# Purification of Arboviruses Grown in Tissue Culture (35725)

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Highly purified virus preparations are a prerequisite for any basic studies on the morphology and biochemistry of the virion. A variety of procedures for the purification of arboviruses has been described; *e.g.*, fluorocarbon treatment (1), protamine sulfate treatment (2, 3), and aluminum phosphate gel chromatography (4), in combination with centrifugation. However, our knowledge about the properties of the virion is as yet insufficient. On the other hand, recent advances in the cultivation of viruses in tissue cultures have made it possible to obtain large amounts of viruses without too much difficulty. This report summarizes experiments in which an efficient recovery of highly purified arboviruses was achieved by zinc acetate precipitation and gel filtration combined with density gradient centrifugation using materials derived from monolayer cultures of an established hamster kidney cell line (BHK-21).

*Materials and Methods. Seed viruses.* The Chikungunya virus [abbreviated as CHIK (5)] African strain (at the 174th suckling mouse brain passage, kindly supplied by Drs. K. Fukai and A. Igarashi, of the Research Institute of Microbial Diseases, University of Osaka, Japan), and the Japanese encephalitis virus (JE) Nakayama strain (kindly supplied by Dr. A. Oya, of the National Institute of Health, Tokyo, and passed through suckling mice intracerebrally for 37 generations in our laboratory) were serially cultivated in BHK-21 cells. After the infected cells were frozen and thawed, the resultant fluid was centrifuged at 1000*g* for 15 min to obtain the supernatant, which was kept at −70° until ready for use.

Type 1 dengue virus (DEN-1) Mochizuki strain (6) was transferred intracerebrally from mouse to mouse. The infected brains (from the 180th to the 185th mouse passages) were homogenized in phosphate buffer saline [(PSB), pH 7.0] (7) containing 0.2% bovine serum albumin [(BSA), Fraction V, Armour Pharmaceuticals, Chicago]. The homogenate was centrifuged at 1000*g* for 10 min, and the supernatant was kept at −70°.

*Tissue culture.* BHK-21 clone 13 cells (8) (kindly supplied by Drs. C. L. Wisseman, Jr. and O. R. Eylar, of the University of Maryland School of Medicine, Baltimore, Maryland) were propagated in 500-ml Roux bottles. For the initial cell growth, Eagle's MEM containing 0.12% sodium bicarbonate, 5% heat-inactivated calf serum, and 6 μg/ml of kanamycin was used. The cells were subcultured every third or fourth day, depending on their growth rate, by use of Versene–trypsin solution. For the virus propagation, the serum was replaced by 0.2% BSA (this culture fluid was designated as BSA medium) in order to eliminate impurities as completely as possible and to avoid any aggregation of viruses (9).

*Buffer solution used for virus purification.* ST buffer composed of 0.13 *M* NaCl in 0.05 *M* Tris–chloride (pH 7.8), and STE buffer composed of 0.13 *M* NaCl and 0.001 *M* ethylenediaminetetraacetate (EDTA) in 0.05 *M* Tris–chloride (pH 7.8) were employed.

*Injection of cells and virus propagation.* Five ml of the seed virus suspension was introduced into 3-day-culture cells and held at 37° for 90 min. The multiplicity of infection (moi) was 10 to 50 plaque-forming units (PFU)/cell for CHIK, 0.1 to 1 for DEN-1, and 1 to 10 for JE viruses.

After infection, the cells were washed twice with warm PBS (pH 7.0) and refed with the BSA medium. The cultures were then incubated at 37° until the fluid was harvested.

*Titration of virus infectivity.* Virus titers

were determined by a plaque method in BHK-21 cells, as developed by H. Aoki, R.O. Eylar and C. L. Wisseman, Jr. (personal communication) as a modification of the original method described by Schulze and Schlesinger (10). Three-day-old monolayer cultures grown in 50-ml dilution bottles were infected with 0.2 ml each of serially diluted viruses and held at 37° for 2 hr. Thereafter, the inocula were removed as completely as possible, and methylcellulose overlay medium (Eagle's MEM containing 1.5% methylcellulose, double strength sodium bicarbonate, 5% heat-inactivated calf serum and 6 μg/ml of kanamycin) was introduced. After incubation at 37° for 4 days (for CHIK), 12 days (for DEN-1) and 5 days (for JE), the overlay medium was washed off with a solution containing 0.14 *M* NaCl, 1% glucose, 0.002% phenol red, and 0.4% KCl (adjusted pH 7.0 with 1 *N* NaOH), then fixed and stained with 2.5% crystal violet solution in 30% ethanol including 1% ammonium oxalate. After being washed with water, the plaques were counted.

*Assay of viral hemagglutinin (HA).* HA titers were determined according to the method of Clarke and Casals (11).

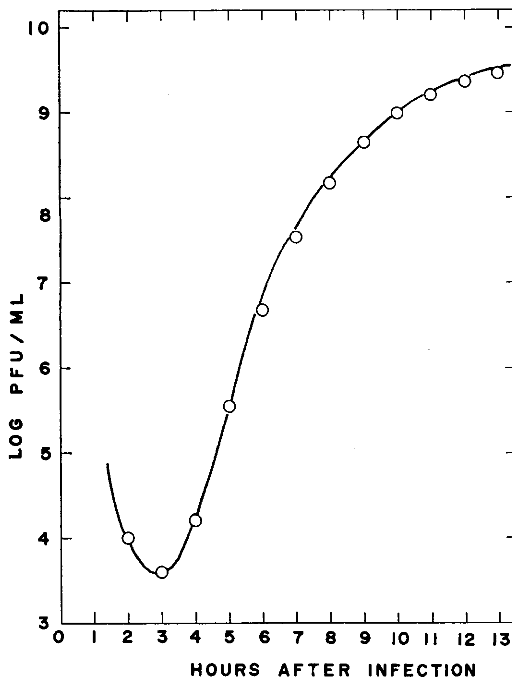

FIG. 1. Growth curve of CHIK virus.

*Protein determination.* Protein amounts were determined according to the method of Lowry *et al.* (12) with BSA as the standard.

*Electron microscopy.* The purified preparations, after being dialyzed against ST buffer *in vacuo* to about 0.5 ml at 4°, were transferred onto a carbon-coated grid by means of a syringe. An 0.5% solution of uranyl acetate (pH 4.3) was added and the excess fluid was removed with a filter paper. The specimens were photographed by a Hitachi 11D electron microscope at a magnification of ×60,000. Particle diameters were determined from the micrographs enlarged at the final magnification of ×300,000.

*Results. CHIK virus.* Most of the experiments were carried out with the CHIK virus. Its growth curve in BHK-21 monolayer cultures with BSA medium is shown in Fig. 1. Usually, the maximum titers (about $10^{9.0}$ PFU/ml) were obtained around 10 hr after infection. Based on the results, the infected culture fluids harvested 14 to 16 hr after infection were used for the purification of the virus.

*Precipitation of virus by zinc acetate.* Following the descriptions by Sokol *et al.* (9) using rabies virus, the optimal conditions for arboviruses were studied by the present authors. The infected culture fluid, freed of cell debris by centrifugation at 1000*g* for 10 min, was mixed with zinc acetate solutions of various concentrations, and adjusted to pH 7.0 with 1 *N* NaOH. The mixture was then centrifuged at 1000*g* for 30 min at 4° and the supernatant fluid was discarded. The pellet was resuspended in saturated disodium EDTA (preadjusted at pH 7.0 by the addition of solid Tris), the volume of which corresponded to one-fiftieth of the starting fluid. The resultant solution was clarified by centrifugation at 1000*g* at 4° for 20 min. Examples of the results obtained are illustrated in Fig. 2. It was shown that 0.05 *M* zinc acetate was optimal for CHIK virus, giving the highest recovery of infectivity. At this step, a fairly good concentration of the virus was achieved, although purity per milligram of protein was not so high (refer to Table I below).

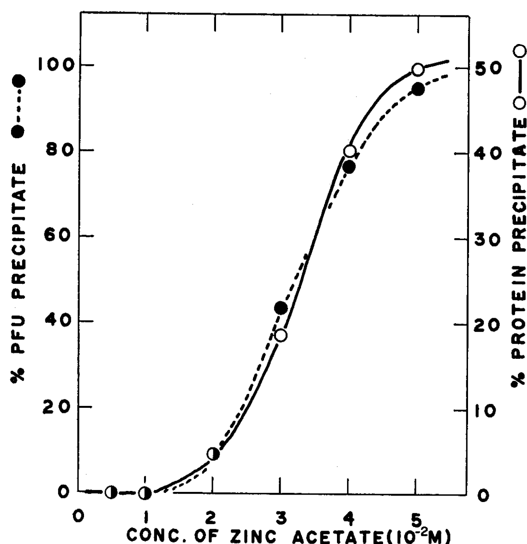


FIG. 2. Precipitation of viruses from infective tissue culture fluids by zinc acetate.

*Filtration through Sephadex G200-Sepharose 6B column.* A column (50 × 2.5 cm) composed of: (upper) 40-cm Sepharose 6B (particle size 40 to 210 μ; Pharmacia Fine Chemicals, Uppsala) and (lower) 10-cm Sephadex G200 (particle size 40 to 120 μ; Pharmacia Fine Chemicals, Uppsala), which was shown in our preliminary tests as most suitable for separation of virus and protein, was equilibrated with ST buffer. Virus suspension (6 to 10 ml), concentrated by the zinc acetate precipitation as described above, was passed through the column and eluted upward with ST buffer at a flow rate of 0.7 ml/min. Most of the virus was eluted in 30 ml immediately following void volume (approx 60 ml), and most of the proteins and low molecular weight impurities were removed (see Fig. 3). At this step, the preparation was fairly pure (see Table I), but it still contained some BSA and host cell components when the authors examined it by an electron microscope. These impurities were removed by sucrose density gradient centrifugation.

*Sucrose density gradient centrifugation.* The filtrate through Sephadex G200–Sepharose 6B column was condensed in a collodion bag (Sartorius-Membranfilter GmbH, Göttingen) to be 0.3 to 0.5 ml (usually 1/50 to 1/100 reduction in volume). Three-tenths ml of the samples, laid on 4.5 ml of linear 10 to 40% (w/v) gradients of sucrose in STE buffer, was centrifuged at 100,000*g* for 90 min at 4° in an RSP 40 rotor of a Hitachi 65P centrifuge. Fractions for analysis were collected through a hole punctured at the bottom of the tube. The infectivity and HA activity coincided with each other, forming one peak which corresponded to a single visible band. It was indicated that the purified CHIK virus was homogeneous with respect to the infectivity and HA activity. At this step, most of the impurities were sedimented either faster or slower than the viruses, so that they could be separated from

TABLE I. Purification of Chikungunya Virus.[a]

| Sample | Vol (ml) | Amt. of protein/ml (mg) | PFU/ml ($\times 10^9$) | PFU/mg of protein ($\times 10^8$) | Virus recovery rate (%) |
|---|---|---|---|---|---|
| Infective tissue culture fluid | 300 | 2.67 | 2.5 | 9.7 | 100 |
| Precipitate dissolved in EDTA solution | 6.7 | 62.5 | 98 | 11 | 87 |
| Pooled fractions after filtration through column | 2.1 | 1.87 | 75 | 401 | 21 |
| "Band"[b] after centrifugation in sucrose density gradient | 10.3 | 0.04 | 8.5 | 2120 | 12 |

[a] The procedures outlined in Fig. 4.
[b] Fractions visible as an opalescent band.

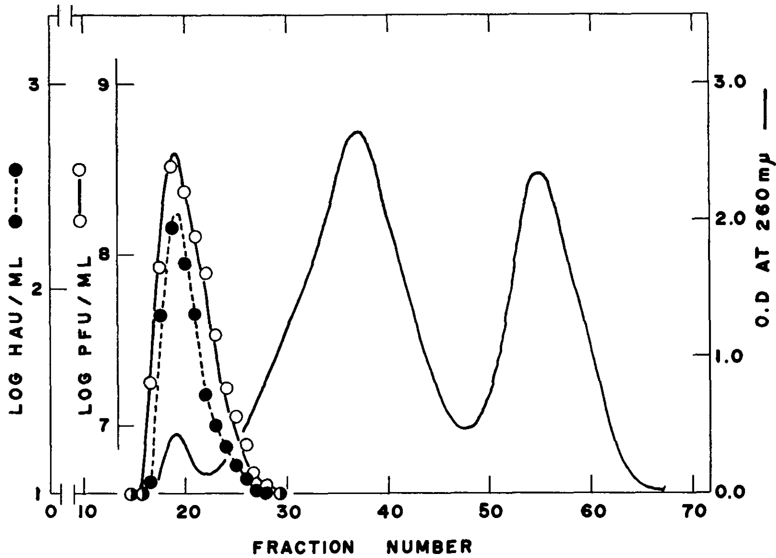


FIG. 3. Filtration through a Sephadex G200–Sepharose 6B column of the CHIK virus preconcentrated by zinc acetate precipitation.

the virions. The trace turbidity caused by the impurities was seen at the top of the tube.

Based on the above data, the procedures shown schematically in Fig. 4 were regarded suitable for the purification of arboviruses, and examples of the results obtained are indicated in Table I. It is evident that the filtration through Sephadex G200–Sepharose 6B

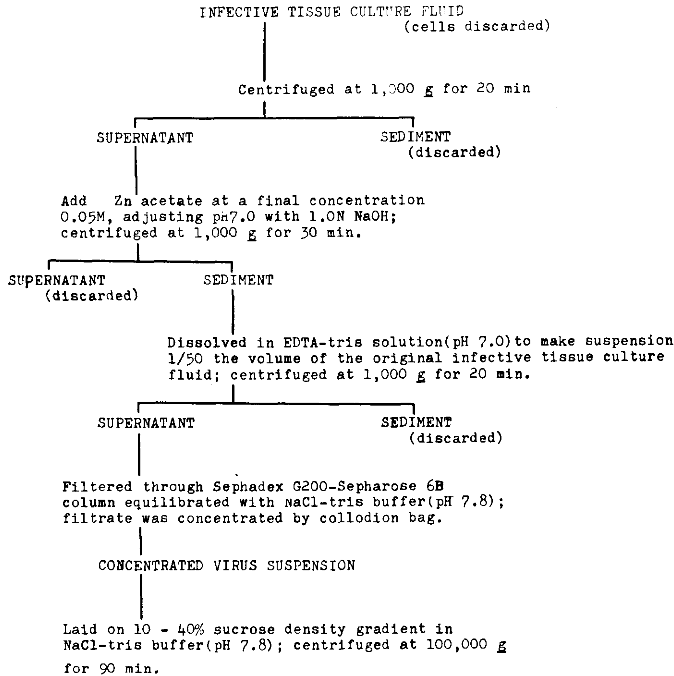


FIG. 4. Schematic presentation of the procedures used for the purification of arboviruses grown in BHK-21 cell cultures.

column is the most efficient step. Virus recovery rates were usually 10 to 20%. The final preparation was 100 to 500 times purer than the original infectious culture fluid.

*DEN-1 and JE viruses.* The abovementioned techniques were applicable to DEN-1 and JE viruses. Infectious culture fluids were harvested 96 to 120 hr after infection for DEN-1 (usually $10^{6.5}$ PFU/ml) and 24 to 36 hr for JE (usually $10^{8.0}$ PFU/ml). Distinct from CHIK virus, the DEN-1 and JE preparations filtered through Sephadex G200–Sepharose 6B column exhibited two peaks of HA activity in the sucrose density gradient centrifugation. Infectivity was found in the rapid-sedimented peak. This fraction was visible as a single band in the case of the JE virus, whereas no such band has been seen so far for the DEN-1 virus.

*Electron micrographs.* Figure 5 shows typical images of the particles, around which spike-like projections are also visible. Clumping of the particles was minimum. The majority of the particles observed had diameters (m$\mu$) of 50 to 60 (CHIK); 50 to 55 (DEN-1); and 45 to 50 (JE).

*Discussion and Summary.* Arboviruses (Chikungunya, dengue type 1, and Japanese encephalitis) cultivated in monolayer cultures of BHK-21 cells grown with Eagle's MEM supplemented with bovine serum albumin were purified by the following procedures: The viruses were precipitated from infectious tissue culture fluids by zinc acetate (0.05 *M*) and were resuspended in saturated ethylenediaminetetraacetate. The suspension was filtered through a Sephadex G200 [(lower) 10 cm]–Sepharose 6B [(upper) 40 cm] column (2.5 cm in diam) by upward elution. The filtrate was concentrated in a collodion bag *in vacuo*. The final step was sucrose density gradient centrifugation. The procedures were comparatively short and straightforward, and about 100 to 500 times purification factors were obtained consistently. The CHIK virus preparation after the sucrose density gradient centrifugation was demonstrated as a single peak, the infectivity and HA activity coinciding with each other; whereas the DEN-1 and JE viruses were shown to be heterogeneous. The problems of whether such a difference reflects some basic characteristics of groups A and B viruses need further investigation. Electron microscopic pictures of the preparations revealed spherical particles of (m$\mu$): 50 to 60 (CHIK), 50 to 55 (DEN-1), and 45 to 50 (JE). These images are compatible, in general, with those previously reported by Igarashi *et al.* (CHIK virus) (13, 14); Matsumura and Hotta (DEN-1 virus) (15); Smith *et al.* (DEN-2 virus) (16); Takaku *et al.* (JE virus) (17); and Nozima *et al.* (JE virus) (18); although the origin of the viruses and the purification methods are not necessarily the same. The present purification procedures can probably be applied with success to other arboviruses in general. Further studies on the biochemical and biophysical nature of the purified virions are underway and will be reported later.

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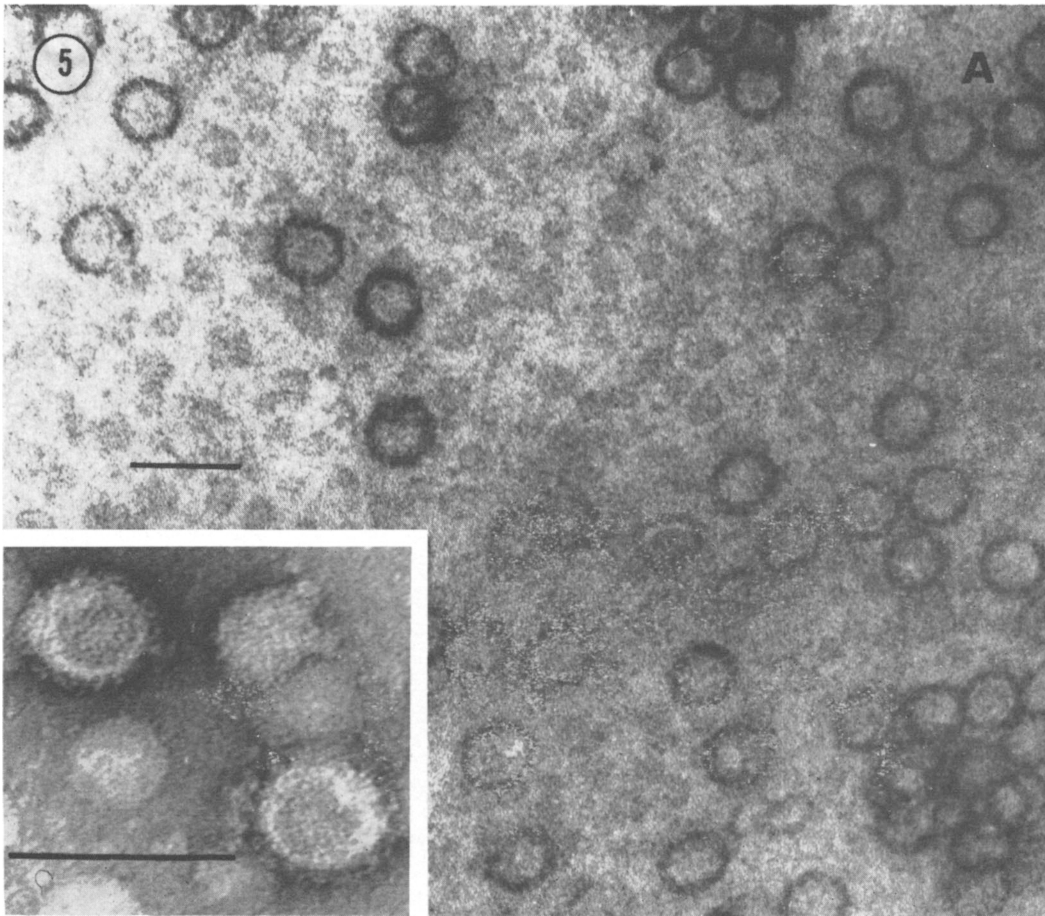
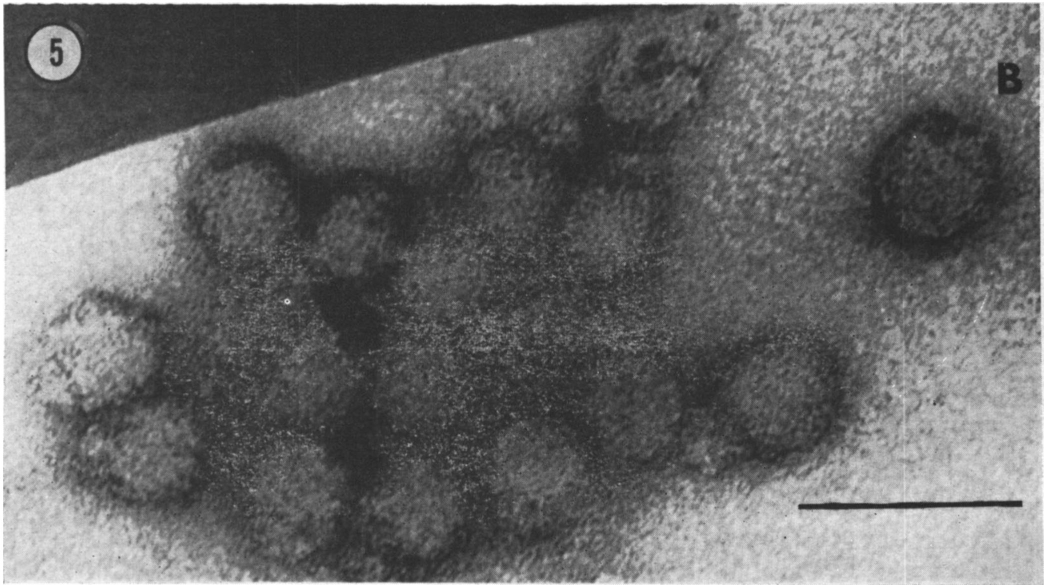
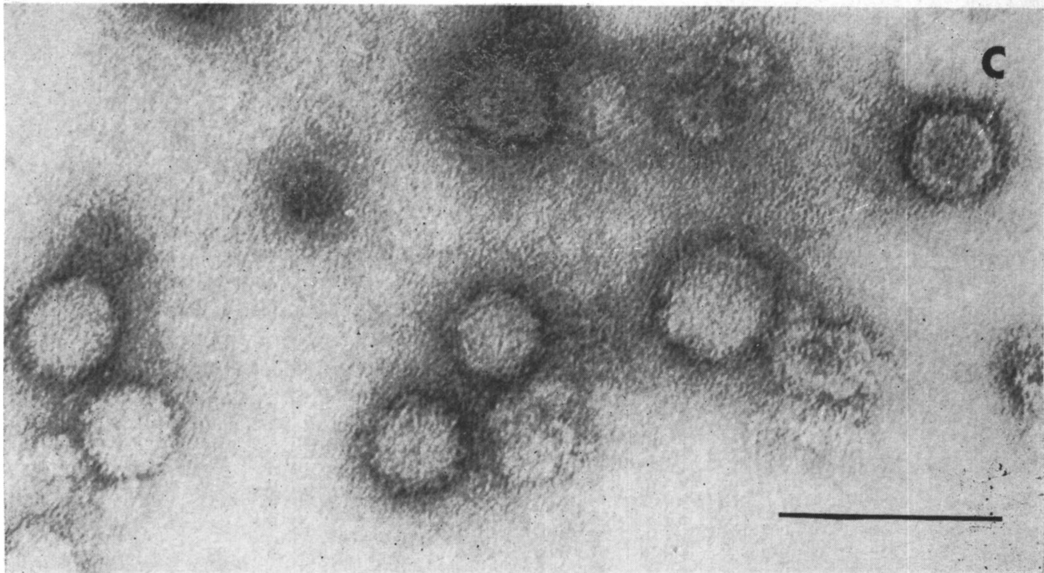



FIG. 5. Electron micrographs of purified arboviruses. After sucrose density gradient centrifugation, the virus-containing fractions were dialyzed against ST buffer to removed sucrose. The scales represent 100 mμ. (A) CHIK virus; ×150,000; insert is ×300,000. (B) DEN-1 virus; ×300,000. (C) JE virus; ×300,000.

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