Quantification of recombinant core-like particles of bluetongue virus using immunosorbent electron microscopy

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Abstract

Immunosorbent electron microscopy was used to quantify recombinant baculovirus-generated bluetongue virus (BTV) core-like particles (CLP) in either purified preparations or lysates of recombinant baculovirus-infected cells. The capture antibody was an anti-BTV VP7 monoclonal antibody. The CLP concentration in purified preparations was determined to be 6.6×10^{15} particles/l. CLP concentration in lysates of recombinant baculovirus-infected cells was determined at various times post-infection and shown to reach a value of 3×10^{15} particles/l of culture medium at 96 h post-infection. The results indicated that immunosorbent electron microscopy, aided by an improved particle counting method, is a simple, rapid and accurate technique for the quantification of virus and virus-like particles produced in large scale in vitro systems. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Immunosorbent electron microscopy; ISEM; Airfuge; EM-90 rotor; Bluetongue; BTV CLP; Monoclonal antibody; Particle counting

1. Introduction

Bluetongue virus (BTV) is the prototype virus of the genus *Orbivirus* in the family *Reoviridae*. The virus contains ten double-stranded RNA genome segments each of which codes for a single protein. Seven of the proteins are structural and form a double-shelled particle. Proteins VP3 and

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VP7 constitute an icosahedral inner capsid that is surrounded by an outer layer composed of proteins VP2 and VP5. In addition to the structural proteins, infected cells contain three non-structural proteins the roles of which remain obscure. French and Roy (1990) constructed a dual-recombinant baculovirus containing genes that encode VP3 and VP7 and their expression in insect cell culture resulted in the synthesis of large quantities of VP3 and VP7 and the concomitant formation of core-like particles (CLP) that lack RNA. CLP have the potential to be used as vaccines against bluetongue and other diseases. Other antigens linked to the VP7 protein, have demonstrated increased antigenicity when presented as part of CLP (Belyaev and Roy, 1992; Pearson and Roy, 1993).

However, if CLP are to be considered seriously as vaccines, it is important to be able to quantify the yield of such particles. Quantification of VP3 and VP7 (or VP7-fusion proteins) is not sufficient to indicate the yield of intact CLP present in lysates of infected cells. Existing electron microscopy (EM)-based procedures are cumbersome and difficult to optimise. In this paper, we report the development of an immunosorbent EM procedure for quantifying CLP particles present in crude samples and which precludes a requirement to purify CLP particles prior to quantification.

Immunosorbent electron microscopy (ISEM) is a useful method for the identification and quantification of viruses. Derrick (1973) used parlodion-filmed, carbon-coated grids with anti-potato virus Y (PVY) or anti-tobacco mosaic virus (TMV) antisera to quantify PVY and TMV in crude leaf extracts. He found a linear relationship between the log of the number of TMV particles attached to serologically-specific grids and the dilution of a crude extract of TMV-infected tobacco tissue. Other researchers have also used ISEM to detect and differentiate between different viruses. Lewis (1990) investigated serological differences between strains of Norwalk-like virus (NLV) from five different outbreaks using anti-NLV-coated grids. Dea and Garzon (1991) identified coronaviruses from various species in clarified faecal specimens and Gupta et al. (1994) demonstrated that a close relationship between

Brinjal necrotic mosaic virus and other strains that were trapped by antisera on a collodion-filmed and carbon-coated grid.

ISEM was evaluated for the quantification of recombinant baculovirus-generated BTV CLP (hereafter referred to as CLP) using monoclonal (MAb) and polyclonal antibodies to the core proteins VP7 and VP3.

2. Materials and methods

2.1. Virus and cells

Spodoptera frugiperda cells (Sf9) were obtained from the American Type Culture Collection (ATCC CRL 1711) and adapted for growth in SF900 II serum-free medium (GIBCO, NY, USA). The cells were grown at 28°C either in monolayer culture or in suspension in 100 ml medium/250 ml Duran Erlenmeyer flask placed on an orbital shaker operating at 100 rpm. Recombinant *Autographa californica* nuclear polyhedrosis virus (AcNPV) co-expressing BTV core proteins VP7 and VP3 (CLP-AcNPV) was obtained from the NERC Institute of Virology and Environmental Microbiology, Oxford, UK (French and Roy, 1990). Recombinant *Autographa californica* nuclear polyhedrosis virus expressing β-galactosidase (β-gal-AcNPV) was purchased from Amrad, Australia. Stock preparations of CLP-AcNPV were propagated by infecting monolayer cultures of Sf9 cells at a multiplicity of infection (MOI) of two plaque forming units (PFU) per cell. Progeny virus (2×10^8 PFU/ml) was harvested 4 days post-infection.

2.2. Monoclonal and polyclonal antisera

Anti-VP7 MAb 20E9, 20A11 and 20D11 were raised in mice to sodium dodecyl sulphate-treated BTV-1, using the procedure described by Lunt et al. (1988). Hybridoma culture supernatants were used at dilutions of 1:100 (20E9, 20A11) and 1:10 (20D11). Polyclonal anti-VP7 antiserum was raised in sheep against yeast-expressed VP7 (Martyn et al., 1990) and used at a dilution of 1:500. Ovine polyclonal antiserum was raised to core

particles of BTV-1 isolated from virus-infected cells and purified by equilibrium density centrifugation in cesium chloride (personal communication, B.T. Eaton). The ovine antiserum was used at a dilution of 1:500. Polyclonal anti-VP3 antiserum was raised in rabbits to polyacrylamide gel-purified VP3 of BTV-1 expressed in *Escherichia coli* as described previously (Wang et al., 1996). After affinity purification using a protein-G column, the anti-VP3 serum was used at a dilution of 1:1000.

2.3. Infection of Sf9 cells with CLP-AcNPV

Sf9 cells were suspended in 100 ml SF900 II medium at a concentration of $3–4 \times 10^5$ cells/ml. Cell density and viability were monitored by counting in the presence of 0.2% Trypan Blue. At a density of 2×10^6 per ml, cells were infected with CLP-AcNPV at a MOI of two PFU/cell. Aliquots were removed at regular intervals post-infection and stored at $-20°C$.

2.4. CLP production, purification and analysis

The method used to purify CLP was adapted from that described by French and Roy (1990). Sf9 cells in suspension (4×10^6 cells/ml) were infected with CLP-AcNPV at a MOI of two PFU/cell and incubated at 28°C for 48 h. Cells were pelleted by low-speed centrifugation ($150 \times g$) and resuspended at a concentration of 2×10^7 cells/ml in 50 mM Tris–HCl (pH 8.0) containing 0.5% NP-40. After 30 min on ice, the lysate was spun at 2000 rpm ($300 \times g$) for 10 min to remove nuclei. CLP in the lysate were purified by ultracentrifugation in self-forming 39% (w/v) cesium chloride density gradients at $140\,000 \times g$ for 18 h (Beckman L80). CLP banded at a density of 1.307 g/cm³ and were analyzed by electrophoresis in 10% SDS-PAGE (French and Roy, 1990), Western immunoblotting and electron microscopy.

2.5. Airfuge technique

Airfuge analysis was carried out as described by Zheng et al. (1996). Briefly, the sector core of the EM-90 rotor (Beckman Instruments, Palo Alto, CA) was lubricated with silicon vacuum grease and 55 µl aliquots of serially diluted solutions of CLP were centrifuged ($118\,000 \times g$, 30 min) onto thin-bar 400 mesh copper grids that had been filmed with 0.5% collodion pre-treated with polylysine. The supernatants were removed and grids stained for ultrastructural examination.

2.6. Immunosorbent electron microscopy (ISEM)

Electron microscope grids (400 mesh thin-bar copper) were filmed with either 1% formvar or 1% collodion and carbon-coated. Other grids were filmed with evaporated carbon and contained no plastic films. Grids were coated with MAb 20E9 at dilutions of 1:50, 1:100, 1:250, 1:500, 1:1000, 1:2000 and 1:4000 in either phosphate buffered saline (PBS) (pH 7.4), 50 mM Tris–HCl (pH 8.0) or 100 mM carbonate buffer (pH 9.6). Volumes (10 µl) of antibody were deposited onto grids and incubated for 10, 15 or 30 min at room temperature, 4 and 37°C. Normal mouse IgG, buffer only, and grids only were used as controls. The grids were washed with double distilled water, 50 mM Tris (pH 8.0) or PBS (pH 7.4) (3×5 min). Grids were then blocked with 20 µl of 1% bovine serum albumin (BSA) in PBS (pH 7.4) for 10 min.

Cesium chloride gradient-purified CLP and crude CLP in lysates of AcNPV-CLP-infected cells were analysed by ISEM. Purified CLP were serially diluted six times in a lysate of uninfected Sf9 cells starting at 1:10. The lysate was generated by treating uninfected cells with 50 mM Tris–HCl (pH 8.0) containing 0.5% NP-40. Infected cells were lysed by addition of NP-40 to 0.5% and lysates were stored at $-20°C$. Following thawing, the lysates were centrifuged at low speed (2000 rpm, $300 \times g$) and 5 µl aliquots added to pre-treated grids prepared as described above for 10, 30 or 60 min at room temperature, 37 and 4°C. Uninfected and β-Gal-AcNPV-infected cell lysates and media only were used as controls. After incubation with shaking, the grids were washed in double distilled water and stained with 10 µl of 2% aqueous uranyl acetate or 2% sodium phosphotungstate (containing 30 µg/ml bacitracin) for 1 min.

Preparations were examined at a magnification of 2500 with a JEOL-100 transmission electron microscope. Areas for examination were selected at random and recorded in the form of electron micrographs. Three to five micrographs were recorded per sample and each sample was prepared in duplicate. Quantification of CLP was performed by counting ('handy counter') the number of CLP per negative film (imaged in a microfiche at $\times 50$ magnification).

2.7. Determination of CLP concentration in crude cell culture lysates

The concentration of purified CLP was determined by ISEM ('binding concentration') and by the airfuge technique ('original concentration') and the capture efficiency was determined by dividing the 'original concentration' by the 'binding concentration'. The number of crude CLP per negative film was determined by the ISEM method and the concentration of particles calculated by multiplying the number of CLP by the capture efficiency.

3. Results

3.1. Determination of CLP concentration

Purified CLP were used to generate a calibration standard. The concentration of CLP was calculated using the airfuge technique (Zheng et al., 1996). Fig. 1 shows a linear relationship between the number of particles counted and the dilution of CLP. Using the formula described previously to calculate particle numbers (Zheng et al., 1996), the concentration of purified CLP was determined to be 6.6×10^9 per μl.

3.2. Determination of the binding efficiency

Serial dilutions of purified CLP were made in uninfected Sf9 cell lysates and 5 μl aliquots of each dilution added to grids coated with a 1:100 dilution of antibody 20E9. Fig. 2(A) shows that CLP were distributed evenly on the antibody-coated grids. A linear relationship was obtained

between the number of particles and their dilution from 1:10 to 1:160 (Fig. 2B). Regression analysis indicated that there were 2600 particles bound to the grid/μl of purified CLP. The ratio of bound to total CLP (binding efficiency) was $6.6 \times 10^9/2.6 \times 10^3$ or 2.54×10^6.

3.3. Optimisation of ISEM procedure

Several factors can affect the quantification of virus particles by ISEM. These include nature of the support film, specificity and concentration of antibody, washing procedure and staining solution. In our study, results indicated that there was no difference in the CLP capture efficiency with either which carbon-coated, formvar-filmed or carbon-coated, collodion-filmed grids that had been coated with MAb 20E9 (data not shown). Carbon-filmed grids were found to be unstable as they frequently broke during incubation and washing steps.

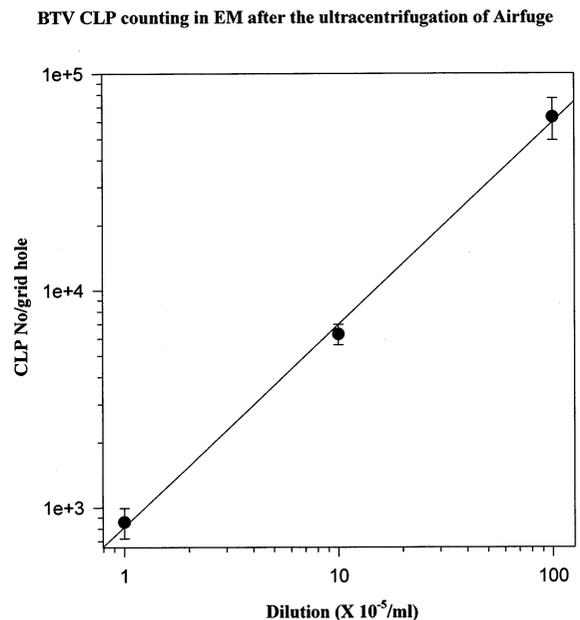**BTV CLP counting in EM after the ultracentrifugation of Airfuge**

Fig. 1. Determination of concentration of purified recombinant BTV core-like particles (CLP) using the Airfuge-EM-90 rotor technique. Purified CLP were deposited onto grids filmed with 0.5% collodion pre-treated with polylysine. The graph shows the numbers of CLP (numbers per micrograph film/plate) against dilution of CLP. The error bars represent the standard error on the mean ($n = 6$) at each dilution.
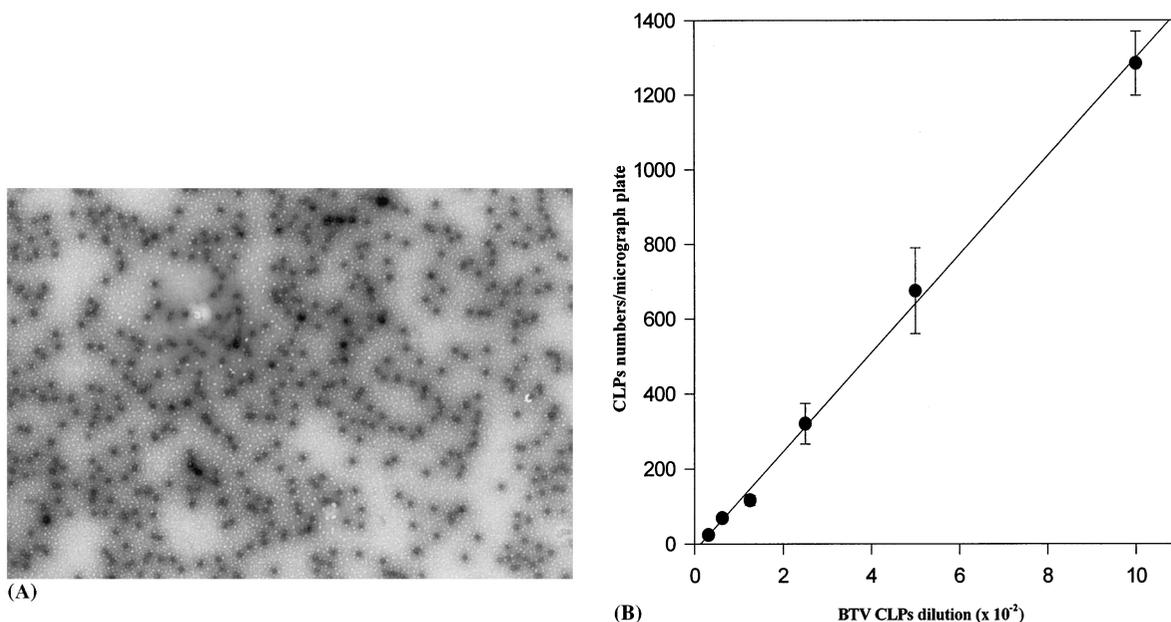
Fig. 2. (A) Negative contrast image of purified CLP captured on a MAb 20E9-coated grid. The particles are evenly distributed on the grid substrate. The particles were photographed at $\times 2500$ with a JEOL-100 electron microscope. (B) Graph of CLP number per film against dilution of CLP. Particles were counted with microfiche at $\times 50$ times magnification. The error bars represent the standard error on the mean ($n = 6$) at each dilution.

To determine the optimum antibody dilution, the number of CLP bound was determined at a range of antibody dilutions. For MAb 20E9, dilutions of 1:100 to 1:1000 were equally effective in capturing CLP (Fig. 3). Of the various antibodies tested, MAb 20E9 captured the largest number of CLP (Fig. 4).

The best MAb 20E9 coating and CLP adsorption times were found to be from 10 to 30 min. Adsorption times greater than 30 min generated background that was most likely attributable to adsorbed hybridoma cell debris. In virus capture experiments, 10 min was found to be optimum. Incubation periods beyond 10 min generated background that could be attributed to the impurity of the preparations i.e. non-specific adsorption of cell debris to the substrate.

In the washing steps, various buffers and water were tested to assess whether the washing buffer facilitated removal of CLP from the grids. We found that the type of buffer used did not have a deleterious effect and thereafter we used double distilled water.

3.4. Quantification of CLP in crude samples

Aliquots of 5 µl of 1:2 dilutions of crude CLP culture samples, prepared at various times post-infection, were added onto MAb 20E9-coated grids. The numbers of CLP were determined as described for purified CLP. Fig. 5(A) shows negative-contrasted CLP from a crude sample captured on a grid coated with MAb 20E9. The concentration of MAb 20E9-captured CLP increased with time post-infection (Fig. 5B). The maximum yield of CLP (3.03×10^{15} per l) was obtained at 96 h post-infection.

4. Discussion

ISEM is a technique that captures specific antigens and can be used to quantify viruses from a range of biological samples. Other techniques such as adsorption, centrifugation (e.g. the use of airfuges) and the spraying of particles directly onto grids all have the disadvantage that both
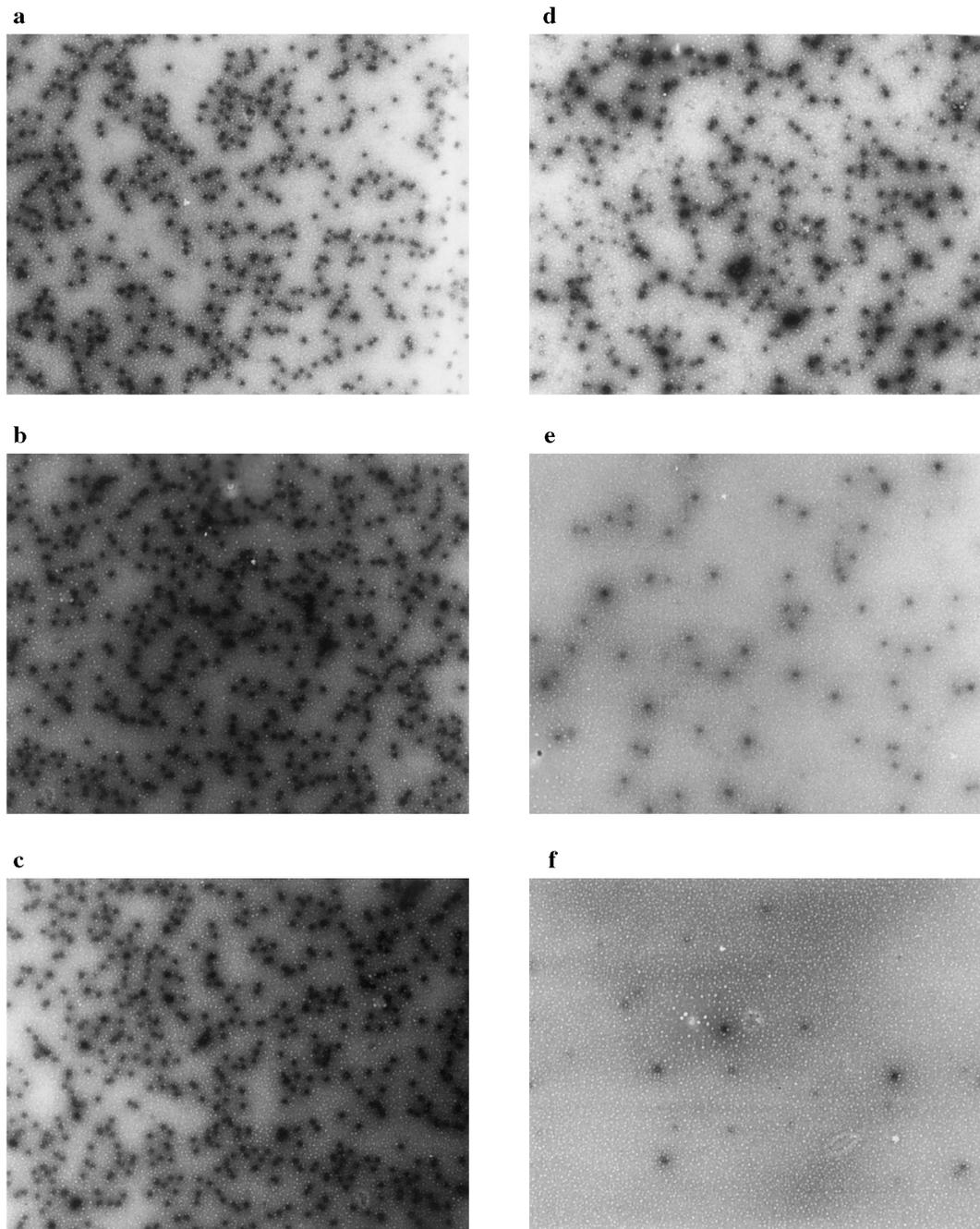
a

Fig. 3. Numbers of CLP captured by grids coated with different dilutions of MAb 20E9 (a) 1:100; (b) 1:250; (c) 1:500; (d) 1:1000; (e) 1:2000 and (f) 1:4000.
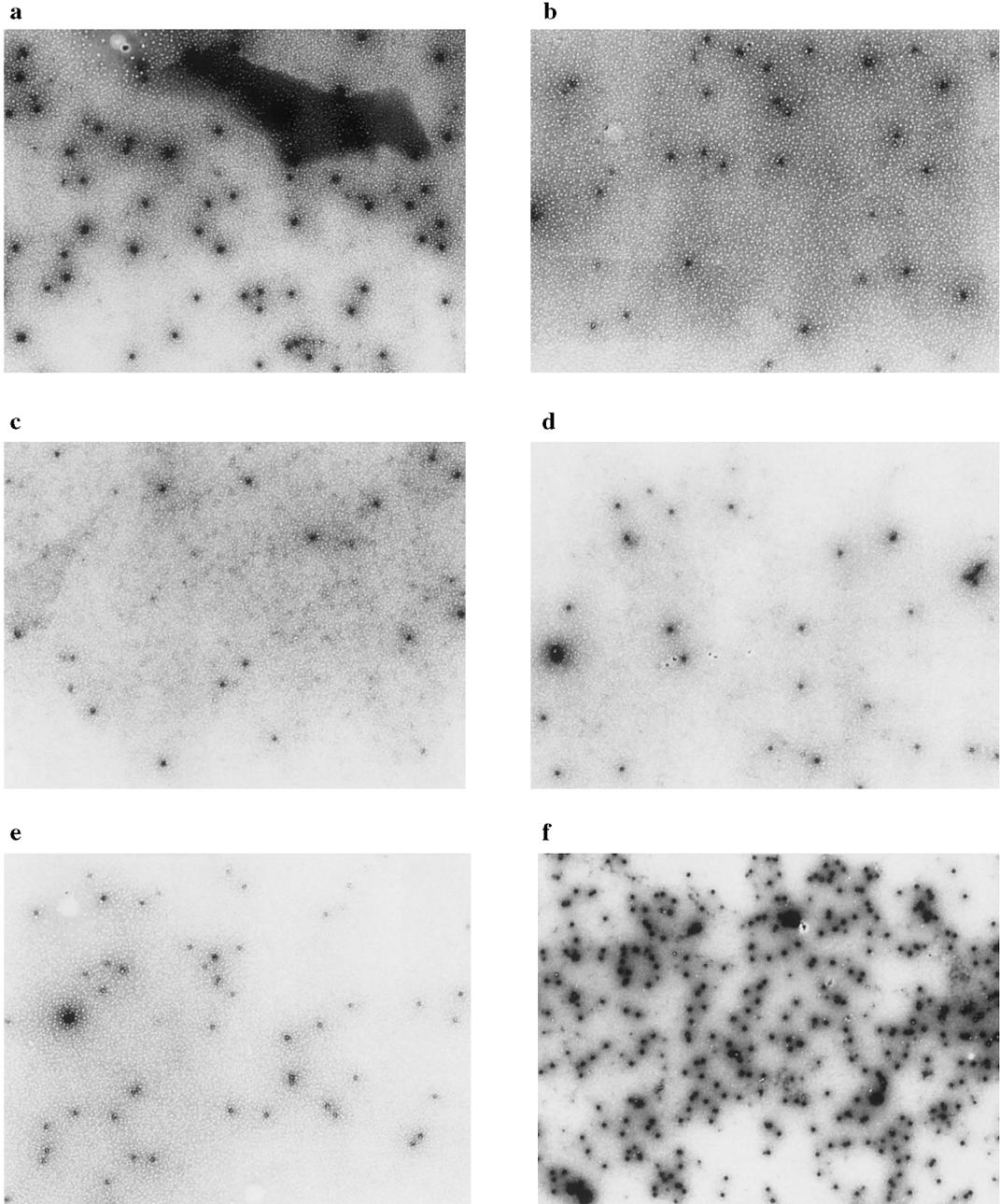
Fig. 4. ISEM of CLP using antibodies (a) MAb 20A11; (b) MAb 20D11; (c) ovine anti-VP7; (d) ovine anti-BTV core particle; (e) rabbit anti-VP3 and (f) MAb 20E9. CLP were used at a 1:10 dilution.

target and non-target particles/antigens become adsorbed to the grid substrate.

Of the factors/variables that have been reported to influence the efficiency of ISEM (support films,

dilution of antibody, incubation time and temperature, washing media, and staining solution), support films and washing media were found to be of little to no consequence. We found that carbon-
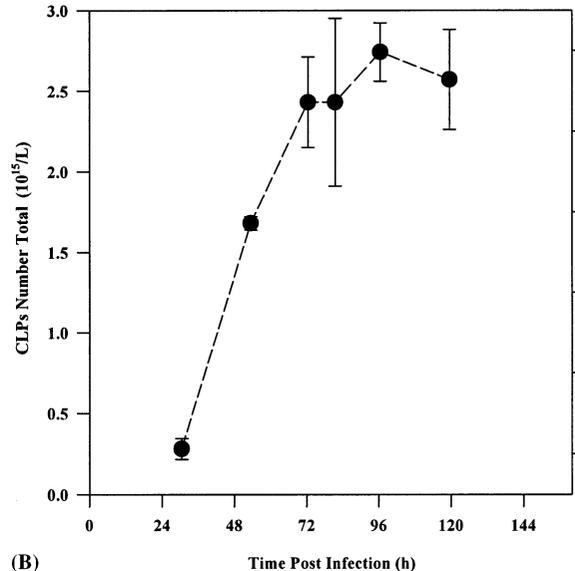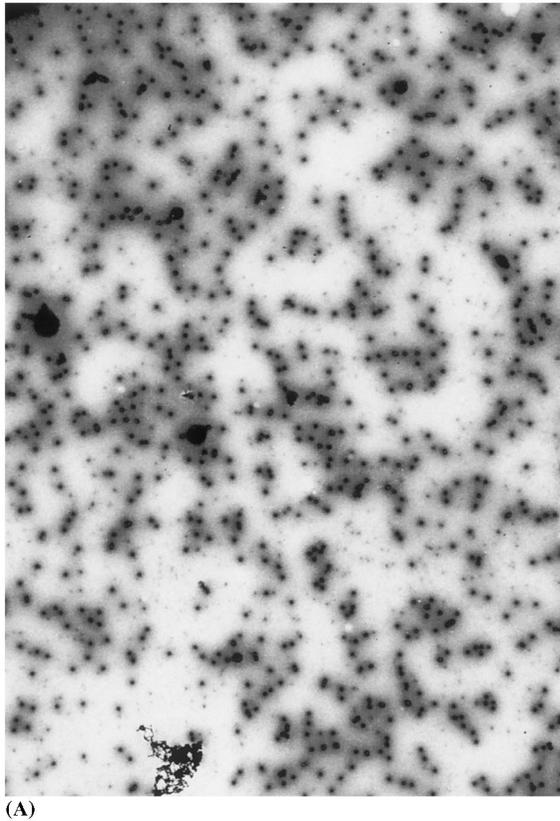
Fig. 5. (A) CLP in the crude culture sample at 96 h post-infection were captured by MAb 20E9. (B), The yield of CLP was quantified by immunosorbent electron microscopy. The concentration of CLP/l of culture medium against time post-infection. The error bars represent the standard error on the mean ($n = 6$) at each sample.

coated, formvar-filmed grids had good stability and adsorption characteristics for antibody binding. This result is consistent with the results of Milne and Luisoni (1977) but different to those obtained by Derrick and Brlansky (1976). In the latter study the authors stated that formvar-filmed grids, with or without carbon-coating, were unsuitable for ISEM. The authors reported the best results were obtained with collodion-filmed, carbon-coated grids. The results from this study indicated that either film substrate could be used with equivalent efficiency.

Potassium phosphotungstate (PTA) and uranyl acetate (UA) are commonly used as negative contrast stains. In this study PTA resulted in the disruption of CLP whereas UA maintained their integrity in addition to generating high contrast

images. The choice of antibody was critical to the overall efficiency of the ISEM technique. The best antibody for capturing CLP was MAb 20E9 and its efficiency may be attributable to its specificity and the accessibility of the 20E9 epitope (Wang et al., 1996). The anti-VP7 polyclonal antibody would be expected to recognise multiple epitopes but some are almost certainly inaccessible in CLP, thereby decreasing the capture efficiency of the technique. VP3 is not expressed on the surface of CLP or core particles and therefore anti-VP3 polyclonal antibody would be expected to be inefficient as a capture antibody for CLP.

Some reports (e.g. Milne and Lesemann, 1978; Lesemann et al., 1980) state that the concentration of antibody affects the efficiency of particle capture. The data from this study indicated that

MAb 20E9 dilutions of 1:100–1:1000 yielded similar results for purified CLP while dilutions of and beyond 1:2000 led to a reduction in the number of captured CLP. These results may be explained in terms of coating density and steric hindrance. At dilutions of and beyond 1:2000 steric hindrance may no longer be an obstacle to CLP binding and the density of antibody adsorption is reduced.The data presented in this paper indicate that the ISEM technique may be useful for quantification of viral antigens in diagnosis and the production of virus vaccines. Accurate quantification is dependent upon determining the capture efficiency.

Acknowledgements

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