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Bovine Enteric Coronavirus Structure as Studied by a Freeze-drying Technique

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SUMMARY

A strain of bovine coronavirus (F15) was studied by electron microscopy using a freeze-drying technique. Purified coronavirus preparations show three different categories of image: (i) 'blackberry-like' virions, (ii) virions with a smooth depression at their surface, and (iii) apparently broken particles showing very clearly the areas of spike insertion in the virus membrane. Virus projections resemble 'mushrooms' with the 'stalk' inserted at the virus membrane. A model of the virion structure is proposed.

Coronaviruses are a group of enveloped RNA-containing viruses that have a unique, typical morphology. They cause a variety of diseases in animals, including avian infectious bronchitis, feline infectious peritonitis, mouse hepatitis, transmissible gastroenteritis in pigs, and diarrhoea in young calves. In humans, they are responsible for some respiratory diseases and acute enterocolitis (for review, see Tyrrell *et al.*, 1975).

Coronavirus virions have usually been described, after negative staining, as large pleomorphic particles showing characteristic bulbous projections which form a corona around the virus core (Robb & Bond, 1979). Several measurements have revealed a range in total diameter of the virions from 70 nm to 200 nm. The surface projections have been observed to vary in shape and size, although the length is normally between 12 nm and 25 nm, and usually form a single fringe radiating from the virus core in all coronaviruses with the exception of bovine enteric coronavirus (BECV) and diarrhoea virus of infant mice (Sugiyana & Amano, 1981), where two layers of projections have been described (Caul & Egglestone, 1977; Robb & Bond, 1979). The distal ends of the spikes are clearly visible on the micrographs but the tails are only occasionally seen.

Although negative staining has permitted some critical analysis of these viruses, uncertainty remains with respect to some details of their ultrastructure, especially regarding the real morphology of the projections and the relationship between the projections and the virus surface. Nermut & Franck (1971) have proved that with the freeze-drying method the size and architecture of enveloped viruses can be established with precision. In the present study we used this technique to define some of the uncertain morphological aspects of BECV virions, and on the basis of a more accurate and reproducible image of the virus particles, we propose a model based on our observations.

BECV, strain F15, was grown in HRT 18 cells as previously described (Laporte *et al.*, 1980; Laporte & Bobulesco, 1981). Virus was purified on a 20 to 45% (w/w) sucrose gradient (Beckman SW27 rotor for 90 min at 45000 rev/min, 4 °C). The virus band was collected and pelleted after dilution in water (Beckman SW25 rotor for 90 min at 45000 rev/min, 4 °C). The pellet was resuspended in distilled water and layered on to a 20 to 60% (w/w) sucrose gradient (Beckman SW27 rotor for 17 h at 25000 rev/min). Gradient fractions were collected and absorbance at 254 nm measured (ISCO U.A.5 density gradient fractionator). The refractive index of the fractions was read with a Zeiss refractometer. Rotavirus particles, used in some experiments as controls, were obtained and purified as previously described (Roseto *et al.*, 1979).

Purified virus particles were placed on to collodion-coated grids for a few minutes and then washed in 0·1 M-ammonium acetate buffer pH 6·2, excess buffer was blotted and the grids were immediately dipped in nitrogen slush (−210 °C). They were then clamped in a copper specimen holder immersed in liquid nitrogen and transferred to the vacuum chamber of a Reichert cryofract apparatus (Escaig & Nicolas, 1976). Ice sublimation was performed at −80 °C at a pressure of about 5×10^{-8} Torr. Virus particles were shadowed with platinum at a single angle (45°) for about 15 s, coated with a carbon film, and warmed to room temperature with dry

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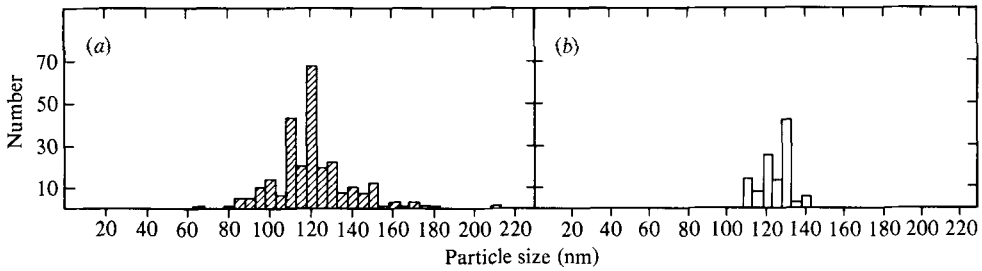


Fig. 1. The size of coronavirus particles as determined by (*a*) negative staining (the mean particle diam. of virions is 120·43 ± 15·45 nm, N = 240) and (*b*) freeze-drying (the mean particle diam. of virions is 130 ± 10·3 nm, N = 130).

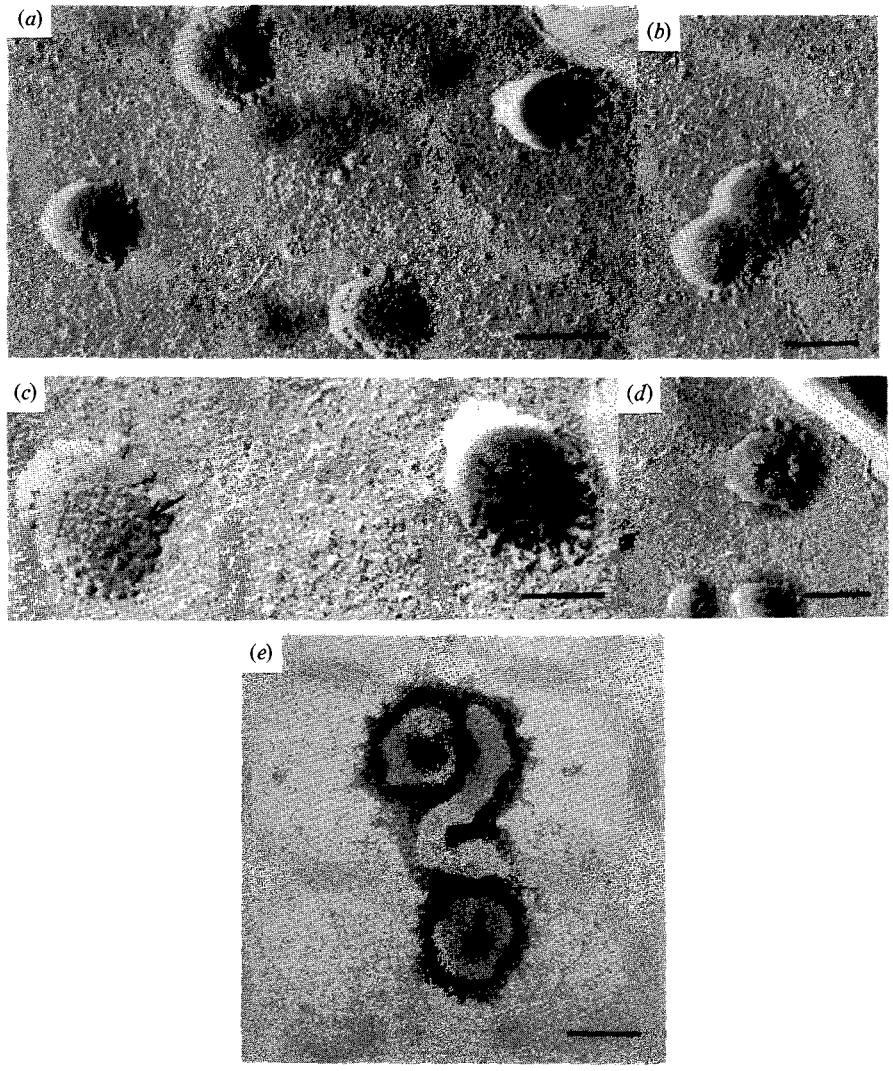
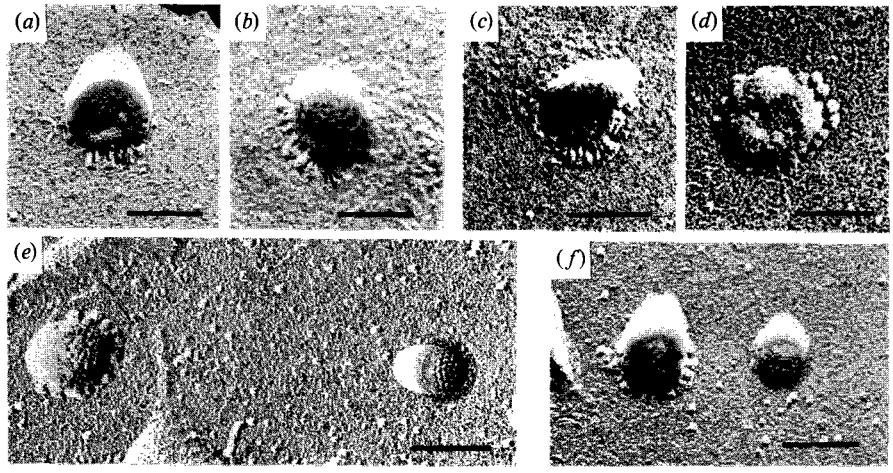


Fig. 2. (*a, b*) Coronavirus particles treated by freeze-drying showing the 'blackberry-like' morphology. (*c*) Enlargement of part of (*a*) clearly showing the characteristic projections of coronavirus, the uneven surface probably formed by the highly packed projecting heads (left arrow), and also showing the smooth central depression (right arrow). (*d*) Typical particle showing the smooth depression (arrow). (*e*) Coronavirus particles showing typical pleomorphy after negative staining. Bar markers in (*a, b*) and (*d, e*) represent 100 nm; bar marker in (*c*) represents 50 nm.



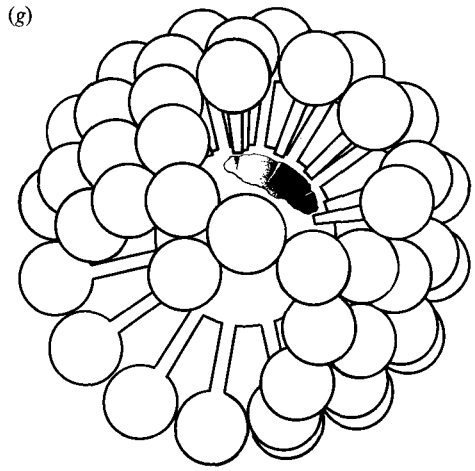


Fig. 3. (*a* to *d*) Partly broken coronavirus particles, showing the insertion of undamaged projections into the virus membrane (size, 27 nm in length). (*e, f*) Comparison of coronavirus and rotavirus structure prepared by the freeze-drying technique. (*g*) Speculative model of coronavirus particle showing projections inserted in the virus membrane. The central depression is considered to be a smooth zone. In the model, the arrangement of the projections is not symmetrical. All bar markers represent 120 nm.

nitrogen. The different thicknesses of platinum and carbon deposits were estimated to be 1·5 and 2 nm respectively, using a 'Kronos' quartz film thickness monitor.

Examination of purified BECV preparations after negative staining showed typical pleomorphic particles, with diameters ranging from 65 nm to 210 nm (Fig. 1*a*), and with a mean value of 120·43 ± 15·45 nm. Using the freeze-drying technique the virion population was less pleomorphic and more homogeneous. Particles were, in general, spherical with an average diameter of 130 ± 10·3 nm (Fig. 1*b*). Three different categories of image were observed. (i) Virions which exhibited a complete corona of spikes and an uneven surface probably formed by the highly packed heads of these projections (Fig. 2*a* to *c*); these 'blackberry-like' virions constituted the majority of particles. (ii) Virions with a characteristic smooth depression with a mean diameter of 44 nm and representing nearly 25% of the virion surface (Fig. 2*c, d*); these virions constituted about 30% of the particles. (iii) Apparently broken particles clearly showing undamaged projections and the area of spike insertion at the virus membrane (Fig. 3*a* to *c*); the projections are well-defined and look like 'mushrooms' with a 'cap' (13 nm in diam.) and a 'stalk' (approx. 14 nm long), and do not form a double fringe as is sometimes observed in negative-stained virions. These are the least common particles.

The freeze-drying technique we employed to examine BECV offers some advantages over negative staining, especially as particles seem to be better preserved with regard to shape and size, and since single-sided images can be obtained instead of superimposed ones (Roseto *et al.*, 1979). In this context, the most interesting observations made in this study are related to the shape and size of the virus particles themselves, and to the structure of the bulbous projections.

From our observations BECV particles are less pleomorphic than it has been previously assumed. In fact, in our preparations the particles show a rather homogeneous morphology with an approximately spherical shape and an average diameter of 130 nm. The 'blackberry-like' appearance of the virions is mainly suggested by the corona of spikes visible at the periphery of the particles. For the rest of the virion surface, and with the coating angle used in our experiments, the thickness of the metal layer precludes a good definition of the knobs. However, it seems reasonable to assume that the highly packed heads of the projections which arise from the smooth internal membrane, would form the uneven surface visible in the pictures beyond the corona of spikes.

One interesting observation we have made in this study was of the presence of a central depression at the surface of about 30% of the particles. This depression could be an artefact but it may correspond to the tongue- or flask-shaped structure described by Bingham & Almeida (1977) after negative staining. As no such type of depression was seen in the rotavirus particles added as a control to the BECV particles, we conclude that our observation was not due to an artefact of preparation.

The freeze-drying technique has enabled us to determine the morphology of the surface projections of coronaviruses, which is different to their appearance after negative staining. At least four different types of surface projection have been described previously in the literature. One type has projections with a petal-shaped appearance (Caul & Egglestone, 1977), a second type of projection resembles a teardrop-like knob attached to the particle by a thin stalk (Sugiyana & Amano, 1981), and a third type, in which the projections appear to be fairly large spherical knobs, has been reported for a coronavirus-like agent associated with seabirds (Traavik *et al.*, 1977). Finally, filiform projections without well-defined extremities have been reported for transmissible gastroenteritis virus (Underdahl *et al.*, 1974). Our results with BECV using the freeze-drying technique show very clearly a single form of projection which looks like a mushroom and has an average length of about 25 nm.

These observations and our theoretical calculations allow us to propose the model for BECV depicted in Fig. 3(*g*). This is a model of a spherical particle covered with highly packed surface projections inserted at the virus membrane. The projections are assumed to attain a maximum number of 110 per particle and the heads to be in close contact with each other. The validity of this model should be verified by extending the application of the freeze-drying technique to other coronaviruses from different species.

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