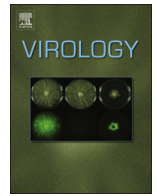




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## A new nidovirus (NamDinh virus NDiV): Its ultrastructural characterization in the C6/36 mosquito cell line



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### ABSTRACT

We describe the ultrastructure of the NamDinh virus (NDiV), a new member of the order *Nidovirales* grown in the C6/36 mosquito cell line. Uninfected and NDiV-infected cells were investigated by electron microscopy 24–48 h after infection. The results show that the viral nucleocapsid-like particles form clusters concentrated in the vacuoles, the endoplasmic reticulum, and are scattered in the cytoplasm. Mature virions of NDiV were released as budding particles on the cell surface where viral components appear to lie beneath and along the plasma membrane. Free homogeneous virus particles were obtained by ultracentrifugation on sucrose gradients of culture fluids. The size of the round-shaped particles with a complete internal structure was 80 nm in diameter. This is the first study to provide information on the morphogenesis and ultrastructure of the first insect nidovirus NDiV, a missing evolutionary link in the emergence of the viruses with the largest RNA genomes.

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### Introduction

NamDinh virus (NDiV), named after a Vietnamese province, was isolated from mosquitoes and is yet to be linked to any pathology. Recently, molecular biology methods were used to identify this mosquito-borne virus as possessing a 20.192 kb genome, the largest genome among non-segmented single-stranded RNA viruses of insects, and its characteristics have defined NDiV as the prototype of a new member of the order *Nidovirales* (Nga et al., 2011). Members of the *Nidovirales* have a wide range of hosts including crustaceans, fishes, birds and a variety of mammals (Siddell et al., 2005). They included the *Coronaviridae*, *Arteriviridae* and *Roniviridae* families that are known to re-arrange host cell membranes for the purpose of replication and assembly. However, many details of these processes remain obscure, and knowledge of the ultrastructural characterization and the various steps in the replication process of these viruses in cell culture is still very limited. Using improved cryo-fixation methods, electron tomography and immunogold electron microscopy (EM), (2012) showed recently that the arteriviral RNA is present within double-membrane vesicles, but no obvious communication with the cytoplasm was detectable. These authors

also investigated the structure of the nidovirus-induced membrane modifications and found that these viruses transform the endoplasmic reticulum (ER) into a reticulo-vesicular network that integrates numerous inter-connected double-membrane vesicles (Knoops, 2011). Here we have applied EM methods to investigate the morphological and ultrastructural features of NDiV grown in C6/36 mosquito cells via ultrathin sections, negative staining and immunogold labeling. The results provide useful information regarding NDiV, the first insect nidovirus identified in mosquitoes.

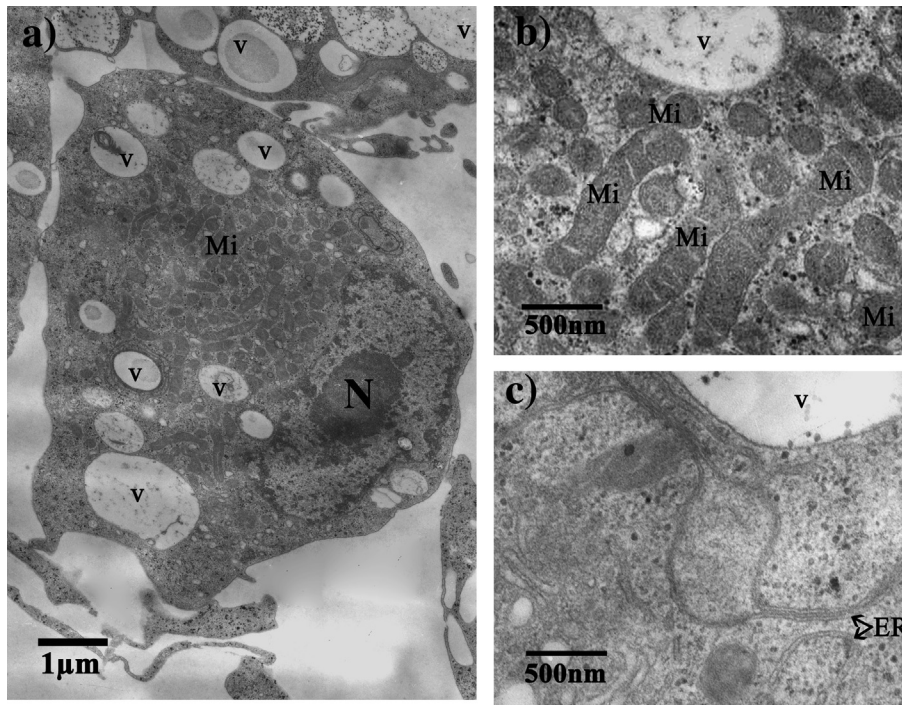
### Results

C6/36 mosquito cells possess typical round shape with the nucleus remaining on one side of the cell throughout 3 days of observation (Fig. 1a). At higher magnifications, it is easy to recognize numerous mitochondria, vacuoles (Fig. 1a and b), and the ER (Fig. 1c) in the cytoplasm. By EM, the control culture did not appear contaminated with other mosquito viruses.

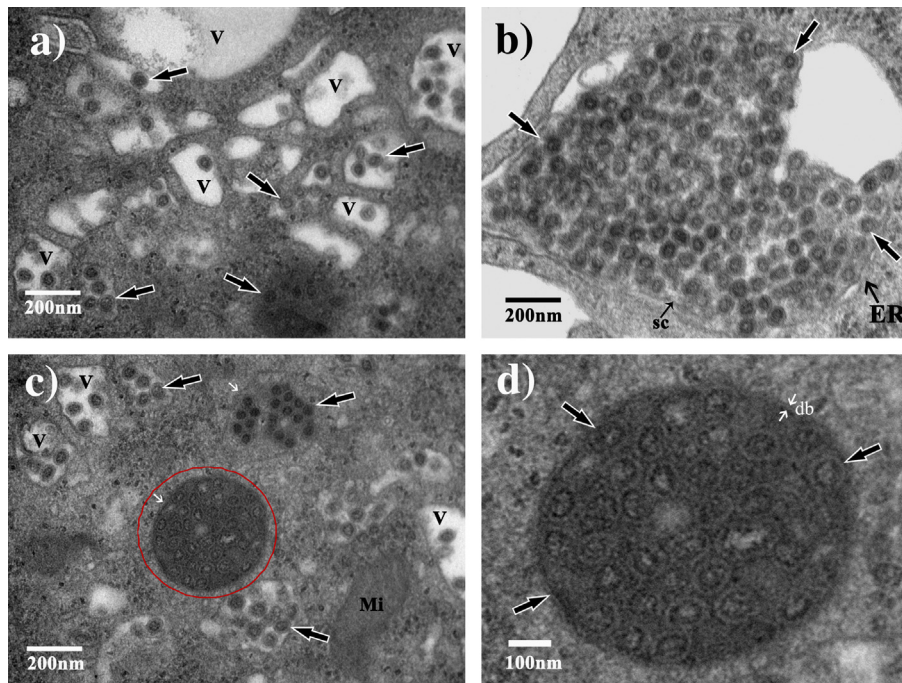
Twenty-four hours after infection, the shape of the cells did not differ from that of control cells and EM observations did not reveal intact virions outside the cells. Viral nucleocapsid-like particles appeared accumulated in cell vacuoles (sc) (Fig. 2a) as well as in the ER which showed swollen cisternae (Fig. 2b) in comparison with the ER in normal cells (Fig. 1c). Individual nucleocapsid-like particles appeared round-shaped, homogenous in size and with

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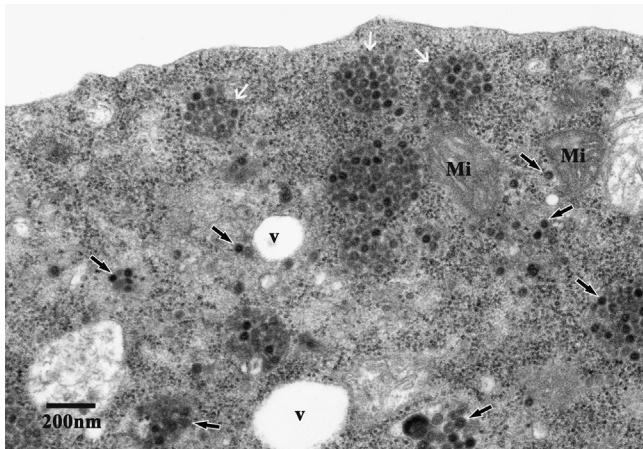
**Fig. 1.** Uninfected C6/36 mosquito cells: (a) a typical round shape and the nucleus (N) are visible on one side of the cell; a great number of vacuoles (v) and mitochondria (Mi) are present in the cytoplasm; (b) higher magnification of numerous mitochondria in the mosquito cell; and (c) clear endoplasmic reticulum (ER).



**Fig. 2.** Assembly of NDIV in the cytoplasm of C6/36 mosquito cells 24 h after infection; (a) viral nucleocapsid-like particles (arrows) of ~50 nm in diameter, accumulate in the vacuoles (v); (b) viral nucleocapsid-like particles (arrows) accumulate in the swollen cisternae of the ER (sc); (c) other vesicles (small white arrows) with granular material are interspersed among the viral nucleocapsid-like particles (arrows); and (d) higher magnification of a vesicle shown in Fig. 2c (red circle) with a double membrane (db), containing dark granular material interspersed among viral nucleocapsid-like particles.

an electron-dense interior containing the viral genetic material. The average diameter of the viral nucleocapsid-like particles was 50 nm. Vesicular structures containing electron-dense material were also detected in the cytoplasm and identified as putative viral nucleocapsid inclusions. These vesicles possessed double-membranes (Fig. 2c and d).

After 48 h of infection, the mosquito cells had changed more dramatically. By EM observation, the cytopathic effect appeared clearly as cell swelling and cell aggregation (data not shown). Abundant NDIV nucleocapsid-like particles appeared as clusters or free in the cytoplasm; no viral nucleocapsid-like particle was identified in mitochondria (Fig. 3) or in the nucleus (data not shown).



**Fig. 3.** A C6/36 mosquito cell 48 h after infection. Viral nucleocapsid-like particles (arrows) are scattered in the cytoplasm and numerous such particles are grouped in vesicles (small white arrows) and lie close to the plasma membrane.

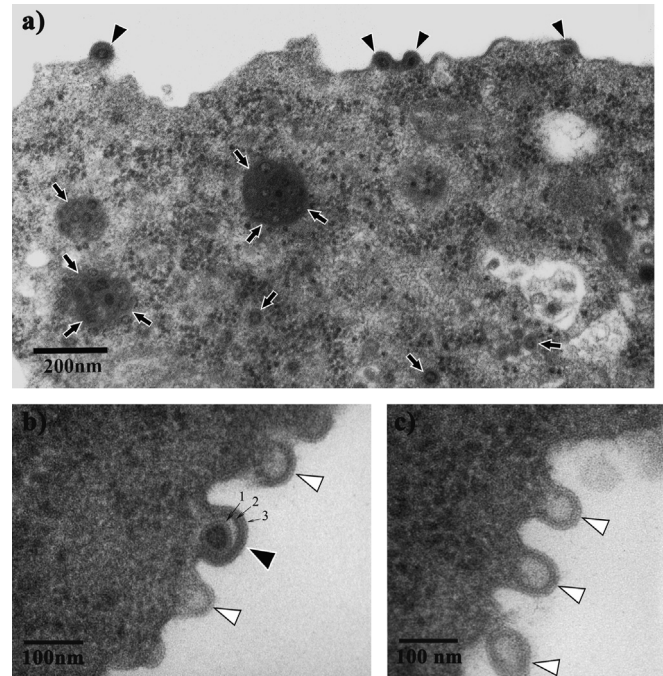
In many cells, 48 h post-infection, the numerous aggregates of viral nucleocapsid-like particles appeared to migrate to the cell surface (Fig. 3). At this time point, budding particles were detectable in some cells as protrusions from the plasma membrane; viral nucleocapsid-like particles appeared close to the plasma membrane where putative proteins of the viral envelope had associated (Fig. 4). In the mature period (48 h after infection; budding process), the NDIV clearly appeared at the level of plasma membrane protrusions: the viral envelope consisted in a trilaminar membrane including the host membrane. At this final stage (just before cell lysis), the virus particles appeared ready to bud (Fig. 4b and c) as complete virions (black arrowheads) or as incomplete virions (white arrowheads).

The viral nucleocapsid-like particles detected in the cytoplasm (white arrows) and accumulated in the ER vesicles (Ves) of 48 h NDIV-infected C6/36 cells, appear immunogold-labeled on ultrathin sections with anti-NDIV polyclonal antibodies, as shown in Fig. 5. No viral nucleocapsid-like particle was found in mitochondria (Mi).

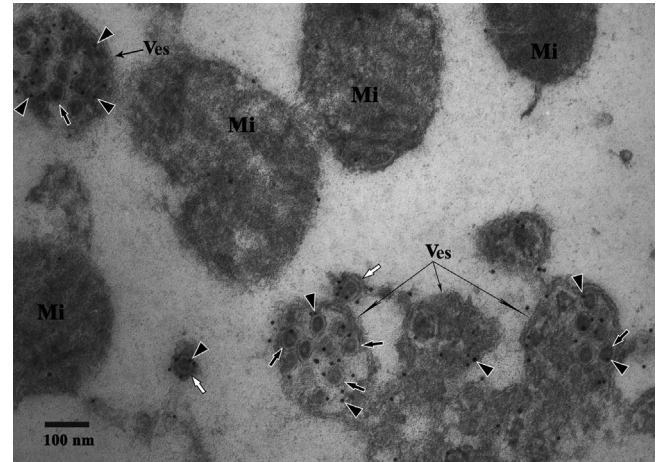
We have been successful in visualizing the virus particles in concentrated fluids by negative staining and immunogold EM. The virions appeared round-shaped with complete (black arrowheads) and incomplete (white arrowheads) virus particles. The diameter of the virus particles was about 60 nm for the incomplete particles and about 80 nm for the complete particles (Fig. 6a). We have measured the size of the smallest and biggest particles of more than 25 particles each per picture, with three pictures. The envelope layer measured 12 nm in width and appeared rather smooth. Higher magnifications of virion images revealed short spikes of NDIV, measuring 3–4 nm in length (framed image in Fig. 6a). Immunogold labeling of non-fixed isolated virions using the polyclonal antibodies indicated a specific antigenic reaction against the viral envelope (Fig. 6b).

## Discussion

NDIV is the first insect nidovirus described, a missing evolutionary link in the emergence of viruses with the largest RNA genomes, and it was identified as the prototype of a new member of the order *Nidovirales* (Nga et al., 2011). This study describes the morphogenesis and ultrastructural characterization of NDIV by ultrathin sections, negative staining and immunogold EM. NDIV replication appeared to occur in the cytoplasm of the host cells as is the case for other single-stranded RNA viruses (Denison, 2008). Viral nucleocapsid-like particles also accumulated in cell vacuoles

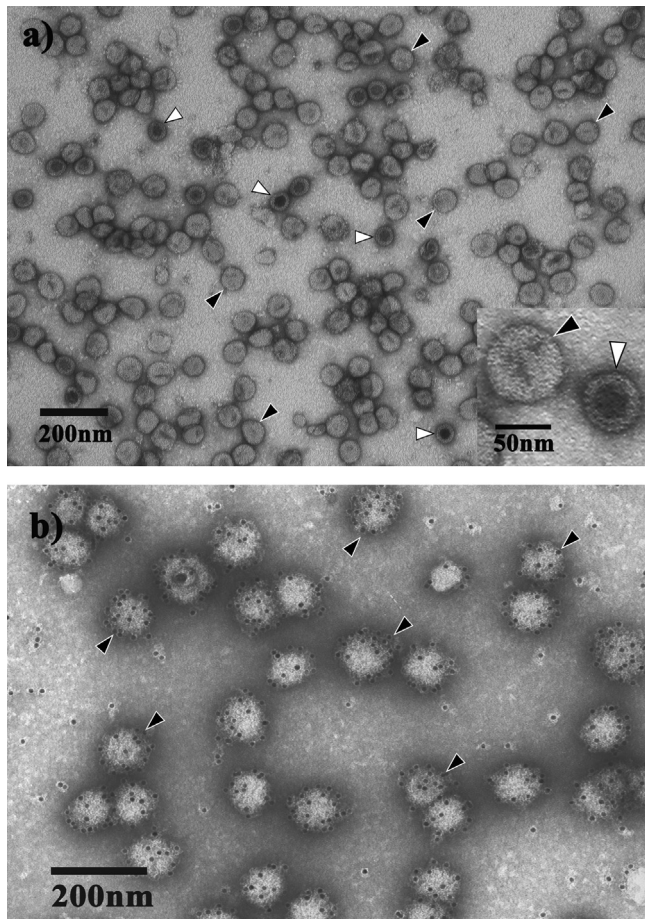


**Fig. 4.** Assembly of NDIV in C6/36 mosquito cells 48 h after infection: (a) viral particles (arrowheads) bud at the surface of the plasma membrane, whereas other viral nucleocapsid-like particles (arrows) are grouped in vesicles; (b) detail of the budding process: viral nucleocapsid-like particles are inserted into plasma membrane protrusions, forming a complete virion (black arrowheads); and (c) incomplete virions also appear without nucleocapsid (white arrowheads).



**Fig. 5.** Immunogold labeling of a thin section of NDIV-infected C6/36 mosquito cell, 48 h after infection. Viral nucleocapsid-like particles (arrows) appear in the cytoplasm (white arrows) and accumulate in endoplasmic vesicles (Ves), labeled with the polyclonal antibodies revealed by 10 nm gold particles (arrowheads).

and in the ER which showed swollen cisternae. Other nidoviruses replicate their genomes in the cytoplasm in association with intracellular membrane rearrangements as single- or double-membrane vesicles (Knoops et al., 2012; Knoops, 2011). However, the exact sites of RNA synthesis and the relationships between these membrane vesicles and the cytoplasm remain undefined. In this study, the nucleocapsid-like particle of NDIV was identified in the host cell cytoplasm as possessing a round shape and an inner core observed in several types of vesicles and in the ER and its viral nature was confirmed by immunogold labeling on thin sections. In contrast to members of the *Coronaviridae* family such



**Fig. 6.** (a) Negative stained preparation of isolated NDIV, concentrated from culture supernatant of 48 h-infected C6/36 mosquito cells, showing complete (black arrowheads) and incomplete (white arrowheads) virions, mainly of spherical form with a diameter of 60–80 nm, respectively. The frame in Fig. 6a reveals short spikes on the virions 3–4 nm long; (b) the immunolabeling of non-fixed virions with the polyclonal antibodies, revealed with protein A conjugated to 10 nm gold particles, surrounds the envelope of the viral particles (black arrowheads).

as SARS-CoV, NDIV particles are formed on membranes of the “budding compartment”, a term used to describe the continuous membranous system from the ER to the Golgi complex. In our study, replication of NDIV in the cytoplasm of C6/36 cells closely follows the ER and vesicular localization, and the viral nucleocapsid-like particle accumulates in endoplasmic vesicles as does the replication complex for coronaviruses. Certain double-membrane vesicles also contain dark granular material interspersed among viral nucleocapsid-like particles (Goldsmith et al., 2004; Knoops et al., 2008; Le et al., 2011), while virions bud from the cytosol. However, we could not identify any opening or pore in these vesicles or in the ER that would allow movement of the viral RNA from the vesicles or ER compartments to the cytosol. Replication of NDIV in C6/36 mosquito cells was followed by a maturation period on the plasma membrane during which viral nucleocapsid-like particles were inserted into “protrusions” of this membrane to export the complete virions and the incomplete virions lacking the nucleocapsid. Assembly and budding of NDIV have characteristics in common with those of other members of several RNA virus families such as the *Togaviridae*, *Rhabdoviridae*, *Paramyxoviridae*, *Orthomyxoviridae* and *Retroviridae* (Ahmad et al., 2011; Haenni and Diaz-Ferrao, 2011; Hunter, 2001; Rota et al., 2003; Zhong et al., 2013). Assembly of NDIV at the plasma membrane obviates the need for the assembled particle to navigate to an additional compartment of the secretory pathway

because the virions are released directly into the external milieu of the cell. For most enveloped viruses, the location within the cell where envelopment takes place is determined by targeting of the viral glycoprotein(s) at that site (Hunter, 2001). However, the proteins of NDIV have only been identified by molecular biology techniques (Nga et al., 2011), so that the biological functions of these proteins are not fully understood. Furthermore, viral nucleocapsid-like particles may be morphologically confused with other structures in the cytoplasm such as coated vesicles, multi-vesicular bodies, perichromatin granules and glycocalyxal bodies. Immunogold EM of ultrathin sections confirmed that these viral nucleocapsid-like particles belong to NDIV and appear in the cytoplasm and endoplasmic compartments of the host cells, whereas no viral structures were found in the mitochondria or the nucleus.

NDIV isolated from C6/36 cells and highlighted by negative staining presented a homogenous spherical shape. Yet NDIV differs from Cavally virus, a novel insect nidovirus characterized in 2011 as an enveloped spherical CoV-like virion with a diameter of 120 nm, and “club-shaped” surface projections (Zirker et al., 2011). NDIV is an enveloped virus, whose projections or spikes on the membrane of the viral envelope remain very short, 3–4 nm long and that can identify and bind to receptor sites on the host cell membrane. In addition to the negative staining method, the NDIV was examined by immunogold labeling that expanded the range of applicability of the negative staining technique (Beesley and Betts, 1985; Nguyen et al., 2001). Immunogold EM makes it possible to detect and locate proteins of antigens in viruses or cells and can help connect a visible structure with a specific *in situ* localization site and establish the distribution of a molecule at high resolution (Beesley and Betts, 1985; De Paul et al., 2012; Hyatt and Eaton, 1993; Nguyen et al., 2001; Kanak et al., 2002). Immunogold EM on unfixed particles of NDIV revealed labeling of the envelope protein(s) by the presence of 10 nm gold particles around isolated virions. In our experiments, NDIVs are labeled with an average of 15 gold particles per virion using polyclonal antibodies prepared against the entire viral particle. This value probably reflects the topographic distribution of the viral proteins in non-fixed virus particles, and the accessibility of specific epitope constituents. However, its resolution is limited by the steric hindrance generated by the packing density of the antigenic sites associated with different constituents of the viral envelope. NDIV possesses four virion proteins (p2a, p2b, p3, and p4) as demonstrated previously but the number of antigenic sites of NDIV is still unknown (Nga et al., 2011). With respect to nidoviruses, information concerning the viral envelope proteins of members of the *Arteriviridae* and *Coronaviridae* families is available (Dubois-Dalcq et al., 1984; Pedersen et al., 1999), but information on the NDIV envelope proteins as well as on the biological functions of these proteins during replication in the host cell requires further studies with the support of specific monoclonal antibodies against the NDIV proteins.

## Conclusion

Using EM methods, this study reports valuable information on ultrastructural aspects of NDIV grown in C6/36 mosquito cells for 24–48 h after infection. The results demonstrate that NDIV possesses a homogenous spherical shape, with a diameter of 60–80 nm. By EM analyses, we show that replication and assembly of NDIV was only detected in the cytoplasm of the host cells in which viral nucleocapsid-like particles appeared in the cytoplasm and in the endoplasmic compartments such as vacuoles, ER, and vesicles, but not in mitochondria. Viral particles were released 48 h post-infection via budding at the surface of the plasma membrane.

## Materials and methods

### Virus inoculation

NDiV obtained from the National Institute of Hygiene and Epidemiology, Hanoi, Vietnam, was identified in four mosquito pools, two from *Culex vishnui* and two from *Culex tritaeniorhynchus*, collected in two provinces of Vietnam. Mosquito handling for virus isolation and propagation in cell culture was described previously (Nga et al., 2011). Virus stock titers were determined via the plaque assay using C6/36 mosquito cells, and stocks were stored at  $-80^{\circ}\text{C}$ . C6/36 cells were infected at a low multiplicity of infection of 0.1, with the virus obtained from limiting dilution endpoints of early-passage supernatants (Zirkel et al., 2011).

### Electron microscopy

#### Ultrathin sections

C6/36 mosquito cells grown at  $28^{\circ}\text{C}$  were infected with  $2 \times 10^4$  pfu/mL of NDiV as described previously (Nga et al., 2011). Twenty-four and 48 h after infection, most of the medium and floated cells were discarded, the cells were detached from the culture flask with a cell scraper, and the cell suspension was centrifuged at 2000 g for 5 min. The pellet was re-suspended in phosphate buffered saline (PBS) pH 7.2 without  $\text{Ca}^{++}$  and  $\text{Mg}^{++}$ , and the suspension was spun at 2000 g for an additional 5 min. The cell pellet was re-suspended and fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer pH 7.2 and kept overnight at  $4^{\circ}\text{C}$ . The sample was then rinsed carefully several times in 0.1 M cacodylate buffer pH 7.2; the final pellet was fixed with 1%  $\text{OsO}_4$  in the same buffer for 60 min and subsequently dehydrated in graded ethanol (50–100%), washed in propylene oxide and infiltrated for 6 h in a 1:1 mixture of propylene oxide and epoxidic resin (Epon). The cells were finally embedded in Epon 812. Ultrathin 70 nm thick sections were obtained with an ultramicrotome (Ultracut UC6, Leica) and stained with uranyl acetate and lead citrate.

#### Immunogold EM of ultrathin sections

C6/36 cells infected for 48 h with NDiV were detached as described above, washed with PBS and the final pellet was fixed in 3% paraformaldehyde in PBS, pH 7.4, for 1 h. After dehydration in graded ethanol (50–100%) the cells were embedded in LR White resin (EMS). After polymerization of the resin for 48 h at  $37^{\circ}\text{C}$ , the ultrathin 70 nm thick sections were collected on nickel mesh grids and processed for immunolabeling.

In the present study, three flasks each containing NDiV-infected C6/36 cells prepared in the same conditions and time points after infection were collected; EM observations were performed on ultrathin sections and at least 10–15 sections were examined per sample per time point.

#### Negative staining and immunogold EM

The virus was concentrated from 48 h-infected cell culture fluid, by centrifugation at 12,000 g for 30 min at  $4^{\circ}\text{C}$ , after which 6.6% polyethylene glycol 6000 and 2.2% NaCl were added to the supernatant. After stirring for 1 h at  $4^{\circ}\text{C}$  and centrifugation at 12,000 g for 1 h, the supernatant was discarded. The virus-containing pellet was re-suspended in saline–Tris–EDTA buffer pH 7.4, sedimented at 250,000 g for 1 h and resuspended a second time. Purified viral material was used for antibody preparation, and EM studies.

For negative staining, 30 ml droplets of NDiV suspensions were put on carbon-coated grids for 10 min to pick up the virus sample; the grids were then fixed in 1% glutaraldehyde for 5 min, washed

several times in 0.1% ammonium acetate and stained with 1% uranyl acetate for 5 min.

For immunogold labeling of the NDiV suspension, the method used was that of Nguyen et al. (2001). Droplets of NDiV suspension (30 ml) were placed on a clean parafilm surface and collodium carbon-coated grids were placed on top of the droplets for 10 min to pick up the virus sample. The grids were washed with PBS and allowed to float on a droplet of PBS with 2% bovine serum albumin (BSA) for 30 min to block non-specific antigenic sites. The grids were then made to react for 30 min with anti-NDiV rabbit antibodies diluted in PBS–0.5% BSA. After careful washing with PBS–0.2% BSA several times for 10 min, the grids were incubated with protein A conjugated to 10 nm gold particles for 30 min. This step was followed by several washes with PBS as above and the grids were fixed with 0.1% glutaraldehyde for 5 min. They were then washed thoroughly with 0.1% ammonium acetate and negatively stained with 1% uranyl acetate for 5 min. The procedure of immunogold EM on ultrathin sections was performed similarly (Hyatt and Eaton, 1993). Control experiments testing the specificity of the immunolabeling were carried out by incubating some of the samples directly with gold-labeled protein A, without previous incubation with specific antibodies. Other control experiments were carried out by incubating some samples with non-specific antibodies. The grids were not allowed to dry during the procedure and always floated on the surface of the drops. All steps were performed at room temperature. The samples were observed through a transmission EM JEM1010-JEOL, operating at 80 kV.

#### Polyclonal antibodies

Polyclonal antibodies were prepared using the entire isolated viral particles as antigen; the dose injected into rabbits was 50 mg NDiV particles diluted in 0.5 ml PBS and mixed with Freund complete adjuvant, in a volume ratio of 1:1 on days 1, 7, 14, and 21 (four booster injections). The dose injected was increased to 150 mg NDiV particles diluted in 0.5 ml PBS mix with Titermax classic adjuvant, in a 1:1 ratio for the fifth booster injection on day 35. Two weeks after the fifth injection, the IgG titer of the antibodies against NDiV was determined by the indirect ELISA method and the IgGs were separated; blood was taken from the rabbit heart, kept at room temperature for 30 min, and then at  $4^{\circ}\text{C}$  overnight. The blood was centrifuged at 2500 rpm for 15 min, and the serum was separated and kept in aliquots at  $-20^{\circ}\text{C}$ .

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