and small dissolved organic matter with ambient reservoir water. Samples for monitoring *in situ* conditions in the reservoir water were also collected during the study period. The experimental design and protocol are described in detail in Šimek et al. (2001). Briefly, samples (250 to 300 ml) were taken from each dialysis bag and reservoir water at 0, 12, 24, 48, 72, and 96 h, fixed with formaldehyde (2% final concentration). Sample aliquots for enumerations using epifluorescence microscopy were examined within 4 h of sampling. For enumerations of giant virus-like particles (VLPs) by transmission electron microscopy (TEM), aliquots were stored at 4°C for 7 d until analysis.

Giant VLPs were enumerated using TEM as described previously (Weinbauer et al. 1993). Briefly, viruses were collected quantitatively onto Formvar-coated, 400 mesh electron microscope grids by centrifugation in a swinging-bucket rotor (Sorval TH-641; 100 000 × *g* for 2.5 h), stained for 30 s with 1% (wt/vol) uranyl acetate, and rinsed 3 times with deionized distilled water. Head-size diameters and tail parameters of giant VLPs were determined from photomicrographs (magnification 30 000 to 140 000×) analyzed under a dissecting lens (10×) (Weinbauer et al. 1993). For counting by using epifluorescence microscopy, VLPs were stained with SYBR Green I (10 000× in dimethyl sulfoxid). Bacteria, HNF and ciliates were stained with 4-6-diamidino-2-phenylindole (DAPI) (final concentration 0.1 µg ml^{-1}) and counted by epifluorescence microscopy. For details of preservation and enumeration see (Šimek et al. 2001). Briefly, plastid-containing flagellates were enumerated in samples preserved with Lugol's solution, cleared with sodium thiosulphate, employing the Utermöhl method and an inverted microscope (Lund et al. 1958).

RESULTS AND DISCUSSION

In the grazer-free treatment (<0.8 µm), bacterial numbers increased between 12 and 48 h (Fig. 1D). Bacterial dynamics in the <5 µm treatment showed different trends. After a slight initial increase, bacterial abundance dropped between 24 and 96 h (Fig. 1E). Compared to the <0.8 and <5 µm treatments, whole-water samples from the reservoir showed negligible changes in bacterial abundance (Fig. 1F). Marked differences in total VLP abundance were found among the <0.8 µm, <5 µm, and reservoir populations (Fig. 1D–F). Total VLP abundance appeared to vary inversely with bacterial abundance. For instance, by the end of the experiment in the heavily grazed <5 µm treatment, the largest numbers of total VLPs (47 × 10^6 ml^{-1}) were detected, along with the lowest bacterial abundance. In contrast, samples from the grazer-free treatment (<0.8 µm), in which bacteria became most abundant, yielded the lowest estimates of total VLP abundance (17 × 10^6 ml^{-1}). As opposed to

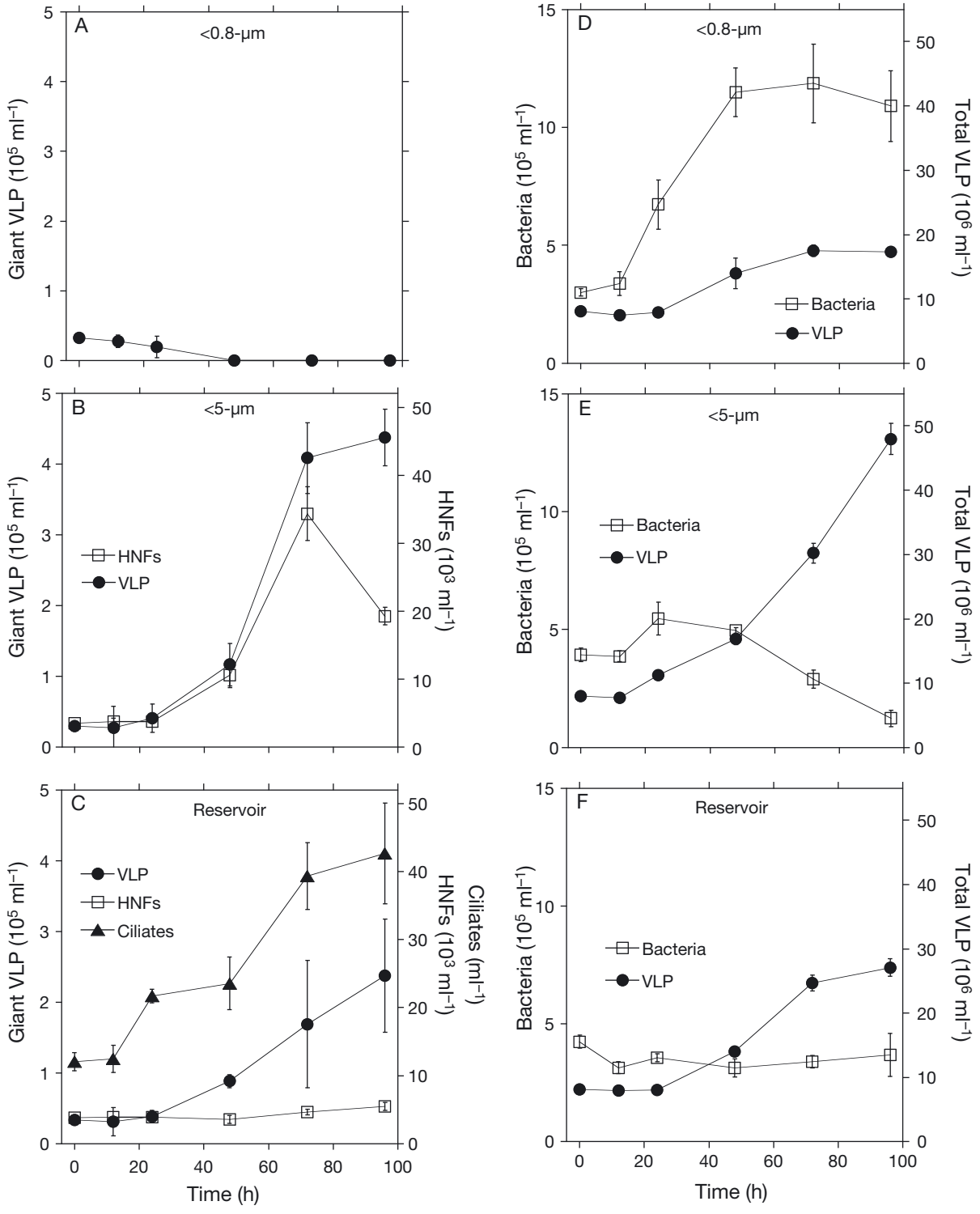

Fig. 1. Time-course changes in the abundance of bacteria, total virus-like particles (VLPs), giant VLPs, heterotrophic nanoflagellates (HNFs) and ciliates in size fractionation treatments (<0.8 and <5 µm) of reservoir water incubated in the reservoir in dialysis bags compared to *in situ* reservoir water: (A) giant VLPs in <0.8 µm treatment; (B) giant VLPs and HNFs in <5 µm treatment; (C) giant VLPs, HNFs and ciliates in unfiltered water; total VLPs and bacteria in (D) <0.8 µm, (E) <0.5 µm treatments and (F) unfiltered water. Bacterial, VLP and HNF and ciliate abundance values are the means of 3 replicates; error bars indicate SD. The values for giant viruses are the means of triplicate subsamples from a single pooled sample representing all 3 replicates; the vertical bars show the SD of triplicate estimates (i.e. 3 TEM grids)

the markedly distinct trends between the grazer-free (<0.8 µm) and grazer-enhanced (<5 µm) treatments, samples from the reservoir differed little from the <5 µm samples. In both reservoir and <5 µm treatment samples, total VLP abundance increased steadily from 24 h to 72 h and then declined slightly; in contrast to the <0.8 µm treatment, in which total VLP abundances were relatively invariant. The total VLP to bacteria ratio ranged from 19 to 93 in the reservoir, which is close to the value of 21 obtained when using flash-freezing of samples in liquid nitrogen from the same environment (Weinbauer et al. 2007).

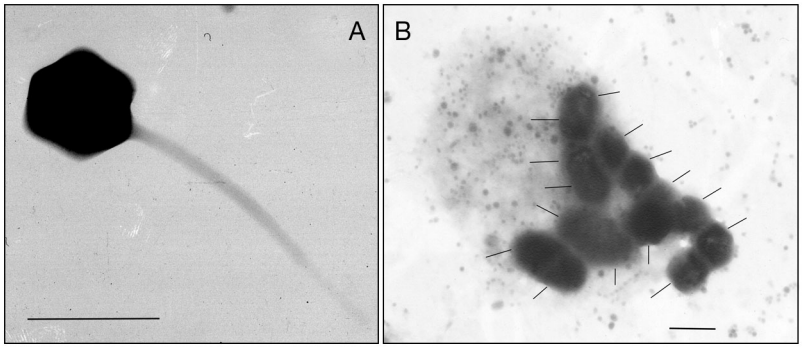

Fig. 2. Electron micrograph of giant tailed viruses (A) and a potentially lysing flagellate (B). Scale bars = 500 nm. Note that the seemingly elongated dimension of some particles is a photographic artifact, which becomes evident by changing the plane of focus during TEM inspection, revealing 2 distinct viral particles. Dashes point to the different VLPs

At the start of the study, *in situ* HNF and ciliate abundances were 3.8×10^3 and 12×10^3 cells ml^{-1}, respectively. HNF abundance increased slightly, whereas ciliate abundance showed a 3.6 fold increase during the 96 h survey period (Fig. 1C). In the 0.8 µm treatment, no HNFs were observed in any samples taken during the experiment. In the 5 µm treatments, HNF abundance increased by about one order of magnitude to values of 34.3×10^3 ml^{-1} but decreased at the end of the incubation; no ciliates were detected in this treatment (Fig. 1B). Plastid-containing, potentially mixotrophic flagellates were present at very low abundances *in situ* (a few *Dinobryon* sp. cells ml^{-1}) and in the experiments. Numbers of photosynthetic flagellates (largely *Rhodomonas minuta*; P. Znachor et al. unpubl.) were also well below 500 cells ml^{-1}, i.e. at the very low abundance typical for the clear water phase (Šimek et al. 2008). The HNF community was dominated by bacterivorous *Spumella*-like chrysophytes. One can assume shifts of a HNF community towards the dominance of HNFs best adapted to a bacterial community developing in experimentally manipulated (<5 µm) treatments without top-down control by larger zooplankton (Šimek et al. 2013). A sudden experimentally induced pulse in bacterial food availability was also proposed as a driving force for a rapid shift of an HNF community towards dominance of several *Spumella*-like phylotypes in the Římov Reservoir (Šimek et al. 2013). Ciliates were dominated by small algivorous prostomes (*Urotricha* spp. and *Balanion* sp.) and omnivorous oligotrichs (*Rimostrombidium* sp. and *Halteria grandinella*), i.e. ciliates with an equivalent cell diameter of 15 to 20 µm.

We found morphologically distinct VLPs with a head diameter of 405 ± 31 nm and a tail or remnants of a tail. The tail appeared flexible, had a diameter of ca. 70 nm and a length of ca. 1100 nm (Fig. 2A). The average head diameter of the total viral community was only ca. 65 nm (M. G. Weinbauer unpubl. data) and thus similar to other limnetic oxic environments, where the average capsid diameter is typically within the range of 60 to 70 nm (Weinbauer 2004). The VLPs constituting the distinct population can be classified as giant viruses, since this class of viruses was initially defined as having capsid diameters >300 nm (Bratbak et al. 1992).

The abundance of the giant VLP population was 3.3×10^4 ml^{-1} *in situ* and increased 7-fold during the survey period (Fig. 1C). The corresponding net production rate (calculated from the slope of a linear regression analysis of ln transformed data) was 0.70 ± 0.235 d^{-1}. In the <0.8 µm treatment with undetectable HNF concentrations, the abundance of giant VLPs decreased during the experiment and fell below the detection limit after 2 d (Fig. 1A). The decay rate was 0.50 ± 0.216 d^{-1}. In the <5 µm treatment, the abundance of giant viruses increased 15-fold (Fig. 1B) corresponding to a net production rate of 1.08 ± 0.288 d^{-1}. Production rates of giant VLPs (calculated as net production rates plus decay rates) were 1.2 d^{-1} in the reservoir and 1.6 d^{-1} in the <5 µm treatment. Absolute values of viral counts by TEM or epifluorescence microscopy might be an underestimation, e.g. due to storage at 4°C (Hennes & Suttle 1995, Wen et al. 2004). However, storage time before ultracentrifugation onto TEM grids was identical for all samples; thus, relative differences between treatments and rates should not be affected by such problems. In addition, large viruses seem to have lower decay rates than the average virus community (Heldal & Bratbak 1991).

Using published data of total VLPs from the reservoir at the sampling time (see also Fig. 1), the proportion of giant VLP as a fraction of total viral abundance *in situ* was ca. 0.3%. As a comparison, estimates of viruses with a capsid diameter of >100 nm in marine and limnetic systems range from <1 to 10% (Weinbauer 2004). The abundance of giant VLPs was correlated (Spearman rank correlation) with total grazing rates ($\rho = 0.95$; $p < 0.0001$) and HNF abundance ($\rho = 0.80$, $p < 0.001$). Other parameters such as total VLP abundance, bacterial abundance and total and cell-specific bacterial production (production data obtained from Šimek et al. 2001) showed no significant correlation with giant VLPs ($p > 0.05$). A multiple regression analysis of these parameters was significant ($r^2 = 0.78$, $p < 0.001$) and confirmed the Spearman rank correlation analysis. The result of the multiple regression analysis was calculated as:

$$VA_g = 0.54 - 0.34 \times FA + 1.91 \times GR \quad (1)$$

where VA_g is abundance of giant VLPs (10^4 particles ml^{-1}), FA is HNF abundance (10^3 cells ml^{-1} d^{-1}) and GR is the grazing rate (10^6 bacteria ml^{-1} d^{-1}). GR was measured by the fluorescently labeled bacteria approach and data were obtained from Šimek et al. (2001).

The following lines of evidence suggest that the giant VLPs infect HNFs: First, large viruses vanished in the HNF-free <0.8 um treatment, indicating that no giant VLPs were produced when no flagellates were present. Second, giant VLP abundances were higher in the <5 µm treatments than in the reservoir, i.e. they were highest at the most elevated HNF abundances. Third, the capsid size of giant VLPs was more than 4 times larger than the capsid size of viruses detected in bacterial cells by TEM in the same samples (M. G. Weinbauer unpubl. data). Fourth, the abundance of large viruses was only significantly related to HNF abundance and grazing rate (explaining 78% of the variation in a statistical sense) and not to bacterial or ciliate parameters, thus suggesting an HNF origin of the giant VLPs. Finally, a TEM picture indicates a eukaryotic rather than a bacterial cell as host (for details see below).

The size of particles in the plankton is a major factor influencing contact rates between viruses and cells (Murray & Jackson 1992). Thus, the large head and tail size of giant VLPs could increase contact rates. This can be seen as adaptations of giant VLPs to low (relative to bacteria) host abundances. In addition, giant VLPs fall well within the optimum size range of food for HNFs (Šimek et al. 2001), particularly when regarding not only capsid size but also tail length. Thus, on the one hand grazing of giant VLPs could be a (moderate) source of C, N and P for HNFs (and ciliates) (González & Suttle 1993, Bettarel et al. 2005) and, on the other hand, grazing could be a defense against lytic infection by giant VLPs (unless infection occurs in the food vacuole). Alternatively, induction of cells with a latent infection as mechanisms of viral production (Massana et al. 2007) cannot be excluded; however, potential induction agents remain unknown.

The potential virus-induced mortality of HNFs (VMF) due to lysis by giant VLPs was calculated as:

$$VMF = 100 \times [(\mu VA_g \times VA_g/BS) / (\mu FA \times FA)] \quad (2)$$

where VA_g and FA are abundances at the start of the *in situ* survey (Fig. 1), μVA_g is the growth rate of giant VLPs (Table 1), µFA is the growth rate of HNF, estimated at 1.59 d^{-1} in the experiment (Šimek et al. 2001) and BS is the burst size, using the value of 70 estimated by Massana et al. (2007). VMF was calculated as 0.57×10^3 cells ml^{-1} d^{-1} (9.4%) *in situ* and 0.71×10^3 cells ml^{-1} d^{-1} (12.5%) in the <5 µm treatment. Viruses typically infect only a small range of eukaryotic hosts (Massana et al. 2007). Thus, the mortality rates have to be considered as a conservative estimate. In addition, our data might be an underestimation, since the loss of infectivity is likely higher than the loss (decay) of particles. Also, the BS we used might be an overestimation, since BS is typically elevated in isolated virus–host systems compared to *in situ* (Børsheim 1993). A TEM micrograph from the <5 µm treatment of our experiment shows a group of VLPs identical in size to the giant VLPs embedded in an organic matrix (Fig. 2B). Note that the seemingly elongated dimensions of some particles is a photographic artifact, which becomes evi-

Table 1. Production and decay rates (d^{-1}) of total and giant VLPs in incubations of filtered (<0.8 and <5 µm) and *in situ* reservoir water. Positive values indicate production, negative values indicate decay. Values for net production/decay are mean ± SD. Production rates in the final column are calculated by adding mean decay rates of particles (as estimated in the <0.8 µm treatment) to net production rates. NA: not applicable

Treatment	Net production of total VLPs (d^{-1})	Net production/ decay of giant VLPs (d^{-1})	Production of giant VLPs (d^{-1})
<0.8 µm	0.38 ± 0.02	−0.5 ± 0.2	NA
<5 µm	0.53 ± 0.02	1.1 ± 0.3	1.6
Reservoir	0.48 ± 0.03	0.7 ± 0.2	1.2

dent by changing the plane of focus during TEM inspection, revealing 2 distinct viral particles. The number of viruses in this presumptive lysing flagellate cell was ca. 15. Using this BS, mortality estimates for the entire flagellate community *in situ* and in the experiments would be 44 to 59%, corresponding to 2.6 to 3.3 × 10^3 flagellate cells ml^{-1} d^{-1} lysed by viruses. Our observations and rate estimates suggest a potentially important role for viruses infecting HNFs. Targeted and intensive studies are needed to deepen our understanding of the dynamics of the viruses of HNFs.

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