Immunosorbent Electron Microscopy for Detecting Apple Chlorotic Leaf Spot and Plum Pox Viruses

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ABSTRACT

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Plum pox virus (PPV) and apple chlorotic leaf spot virus (CLSV) were detected in extracts of leaves of infected trees by immunosorbent electron microscopy (ISEM). Attachment of virus particles to antiserum-coated electron microscope grids provided a test as sensitive as the enzyme-linked immunosorbent assay (ELISA). Confirmation of the identity of the bound particles was obtained by coating further with homologous antibody followed by sheep antirabbit immunoglobulins which increases the sensitivity and facilitates the interpretation of the test. These results confirm the usefulness of ISEM for detecting several viruses and indicate its potential as a rapid and reliable alternative to ELISA for the detection of PPV and CLSV.

For many years, fruit tree viruses have been detected by transmission to susceptible indicator plants in the nursery or in the greenhouse. Detection of viruses by the leaf dip electron microscope technique, sometimes used with herbaceous plants, was generally unsuccessful in woody plants. Before immunoenzymology, serological techniques were applied to only a few viruses in woody plants because of low levels of sensitivity. Immunoenzymatic methods were first used with animal pathogens and one of these techniques, the enzyme-linked immunosorbent assay (ELISA), was applied to plant viruses in 1976 (3,19). Its high sensitivity (1,000–10,000 times more than the usual serological techniques) and ease of use are advantageous in the detection of plant viruses (1,4,12,15). Recently, another sensitive technique, immunosorbent electron microscopy, has been used to detect many plant viruses (2,6,11,18). In a short communication (17), we noted the possibility of using these techniques to detect plum pox virus (PPV), a fruit tree virus that represents a severe danger to the fruit-growing industry.

This paper summarizes the results obtained in the application of immunosorbent electron microscopy for detecting PPV and apple chlorotic leaf spot (CLSV), another virus that induces serious diseases, and describes a modification of the basic technique that facilitates the observation of virus particles and consequently increases its sensitivity.

MATERIALS AND METHODS

Virus isolates. Plum pox virus was investigated in *Pisum sativum* 'Serpette d'Auvergne' and 'Express généreux'; in peach seedlings GF 305 experimentally infected with D_1 and M_1 strains (14); and in naturally infected peach, plum, and apricot trees. Immunosorbent electron microscopy was used to detect CLSV in *Chenopodium quinoa* Willd. experimentally infected with strains In 21 and P 863 of CLSV (10) and naturally CLSV-infected peach and plum trees also were used. Some experiments also were carried out with purified virus prepared according to a method previously described (8).

Antisera. Antisera were prepared against the P 863 strain of CLSV and against the D_1 and M_1 strains of PPV (9,13). Each antiserum had a reciprocal titer of 1,024 by gel double diffusion.

Serology. The technique developed here is referred to as immunosorbent electron microscopy (ISEM) (18). It follows the protocol of Derrick (5) as modified by Milne and Luisoni (16). Antibodies were adsorbed to a carbon-coated grid by floating the grid for 5 min on antiserum diluted 1:100; after washing, the grid was floated for 15 min on an extract from infected plants and the preparation was then stained with 2% uranyl acetate. In most experiments after washing and before staining, virus particles were decorated by floating the grid on diluted (1:100) antiserum; after a second wash, the grid was drained and stained. Inclusion of a second decoration step was advantageous. For this, after the second incubation with antiserum, and before staining, the grids were washed, drained and floated for 10 min on a solution of antirabbit immunoglobulin (IgG) from sheep (Institut Pasteur, Paris) diluted 1:100. Control samples were prepared by the standard leaf dip method. All steps were carried out at room temperature and preparations were examined in a Siemens Elmiskop 101 microscope.

Preparation of the leaf extract. There do not seem to be any universal conditions for the preparation of the plant extract. Most authors recommend grinding with phosphate or tris buffers or water, which are convenient for use with herbaceous tissue. Woody plant tissue often contains inhibitors that interfere with the detection of low concentrations of viruses. This difficulty can be overcome by the use of additives (7,17). Nicotine at 2.5% in water or phosphate buffer was essential for detection of PPV in plum and apricot tissues. To preserve the structure of the CLSV particle, the extraction buffer for ISEM samples was the same as that described for virus purification and for ELISA (7). CLSV-infected leaf material was ground in 0.05 M Tris buffer with 5 mM $MgCl_2$ and 0.2% diaminodipropylamine, pH 8. As indicated above for PPV,

TABLE 1. Effect of coating electron microscope grids with homologous antibody on the number of plum pox virus (PPV) and apple chlorotic leaf spot virus (CLSV) particles observed

Host plants	PPV		CLSV	
	Uncoated grids	Coated grids	Uncoated grids	Coated grids
Herbaceous host	0–5[a]	100–1,000	0	100–1,000
Peach	0–2	20–50	0	10–25
Plum	0	20–40	0	5–10
Apricot	0	10–20	NT[b]	NT[b]

[a] Numbers indicate the average number of particles per mesh adhering to the electron microscope grid. Five meshes were examined for each treatment. One hundred infected samples were examined for each host plant; the same extract prepared from a 10 mm^2 piece of one infected leaf was used for the coated and uncoated grids.
[b] Not tested.

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the buffer must be supplemented with 2.5% nicotine when the sample is from plum or apricot. About 100 samples have been examined for each CLSV-infected host plant. In the case of PPV, for which ISEM is frequently used in our laboratory as a detection method, more than 500 samples have been investigated. When two techniques were compared, the same extract prepared from a piece of one leaf was used.

RESULTS

Detection of CLSV and PPV by ISEM. Virus particles from woody tissue were not readily detectable by the standard leaf dip procedure; only a few elongated particles resembling PPV were found in infected peach leaf samples (Fig. 1A). By comparison, the use of ISEM resulted in an increased number of virus particles

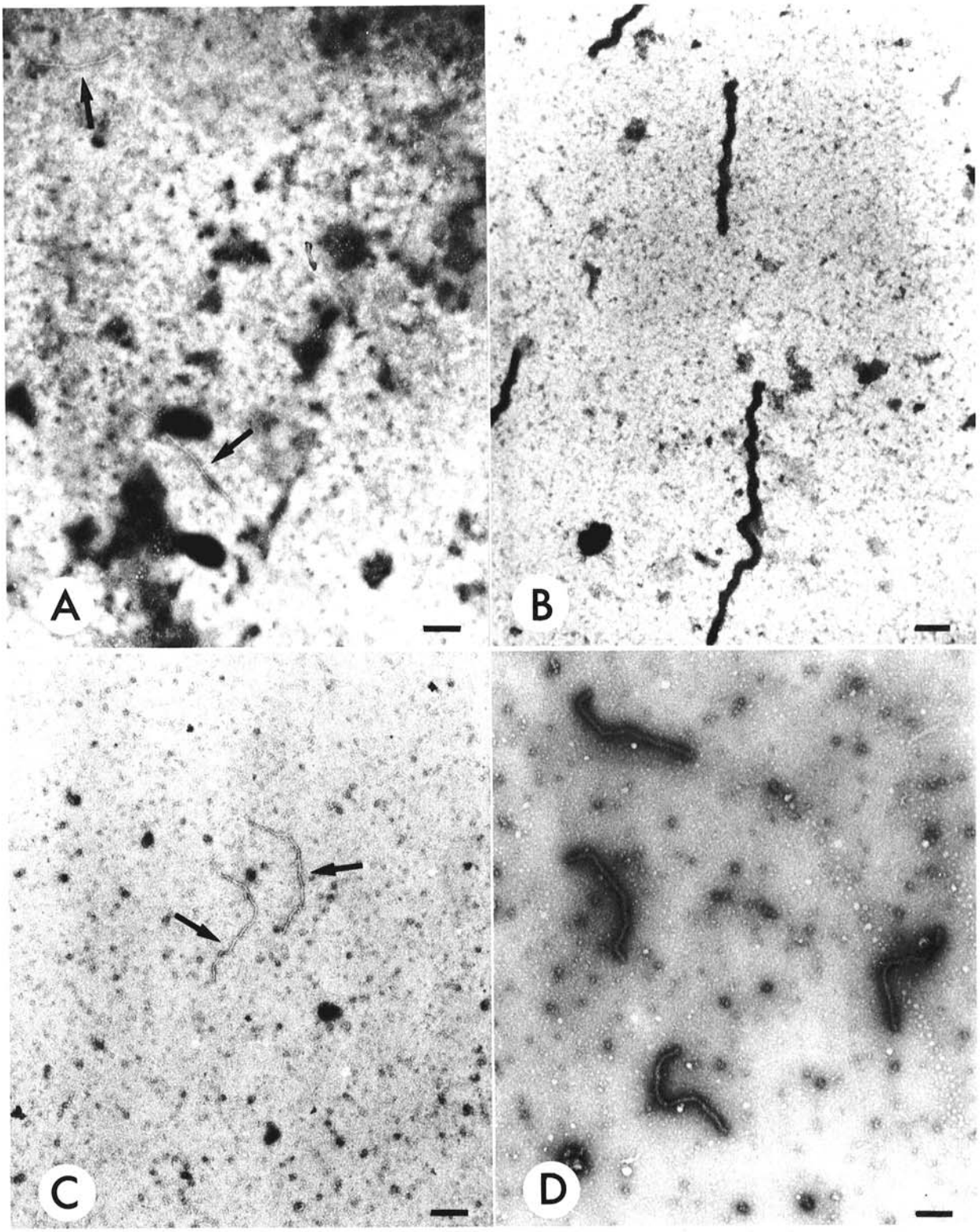

Fig. 1. Immunosorbent electron microscopy (ISEM) of plum pox virus (PPV) and apple chlorotic leaf spot virus (CLSV). **A,** Control grid: leaf dip procedure. PPV-infected peach leaf, two particles (arrows) can be observed (×33,000). Bar = 200 nm. **B,** ISEM (with decoration). PPV-infected plum leaves: presence of several coated particles (×33,000). Bar = 200 nm **C,** ISEM (without decoration). Two CLSV particles (arrows) from infected peach leaves trapped on a CLSV antibody-coated grid can be observed (×33,000). Bar = 200 nm. **D,** ISEM (with decoration). Antibody-coated CLSV particles from plum leaves (×33,000). Bar = 200 nm.

being trapped on the grid (Table 1) as seen by comparing uncoated and PPV-antibody-coated grids (Fig. 1A,B). In extracts from CLSV-infected woody plants in which no particles were noted in controls without antibodies, 10–50 particles per grid square were observed in ISEM on a 38-μm (400-mesh) CLSV-antibody-coated grid (Fig. 1C), suggesting a protective effect on the particle by antibody coating. In addition, the attachment of antibodies to virus particles in the decoration step increases the apparent width of the particles about three times (Fig. 1B,D) making the observation easier and the diagnosis quicker. Moreover, as some virus particles

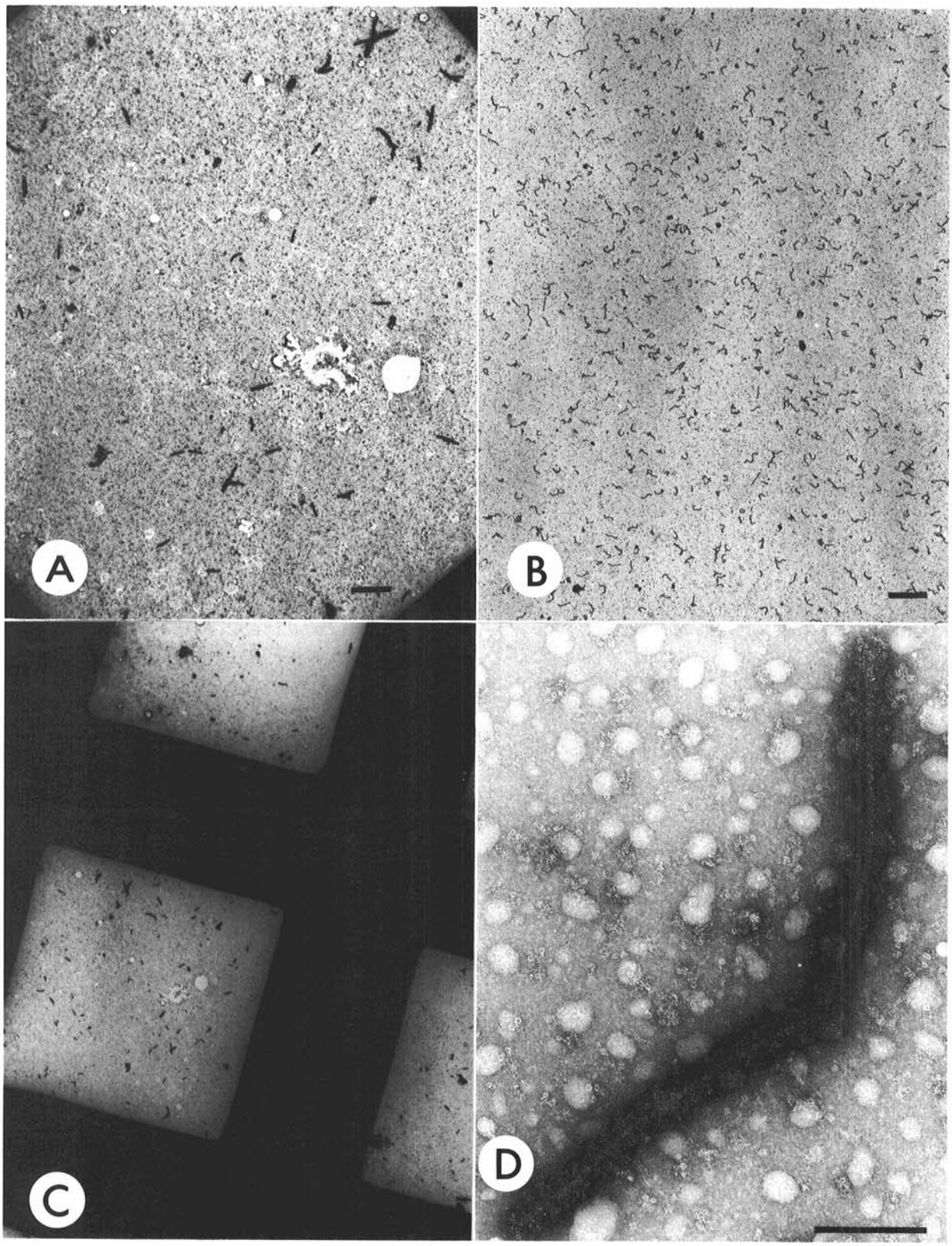

Fig. 2. Immunosorbent electron microscopy of plum pox virus (PPV) and apple chlorotic leaf spot virus (CLSV). **A,** Double antibody-coated PPV particles from peach leaf on a 38-μm (400-mesh) grid (×3,300). Bar = 2,000 nm. **B,** Double antibody-coated CLSV particles from *Chenopodium quinoa* leaf at 48-μm (300-mesh) grid (×3,300). Bar = 2,000 nm. **C,** Double antibody-coated PPV particles from peach leaf (×1,100). **D,** Detail of double antibody-coated PPV (×95,000). Bar = 200 nm.

can be adsorbed directly on the grids without being trapped by antibodies, the decoration confirms the identity of the virus. PPV and CLSV were readily detected in infected peach and plum trees. Apricot trees were found to have PPV, but CLSV was not investigated in this material. Strain specificity was not a problem. Similar results were obtained for PPV with the D_1 and the M_1 antisera. With CLSV and PPV, all the selected strains and natural isolates could be detected. This confirms the results previously reported with ELISA (7) and implies a limited antigenic variation of these two viruses.

Improvement of ISEM by double antibody decoration. As reported above, the number of CLSV or PPV particles observed in woody plants is not as high as in herbaceous plants (Table 1). With a mean number of 10–50 virus particles per grid square and a magnification of ×15,000–20,000 as generally used in ISEM, no more than two to three particles could be observed in the field of view (Fig. 1B,D). A modification of the technique was needed to allow the detection of particles in a larger field at a lower magnification. A "double decoration" technique based on the specific attachment of antirabbit IgG from sheep to the PPV or CLSV antibodies from rabbits was used which gave the PPV and CLSV particles an apparent diameter of 80–95 nm and 50–70 nm, respectively (Table 2). At a magnification of 2,400, the field consisted of one grid square in which double-coated particles readily could be observed (Fig. 2A,B). At magnifications as low as 700, which is the minimum magnification of our electron microscope, double-coated PPV particles could be detected (Fig. 2C).

No interference was noted between the antirabbit IgG from sheep and the antibodies from rabbit which were adsorbed to the grids but not complexed with virus particles. Interaction between these rabbit antibodies and the IgG from sheep did not interfere with the detection of double-coated virus particles (Fig. 2D).

DISCUSSION

Despite low virus concentrations in infected woody plants and the presence of inhibitors, PPV and CLSV could be detected by ISEM with the same sensitivity as ELISA.

The respective sensitivities of ELISA and ISEM were investigated with purified virus suspensions and crude extracts from infected plants. With purified virus suspensions, virus particles at a concentration as low as 5–10 ng/ml were detected by ISEM; similar results were obtained by ELISA. When assaying crude extracts from PPV-infected peach leaves we noticed a perfect correlation between ISEM, ELISA, and a bioassay (17). Similar results were recently obtained using ELISA and ISEM assay for CLSV in infected plum and peach trees. From recent indexing of trees naturally infected with PPV, it was seen that ISEM is sometimes more sensitive and reliable than ELISA, but this could reflect a lower interference of plant inhibitors in the case of ISEM. These results are the first example of the use of ISEM with fruit tree viruses and serve to demonstrate the versatility of this technique. Preliminary adsorption of antibodies to carbon-coated grids (5) increases the number of trapped particles. Antibody coating of the trapped particles confirms the identification of the virus and subsequent coating by antibody-specific IgG permits the use of a lower magnification and, consequently, the observation of more coated particles. Such a low magnification would not be necessary to detect viruses that are present in higher concentrations in infected tissue. In addition to identification of the virus, antibody coating seems to preserve the virus particles, especially CLSV, from degradation under our experimental conditions of rapid incubation.

Despite the significant improvement presented by the use of sheep antirabbit serum in this technique, it is clear that ISEM, which requires an electron microscope, will not replace ELISA as a field diagnostic method in the future. It will probably find most use where smaller numbers of samples are to be examined and where identification of a particular virus by serology and morphology is required. Moreover ISEM appears to be a little more expensive than ELISA. Nevertheless, ISEM has some clear advantages: the test can be applied to very small samples (ie, 1-mm-square sections of leaf) and, with the conditions described here, the test can be completed within 1 hr; it does not necessitate purification of immunoglobulins and preparation of a conjugate and high-titered specific antisera are not required. It is known that ELISA can be applied even with low-titer antisera, but, in such a case, concentration of the conjugate must be increased. With ISEM, nonspecific antibodies do not interfere with the test, whereas sensitive and reliable detection of PPV and CLSV cannot be obtained in ELISA without a preliminary elimination of nonspecific antibodies (7). Like ELISA, ISEM is very useful for the detection of PPV and CLSV; since these two viruses present limited antigenic variation, serological detection is not strain-discriminating in contrast to the results obtained by biological indexing.

Immunosorbent electron microscopy appears to be an alternative to ELISA and provides a useful diagnostic tool for detection of PPV and CLSV, two viruses for which sensitive, rapid, and reliable tests are needed.

TABLE 2. Increase in apparent diameter of plum pox virus (PPV) and apple chlorotic leaf spot virus (CLSV) particles by single and double decoration

ISEM technique	Diameter (nm)[a]	
	PPV	CLSV
Without decoration	15–17	12–13
With single decoration	40–50	25–33
With double decoration	80–95	50–70

[a] Observations and measurements were carried out with purified virus suspensions (1 μg virus per milliliter); 500 particles were measured.

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