Propagation and Preliminary Characterization of a Chicken Candidate Calicivirus

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SUMMARY

The present paper reports the propagation and partial characterization of calici-like virus particles previously observed in gut homogenates obtained from stunted broiler chicks. Radiolabelling experiments with [^{3}H]uridine showed that the virus possesses an RNA genome and that it can replicate in primary chick embryo fibroblasts in the presence of actinomycin D. *In vivo* studies showed that the virus can be serially passaged in specific pathogen-free chicks and virus particles can be recovered from the gut and excreta of infected birds. The morphological appearance and biophysical properties of the virus were similar to those of feline calicivirus, which supports the view that it should be tentatively classified as a member of the *Caliciviridae*.

INTRODUCTION

Caliciviruses are a group of viruses which can be recognized in the electron microscope after negative staining by the cup-shaped hollows on their surface which form three characteristic patterns depending on whether the particles are orientated along their two-, three- or fivefold axis of symmetry (Burroughs *et al.*, 1978). Initially they were classified within the *Picornaviridae* but subsequent studies revealed sufficient differences in their biophysical and biochemical properties and replication cycle for the International Committee on the Taxonomy of Viruses (Schaffer *et al.*, 1980) to propose that they should be re-classified in a new family, the *Caliciviridae*.

The recognized members of the *Caliciviridae*, vesicular-exanthema of swine virus, San Miguel sealion virus and feline calicivirus (FCV) have been isolated from faecal samples and from the gastrointestinal tract but they are not generally considered to cause diarrhoeal disease (Studdert, 1978). In recent years several 'candidate' caliciviruses have been detected in faecal samples by electron microscopy (EM); two of them (human and bovine) have been clearly demonstrated to cause diarrhoeal disease (Cubitt *et al.*, 1979; Bridger *et al.*, 1984).

In 1981 Wyeth *et al.* reported on the basis of EM observations the presence of calici-like virus particles in gut homogenates obtained from stunted broiler chicks. Other features of the illness were poor feathering and sticky vents.

In this paper we present evidence for classifying this virus as a member of the *Caliciviridae* and describe methods for the propagation of the virus both *in vivo* and *in vitro*.

METHODS

Virus. A gut homogenate (Weybridge 53/81) containing large numbers of chicken candidate calicivirus particles (CCV) was obtained from Mr N. Chettle, Central Veterinary Diagnostic Laboratory, Weybridge, U.K. This material had been stored for several months at 4 °C.

A culture of feline calicivirus (FCV) strain K1 was obtained from Dr F. Brown, Animal Virus Research Institute, Pirbright, U.K. and propagated in a continuous line of feline lung cells at an m.o.i. of 0·1 and harvested after 18 h incubation at 37 °C. Aliquots of material were stored at −70 °C until required.

Canine calicivirus (CaCV) (Schaffer *et al.*, 1985) was obtained from Dr F. L. Schaffer, Naval Biosciences Laboratory, University of California, U.S.A. and propagated in a line of dolphin kidney cells, NBL-10 Sp 1 k (Flow Laboratories).

Human calicivirus (HCV) was extracted from a sample of faeces, as described previously (Cubitt *et al.*, 1979).

Cell culture. Feline lung cells were grown in Eagle's Basal Medium (Wellcome) supplemented with 0·1% sodium bicarbonate, 10% newborn calf serum (Flow Laboratories), penicillin (100 units/ml) and streptomycin (100 µg/ml). Once the cells had formed an almost confluent monolayer, they were washed twice in warm phosphate-buffered saline (PBS) pH 7·4, and kept on serum-free maintenance medium (MM) (Medium 199, 0·1% NaHCO₃) until they were needed.

Cell monolayers of primary chick embryo fibroblasts (CEF) were prepared as described by Morser *et al.* (1973).

All monolayers of dolphin kidney cells, pass 23, were cultured and maintained as described in the Catalog of the American Type Culture Collection (1981).

Propagation of CCV in vitro. Two-hundred µl of gut homogenate (53/81) which had been passed through 0·45 µm and 0·2 µm membrane filters (Flow Laboratories) to ensure that it was free of bacteria was added to 2 ml of Medium 199 containing 2 µg/ml actinomycin D and 10 µg/ml trypsin (1:250; Difco) and inoculated onto a cell monolayer of CEF. After incubation for 1 h at 37 °C the inoculum was removed and the monolayer washed twice with warm PBS. Ten ml of Medium 199 containing 2 µg/ml actinomycin D and 10 µg/ml trypsin was added to the flask and incubation continued for a further 18 h at 37 °C. The supernatant was harvested, centrifuged at 3000 *g* to remove cell debris and then again at 90000 *g* in an MSE 3 × 25 ml rotor for 2 h at 4 °C. The resultant pellet was resuspended in 250 µl of TNE buffer (50 mM-Tris–HCl pH 7·4, 100 mM-NaCl, 1 mM-EDTA).

Virus was purified by layering the resuspended pellets onto a 10 ml, 20 to 45% (w/v in TNE) linear sucrose gradient and centrifugation at 180000 *g* in an MSE 6 × 14 ml rotor for 2 h at 4 °C. Fractions (300 µl) were collected and samples processed and examined as described previously (Cubitt & Barrett, 1984).

The experiments were repeated but with the addition of 100 µCi [³H]uridine (sp. act. 29·7 Ci/mmol, Amersham) to the culture medium.

Propagation of CCV in vivo in specific pathogen-free (SPF) chicks. Fertile SPF eggs were obtained from the Central Veterinary Laboratory, Weybridge and set and hatched at the Central Middlesex Hospital. Day-old chicks were selected, tagged and weighed and divided into three groups of three birds. Birds were inoculated by means of a plastic pipette placed directly into their gullet. One group were fed with 1 ml of filtered gut homogenate (CCV, 53/81) which had been shown by EM to contain large numbers of CCV particles. The second group was fed with a faecal filtrate containing large numbers of human calicivirus particles and the third group with a faecal extract obtained from uninfected birds which had been passed through 0·45 µm and 0·2 µm membrane filters and centrifuged at 100000 *g* for 1 h to ensure that it was free of bacteria and viruses. Each group of birds was reared under identical conditions in heated cages but in isolated cubicles.

Droppings from each group of birds were collected daily and examined by EM for the presence of virus particles and each bird was examined for signs of illness. The weights of birds were recorded at weekly intervals or at the time of death.

The guts and their contents were collected from all the birds at the conclusion of the study (after 3 weeks) and from birds that died during the course of the experiment. The gut contents were removed for EM examination and the gut washed with PBS, before being homogenized in serum-free maintenance medium. The homogenate was mixed with an equal volume of chloroform and shaken on a wrist action shaker for 30 min. The cell debris was removed by centrifugation at 2500 r.p.m. in an MSE bench centrifuge. The aqueous layer was collected and CCV extracted by centrifugation at 90000 *g* and passage through a sucrose gradient as described previously (Cubitt *et al.*, 1979).

Samples of blood were collected from birds at the time of death or at the conclusion of the experiment. The serum was separated, heated at 56 °C and examined for the presence of antibodies to CCV and HCV. CCV was extracted from gut homogenates and HCV extracted from a sample of faeces.

A second series of birds were fed with gut homogenate containing CCV particles which had been obtained from one of the birds which had died after 8 days in the initial study and which had been shown to develop an antibody response to CCV. The methods for administering the virus and rearing the birds were identical to the initial study.

Characterization of chicken 'candidate' calicivirus

The virus used for studying the characteristics of CCV was extracted from gut homogenates and gut contents obtained from experimentally infected SPF chicks. FCV K1 propagated in feline lung cells was used as a representative member of the *Caliciviridae.*

Size and morphology. A portion of gut and its contents from an infected SPF chick was homogenized in 5 ml of maintenance medium and thoroughly mixed with an equal volume of chloroform. The aqueous layer was removed and FCV purified by treatment with 'Arklone' (trichlorotrifluorethane) as described by Brown & Cartwright (1960). The supernatant was centrifuged at 100000 *g* for 1 h at 4 °C in a Beckman SW50.1 rotor. The resultant pellets were resuspended in maintenance medium and 10 µl aliquots placed onto 2% agarose. A carbon–Formvar-coated grid was inverted over each drop and the fluid allowed to diffuse away overnight. The grids were stained with 2% potassium phosphotungstic acid, pH 6·4, containing crystalline bovine catalase. One-hundred particles were measured using the lattice spacing of catalase as an internal standard of length (Wrigley, 1968).

The morphological appearance of CCV particles was recorded on film and a number of particles selected for image enhancement by the Markham rotation technique (Markham *et al.*, 1963) to demonstrate the characteristic appearance of caliciviruses.

Buoyant density in caesium chloride. The method employed was identical to that described previously for human and feline caliciviruses (Cubitt *et al.*, 1979). FCV K1 was run in a parallel gradient as a control. The position of the virus peaks was determined by EM examination of each fraction. The number of virus particles on five grid squares of a 400-mesh copper EM grid were counted and the peak was taken as the fraction containing the most particles.

Sedimentation coefficient in glycerol. The S value in 1·05 to 1·25 g/ml glycerol gradients was determined according to the method of Oglesby *et al.* (1971). CCV and FCV K1 were co-sedimented with poliovirus type 2 in parallel gradients and fractionated after 1, 2 and 3 h runs. The position of the virus peaks was determined by EM examination of the fractions.

Effect of lipid solvents. One ml of gut extract containing CCV and 1 ml aliquots of cell harvests containing FCV K1 were treated with an equal volume of ether or chloroform, mixed and incubated for 2 h at room temperature. The aqueous layer was removed, centrifuged at 100000 *g* for 1 h at 4 °C and the resultant pellet resuspended in potassium phosphotungstic acid and examined by EM to determine whether or not the virus particles had been disrupted.

Serological relationships with characterized and 'candidate' caliciviruses. Immunoelectron microscopy (IEM) tests were carried out at 4 °C overnight in microtitre plates using a serum in agar technique (Cubitt *et al.*, 1979). Sera were coded and titrated in a series of doubling dilutions from 1 :10 to 1 :320. The antibody titre was taken as the highest dilution at which virus particles were seen to be linked by antibody.

Tests were carried out with CCV antigen and hyperimmune antisera raised against vesicular exanthema of swine virus, serotypes A, D and E; San Miguel sealion virus type 1 and FCV K1. Another series of tests were performed using convalescent-phase sera to three antigenically distinct strains of human calicivirus 'Shenley,' 'Portsmouth' and 'Japan' (Cubitt & McSwiggan, 1981); Newbury agents of cattle (Bridger *et al.*, 1984) and porcine enteric calicivirus (Saif *et al.*, 1980).

Reciprocal tests were performed with sera containing antibodies to CCV obtained from infected SPF chicks and FCV K1, HCV or a recently described canine calicivirus (Schaffer *et al.*, 1985) as antigens.

RESULTS

Propagation of CCV in vitro

Chick embryo fibroblasts prepared from 11-day-old embryos were found to support the growth of CCV. Initial experiments showed that incubation for 18 h at 37 °C in medium containing 10 μg/ml trypsin and 2 μg/ml actinomycin D yielded sufficient yields for virus particles to be detected by EM (10^7/ml). Virus particles were morphologically indistinguishable from those in the inoculum and IEM tests confirmed that they were CCVs and excluded the possibility of cross-contamination with FCV K1. Progeny virus in the supernatant was shown to infect CEF cells, indicating that the virus can be passaged *in vitro*. The ability of CCV to replicate in the presence of actinomycin D suggested that it has an RNA genome; direct evidence was obtained by radiolabelling with [^{3}H]uridine in the presence of actinomycin D. The gradient profile is shown in Fig. 1; a peak of radioactive counts was found in fraction 18 (density 1·142 g/ml) which corresponded with the only fraction which was found to contain CCV particles when examined by EM. IEM tests confirmed that the virus was CCV.

The possibility that the peak of radioactivity was due to reoviruses seems improbable, as EM and IEM tests failed to reveal any particles other than CCV. Reovirus obtained from the excreta of SPF chicks could be readily propagated in CEF cells provided actinomycin D was excluded from the medium; however, reoviruses were not detected in cultures inoculated with fractions from the gradients.

In a parallel series of experiments with CCV performed in the absence of trypsin there was no evidence of radiolabelling or cytopathic effects and virus particles were not detected by EM or IEM. It appears, therefore, that CCV will only replicate in the presence of trypsin, that the results were not due to recovery of CCV particles from the inoculum without growth and that possible contamination of the inoculum with reovirus was not influencing the results.
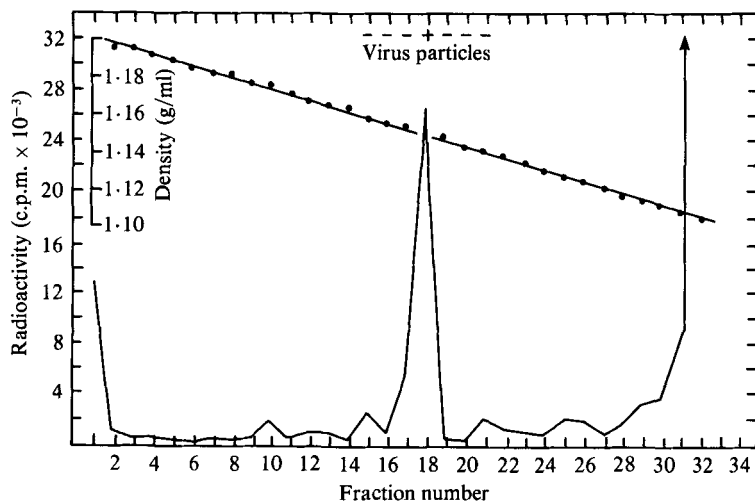
Fig. 1. Gradient profile after velocity centrifugation, demonstrating the incorporation of [³H]uridine into CCV. 'Virus particles' refers to the detection by EM: +, virus detected; −, virus not detected.
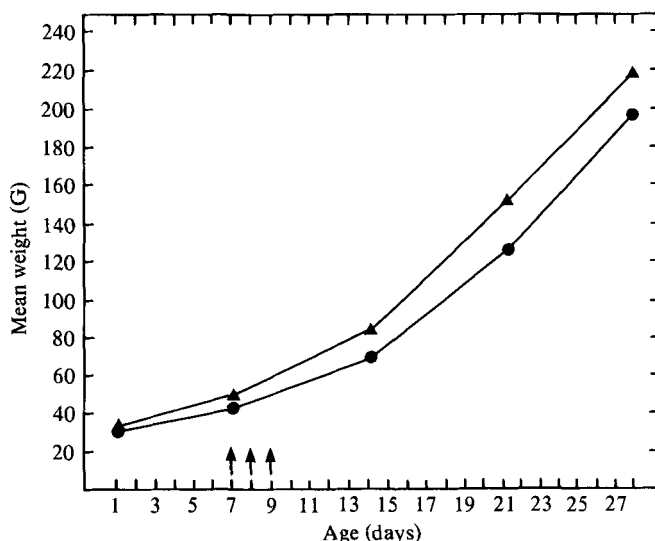

Fig. 2. Growth curve of SPF chicks fed with CCV obtained from the gut of an SPF chick infected with CCV 53/81. ●, Test birds fed with CCV; ↑, calicivirus detected in test bird's droppings; ▲, control birds fed with faecal extract obtained from uninfected SPF chicks.

Propagation of CCV in day-old SPF chicks

All three birds fed with CCV (53/81) failed to thrive. Droppings collected from this group of birds were found to contain reovirus particles from day 3 onwards and calicivirus particles on days 6, 7 and 8. Two of the three birds produced loose droppings and developed sticky vents; one died on day 6 post-infection and the other on day 8. Examination of post mortem material by EM revealed calicivirus and reovirus particles in their gut contents but only calicivirus in gut homogenates. IEM tests on sera from the two birds which died had an antibody titre of 80 to CCV. The third bird survived until the end of the experiment (21 days) but had only attained 75% of the body weight of control birds or those fed with HCV. The antibody titre to CCV in this bird's serum was 20 to 40. Post mortem material from this bird was found to contain reoviruses.

The birds fed with the faecal extract and with HCV developed normally, attaining a weight of 150 g by day 21. All six birds survived and none developed antibody responses to either CCV or HCV. Examination of droppings and post mortem material by EM failed to reveal the presence of any virus particles.

The results of the second series of tests are illustrated in Fig. 2. The eight birds fed with CCV failed to thrive and attained only 83% of the mean weight of the six control birds. In contrast to the initial experiment all the test birds survived and developed only low antibody responses to CCV, 10 to 20. EM examination of droppings from test birds revealed reovirus from day 5 onwards and calicivirus on days 7, 8 and 9.

Examination of post mortem material from birds fed with CCV which were sacrificed on day 28 post-infection, failed to reveal the presence of calicivirus but the material did contain reovirus. The growth curve (Fig. 2) shows that growth was retarded in birds fed with CCV until the second week, after which they grew at the same rate as the control birds. The period of excretion of CCV was only 3 days and the cessation of excretion of CCV coincided with the return to a normal growth rate despite the fact that birds were still shedding reovirus.

Characterization of CCV

Morphology of CCV

The appearance of CCV particles obtained from infected SPF chicks is illustrated in Fig. 3(*a*). The characteristic stain-filled cups and surface morphology of the *Caliciviridae* was apparent. The results of image enhancement by Markham rotation (Fig. 3*b*, *c*) showed the characteristic appearance of a calicivirus orientated along the three- and fivefold axis of symmetry respectively.

Size

The results of measurements of the diameter (furthest point to furthest point) of 100 CCV particles are shown in Table 1. The mean diameter was 36·0 nm, which is similar to the value obtained for FCV, 35·8 nm.

Buoyant density

The buoyant density of CCV determined on four occasions in caesium chloride was between 1·38 and 1·39 g/ml, similar to the value obtained for FCV K1 run in parallel gradients, 1·36 to 1·40 g/ml. The wide range of results obtained for FCV K1 reflects the instability of this virus, the density decreasing with increased length of storage.

Sedimentation coefficient in glycerol

The S value for CCV in glycerol gradients was estimated to be 205S, and 204S for FCV K1 run in a parallel gradient.

Effect of lipid solvents

The morphological appearance of CCV and FCV K1 was unaffected by treatment with ether or chloroform, indicating that lipids do not form an essential structural component of the virions.

Antigenic relationships between CCV and other caliciviruses

The results of IEM tests are shown in Table 2. There was no apparent antigenic relationship between CCV and vesicular exanthema of swine viruses A, D or E, San Miguel sealion virus type 1, feline calicivirus strain K1, human caliciviruses, porcine enteric calicivirus or Newbury agents 1 or 2. However, convalescent-phase sera from infected SPF chicks reacted strongly at a titre of 1:40 to a canine calicivirus (Schaffer *et al.*, 1985).
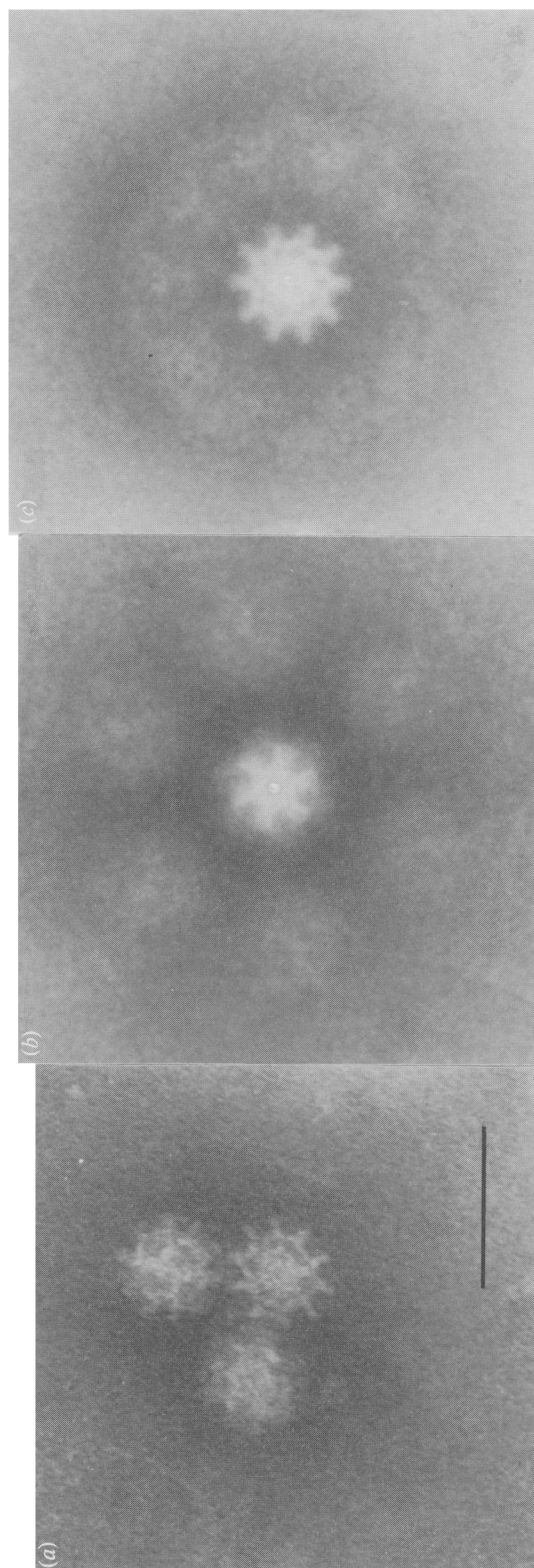
Fig. 3. (a). Electron micrograph of chicken calicivirus particles obtained from a homogenate of gut obtained from a bird infected with CCV 53/81. Virus was stained with potassium phosphotungstic acid. Bar marker represents 50 nm. (b) Markham rotation of CCV particle. N = 3 to demonstrate the characteristic appearance of a calicivirus orientated along the threefold axis of symmetry. (c) Markham rotation of CCV particle. N = 5 to demonstrate the characteristic appearance of a calicivirus orientated along the fivefold axis of symmetry.

Table 1. *Biophysical properties of chicken and feline caliciviruses**

	Mean diameter (nm) ± 1 s.d	Buoyant density (g/ml)	S value in glycerol
CCV	36·0 ± 0·9	1·38–1·39	205
FCV	35·8 ± 1·5	1·36–1·40	204

* Protocols used to determine the measurements are described in Methods

Table 2. *IEM serological relationships between CCV and other members of the* Caliciviridae

	Antigen			
Antiserum	CCV	FCV K1	CaCV	HCV 'Shenley'
CCV	**80**	<20	40	<20
FCV K1	<20	**320**	<20	<20
CaCV	20	<20	**160**	<20
HCV 'Shenley'	<20	<20	<20	**160**
HCV 'Portsmouth'	<20	<20	<20	<20
HCV 'Japan'	<20	<20	<20	20
VESV* A,D,E	<20	<20	<20	<20
SMSLV* 1	<20	<20	<20	<20
Newbury 1,2	<20	<20	<20	<20
PEC*	<20	<20	<20	<20

* VESV, Vesicular exanthema of swine virus; SMSLV, San Miguel sealion virus; PEC, porcine enteric calicivirus.

DISCUSSION

The results of the cell culture experiments demonstrate that CCV can replicate and be passaged in CEF cells provided trypsin is present in the medium, although the yields of virus were low. A similar situation was found with a human calicivirus (Cubitt & Barrett, 1984) and a canine calicivirus (Schaffer *et al.*, 1985) and it has been suggested that the apparently low yields are due to the virus being strongly bound to cell membranes (Schaffer *et al.*, 1985).

The radiolabelling experiments with [^{3}H]uridine in the presence of actinomycin D confirmed that the virus can replicate in CEF cells and demonstrated that CCV has an RNA genome and that nucleic acid synthesis does not involve a DNA-dependent step. Similar results were obtained for a human calicivirus (Cubitt & Barrett, 1984).

Addition of 10 µg/ml trypsin caused cells to detach from the surface of the culture bottles and, therefore, it was not possible to observe cytopathic effects or to develop a plaque assay for CCV.

The results of the *in vivo* experiments provide clear evidence that CCV remains viable for several months when held at 4 °C and that it can be successfully transmitted to and passaged in SPF chicks. The evidence that CCV causes stunting was inconclusive, as affected birds were also found to shed reovirus. However, the two birds that died in the initial experiment were found to be shedding calicivirus and the guts were shown to contain CCV. The rate of growth of birds fed with CCV returned to normal soon after the cessation of excretion in the survivors although they were still shedding reovirus. Furthermore, extensive tests have shown that the reovirus does not cause stunting in SPF chicks and that it is a common gut commensal in healthy broiler chicks (N. Chettle, personal communication). Further studies are planned to establish whether CCV is a cause of stunting.

The morphological appearance of CCV was characteristic of the members of the *Caliciviridae* and bore a strong resemblance to an invertebrate calicivirus (*Amyelosis* chronic stunt virus) described by Hillman *et al.* (1982). The biophysical characteristics (Table 1) were similar to those of FCV K1 which served as a representative member of the *Caliciviridae*. Similar values for the sedimentation rate in glycerol (207S) and for the buoyant density (1·39 g/ml) have been recorded for the prototype calicivirus, vesicular exanthema of swine (Oglesby *et al.*, 1971). These

results together with the evidence that CCV has an RNA genome support the view that chicken 'candidate' calicivirus should be tentatively classified within the *Caliciviridae*, although further biochemical studies on the RNA and proteins of CCV will be necessary to prove conclusively that the virus is a calicivirus.

Further work is now in progress to obtain sufficient yields of chicken calicivirus in order to determine the nature and number of the virus-specified polypeptides and to develop tests for the investigation of stunting syndrome in chickens.

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