

## THE ISOLATION AND CULTIVATION OF CALF ROTAVIRUS IN THE REPUBLIC OF SOUTH AFRICA

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### ABSTRACT

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Calf rotavirus was cultivated and propagated in tissue culture from faeces of 3-week-old calves suffering from severe diarrhoea. Criteria for viral involvement were: production of cytopathic effects in primary foetal calf kidney cells, specific fluorescence, and identification of the agent by means of electron microscopy. In a limited serological survey the majority of the cows on an infested farm were found to possess neutralizing antibodies to the local rotavirus strain.

### Résumé

#### ISOLEMENT ET CULTURE DU VIRUS ROTA DU VEAU DANS LA RÉPUBLIQUE D'AFRIQUE DU SUD

On a cultivé et propagé en culture de tissus le virus rota de veau extrait de fèces de veaux âgés de 3 semaines et atteints de diarrhée aiguë. Les critères qui ont fait conclure à une action du virus étaient: la production d'effets cytopathiques dans les cellules rénales primaires de foetus de veau, la fluorescence spécifique, et l'identification de l'agent au microscope électronique. Au cours d'une enquête sérologique restreint on a constaté que la majorité des vaches dans une ferme infestée possédaient des anticorps neutralisant de la souche locale du virus rota.

### INTRODUCTION

Rotavirus (reo-like virus) has been isolated from neonatal calves suffering from diarrhoea by Mebus, Stair, Underdahl & Twiehaus (1971), Fernelius, Ritchie, Classick, Norman & Mebus (1972), and Bridger & Woode (1975). The pathogenicity of the virus was demonstrated in colostrum-deprived calves by Woode, Bridger, Hall & Dennis (1974). Further evidence of the significance of rotavirus is supported by the work of Mebus, Underdahl, Rhodes & Twiehaus (1969) and Welch (1971), who found it to be the only detected agent in faeces of diarrhoeic calves.

This study is a record of the successful isolation and cultivation in tissue culture of a rotavirus from an outbreak of diarrhoea among calves on a farm in the Transvaal, Republic of South Africa.

### MATERIALS AND METHODS

#### Tissue culture

Primary foetal calf kidney (FCK) cells were prepared by trypsinization in the cold (Paul, 1973), and monolayer cultures, prepared in tubes, were used on the 4th day. In one instance, the cells were inoculated while in suspension and later transferred by pipette into the tubes. The growth medium consisted of Hanks' balanced salt solution (BSS) with 0.5% lactalbumin hydrolysate and 10% bovine serum known to be negative for antibodies to the Nebraska strain of rotavirus. The maintenance medium, Hanks' BSS without serum, contained penicillin 200 IU, streptomycin 200 µg, colistin 200 IU and amphotericin B (Fungizone) 2.5 µg per ml. LLC-MK2\* cells at the 70th subculture, grown in Eagle's medium containing serum and antibiotics as described for Hanks' medium, were used to cultivate the virus by adding trypsin to the medium, as described by Babiuk, Mohammed, Spence, Fauvel & Petro (1977). The cells were washed twice with Hanks' medium and subsequently treated for 1 h at 37 °C with medium containing 25 µg trypsin per ml. The latter medium

was then decanted, virus suspension containing the same concentration of trypsin added and, after 1 h incubation at 37 °C, replaced by Eagle's medium containing 2.5 µg trypsin per ml. The cultures were examined daily under the microscope.

#### Virus isolation

The inoculum consisted of faeces from calves aged 2-3 weeks suffering from severe diarrhoea. Five samples from 2 farms, Farm A belonging to the Johannesburg Municipality and Farm B, near Krugersdorp, both in the province of the Transvaal, were selected because the history of the outbreak was known and rotavirus particles had been detected by means of electron microscopy. A 20% suspension of faeces was prepared in Hanks' BSS which contained penicillin 400 IU and streptomycin 400 µg per ml. The suspension was homogenized by means of a 10 ml syringe, sonified for 3 minutes, kept at 4 °C for 4 h and centrifuged at 2 000 rpm for 10 minutes. The resultant supernatant fluid was used to inoculate the FCK cells. The tissue culture tubes were inoculated with 0.2 ml of the faecal suspension, incubated for 1 h at 37° and washed twice with Hanks' medium before 1 ml of the maintenance medium was added. Cultures, harvested on the 5th day after inoculation, were followed by 2 blind passages. For the release of cell-associated viruses, the cells were rapidly frozen and thawed 3 times. Five serial passages were then made, and the cultures examined for cytopathic effect. In addition, the cultures of the 3rd and 4th passages were examined by immunofluorescence and electron microscopy.

Subsequently, the virus was adapted to the LLC-MK2 cells and serially passaged, trypsin being included in the medium, as described above.

#### Electron microscopy

With some modification, the virus purification technique of Petric, Szymanski & Middleton (1975) was employed. A 20% suspension of faeces in phosphate pH 7.2 was homogenized for 2 minutes with an Ultra-Turrax homogenizer and centrifuged at 1 500 rpm for 15 minutes. The supernatant fluid was

\* Lilly Laboratory Corporation—Monkey Kidney 2 CCL 7, 1

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centrifuged at 5 000 rpm for 1 h in a refrigerated centrifuge, after which equal volumes (50 ml) of supernatant fluid and Freon (1,1,3-trichlorotrifluoroethane) were homogenized in the cold for 30 seconds and immediately centrifuged at 3 000 rpm for 30 minutes at 4 °C. The aqueous phase of the Freon extraction was removed and centrifuged at 25 000 rpm in a Spinco centrifuge, using the SW27 rotor. Six samples, each of 37 ml, were processed at a time. The pellets were resuspended in phosphate buffer and negatively stained by mixing the suspension with an equal volume of 3% phosphotungstic acid at pH 6. A drop of this mixture was transferred to a formvar carbon-coated grid, the excess fluid being drained off by being touched with a filter paper. After being air-dried, the grids were examined in a Siemens Elmiskop 102 electron microscope. Infected FCK cells of the 4th passage showing a considerable cytopathic effect (CPE), and LLC-MK2 cells, in which the virus had been further passaged 4 times with the trypsin treatment, were negatively stained, as described by Almeida & Howatson (1963). The infected cells were pelleted by low speed centrifugation, disrupted with distilled water, stained and examined, as described above.

The preparation and examination of sectioned FCK and LLC-MK2 infected with the virus were carried out, as described by Lecatsas (1968).

*Fluorescent antibody staining (FA)*

The direct FA staining method (Mebus *et al.*, 1969) was used to identify the virus in the infected FCK cells of the 3rd and 4th passage. The rotavirus and coronavirus conjugate\* was kindly supplied by Mme A. M. Delvaux\*\*. The indirect method of immunofluorescence (Cunningham, 1973) was used to conduct the limited serological survey. The majority of the sera were tested at 1:4 dilution and some were diluted to 1:128. Infected LLC-MK2 cells were fixed in cold acetone for 10 minutes, washed with phosphate buffer (PBS), air-dried and covered by a drop of test serum. The slides were incubated for 30 minutes at 37 °C in a moist chamber, washed twice for 10 minutes in PBS and air-dried before addition of 1 drop of 1/20 diluted labelled anti-bovine immunoglobulin prepared in rabbits\*\*\*. The slides were again incubated in a moist chamber for 30 minutes at 37 °C, washed twice for 10 minutes with PBS, air-dried and mounted.

RESULTS

*Cytopathic effect*

During the first 2 passages, microscopy revealed no discernible change in the inoculated FCK monolayers. After the 3rd serial passage, however, 3 groups of cultures inoculated with the samples of Farm A showed CPE on the 4th day. When they were harvested for further subculturing and FA staining after 6 days, the percentage ( $\pm 10\%$ ) of affected cells was low. In the 4th passage, however, the CPE appeared on the 3rd day and  $\pm 50\%$  of the cells were affected by the 6th day. There was no variation in the development of CPE between the cultures infected in suspension and those in the monolayer state. The virus concentration in these cultures never exceeded  $10^{5.5}$  logs per ml.

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The affected cells were swollen and rounded, and formed well-demarcated foci which were leathery in appearance. The size of the foci increased, but the CPE never reached 100%. The affected cells remained attached to the glass after 8 days at the 5th passage. The samples of Farm B failed to induce CPE at the 5th passage and the test was discontinued.

*Electron microscopy*

Particles resembling rotavirus were detected in the infected FCK cells of the 4th passage and in the trypsin treated LLC-MK2 cells. The virus concentration in the FCK cells was low, while masses of virus could be seen in the LLC-MK2 cells. The electron micrographs of the virus in the latter cell type are presented in Fig. 1-4.

In Fig. 1, a group of negatively stained particles is shown, some of which possess double capsids and have a diameter of  $\pm 68$  nm (upper arrow), while others have single capsids and are  $\pm 55$  nm in diameter (lower arrow). In Fig. 2, a uniform group of double capsidated particles with smooth surfaces is shown. In thin sections, the particles were almost invariably seen in the rough endoplasmic reticulum which appeared swollen (Fig. 3 & 4). Various developmental stages of the particles within these vesicles can be seen in Fig. 3.

*Fluorescent antibody staining*

Infected FCK cells at the 3rd passage level showed fluorescence after staining with the conjugated antiserum against reo-like virus, but not with the conjugated antiserum against the coronavirus. The low percentage of fluorescing cells corresponded to the slight CPE seen in the cultures under the light microscope. This percentage, however, increased with the higher passage levels, as did the CPE. The fluorescence was confined to the cytoplasm and was of a diffuse type, similar to that described by Bridger & Woode (1975).

The majority of the cattle sera (90%) from the infested farms stained infected cells brightly at 1:4 dilution. Several sera giving bright fluorescence at 1:4 also produced positive results at higher dilutions (Table 1). Sera of cows from farms with no calf scouring history and from colostrum-deprived calves were negative in the FA test.

TABLE 1 Demonstration of antibodies to rotavirus in cattle sera from an infested herd.

Cow No.	Serum dilutions					
	1:4	1:8	1:16	1:32	1:64	1:128
546.....	+++*	+++	+++	+++	+	+
R532.....	++++	+++	++	+	+	-
R493.....	++++	++++	++	+	+	-
R557.....	++++	++	++	+	+	+
R403.....	+++	++	+	+	-	-
R509.....	++++	+++	+++	++	+	+
R384.....	+++	++	++	+	-	-
S61.....	++++	++	+	-	-	-
R542.....	++	++	+	+	-	-
R368.....	+++	++	+	+	+	-

\* Fluorescence obtained was graded from 1-4 according to brightness

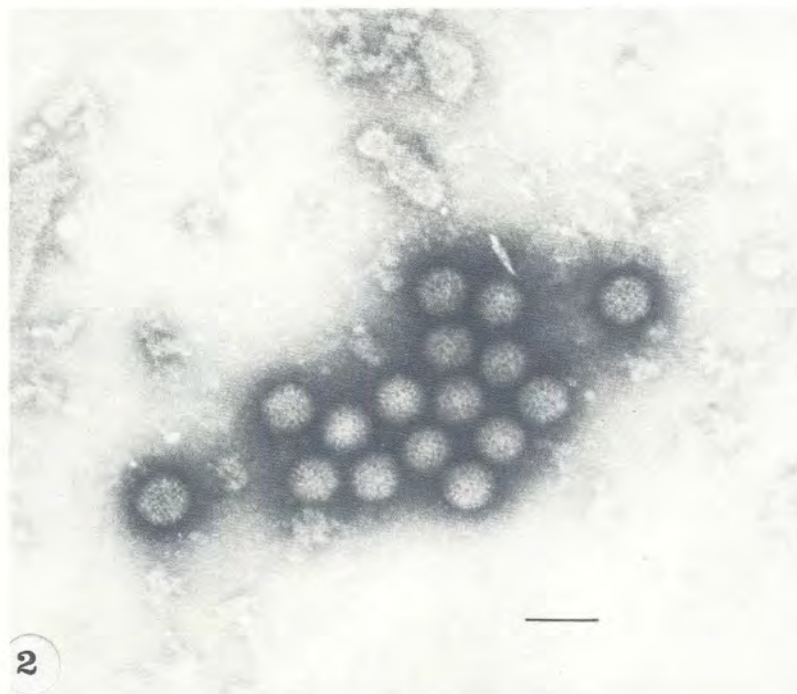
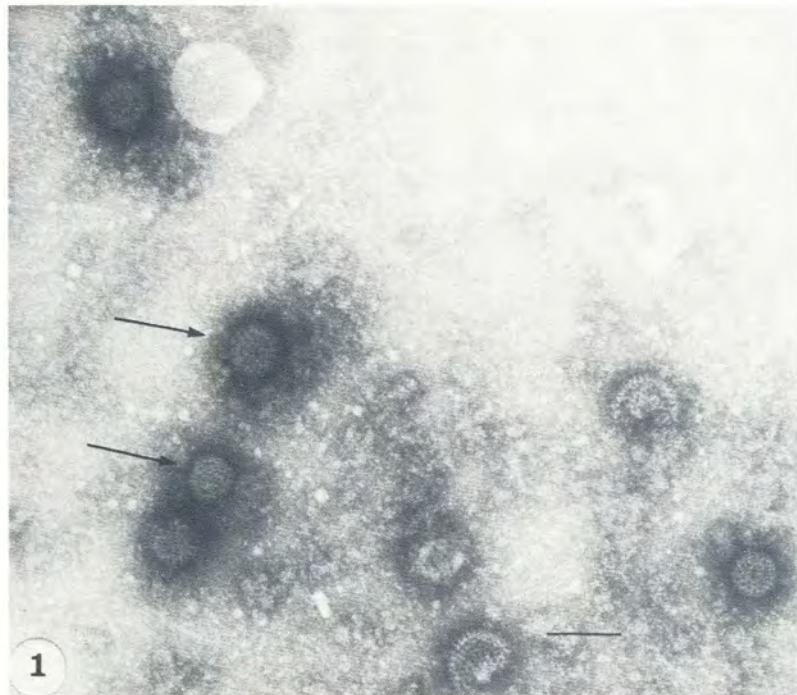


FIG. 1 68 nm particle with double capsid and smooth surface (upper arrow) and 55 nm particle with single capsid and rough surface (lower arrow), negatively stained with phosphotungstate  
FIG. 2 A uniform group of particles with double capsids (negatively stained). Bar equal to 100 nm

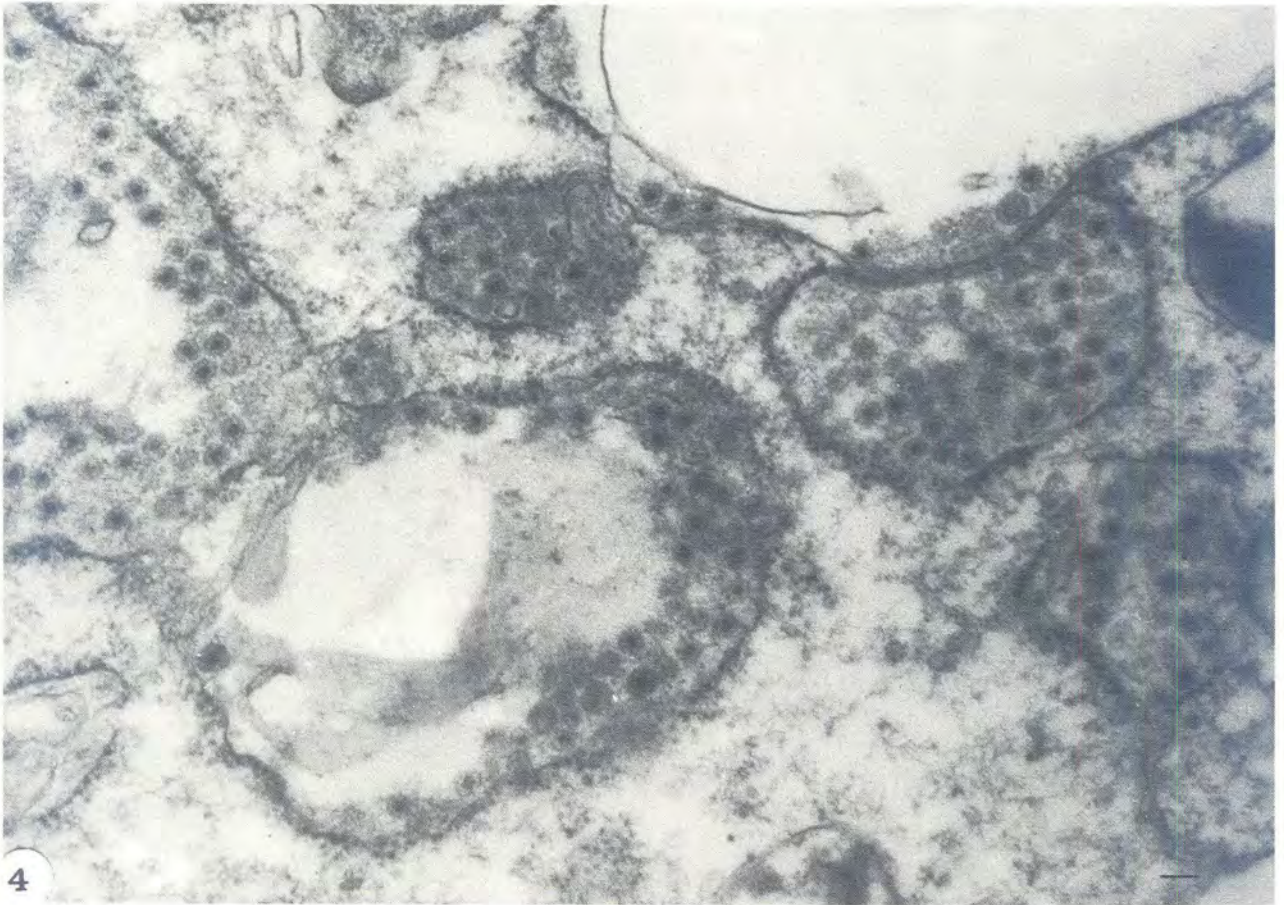
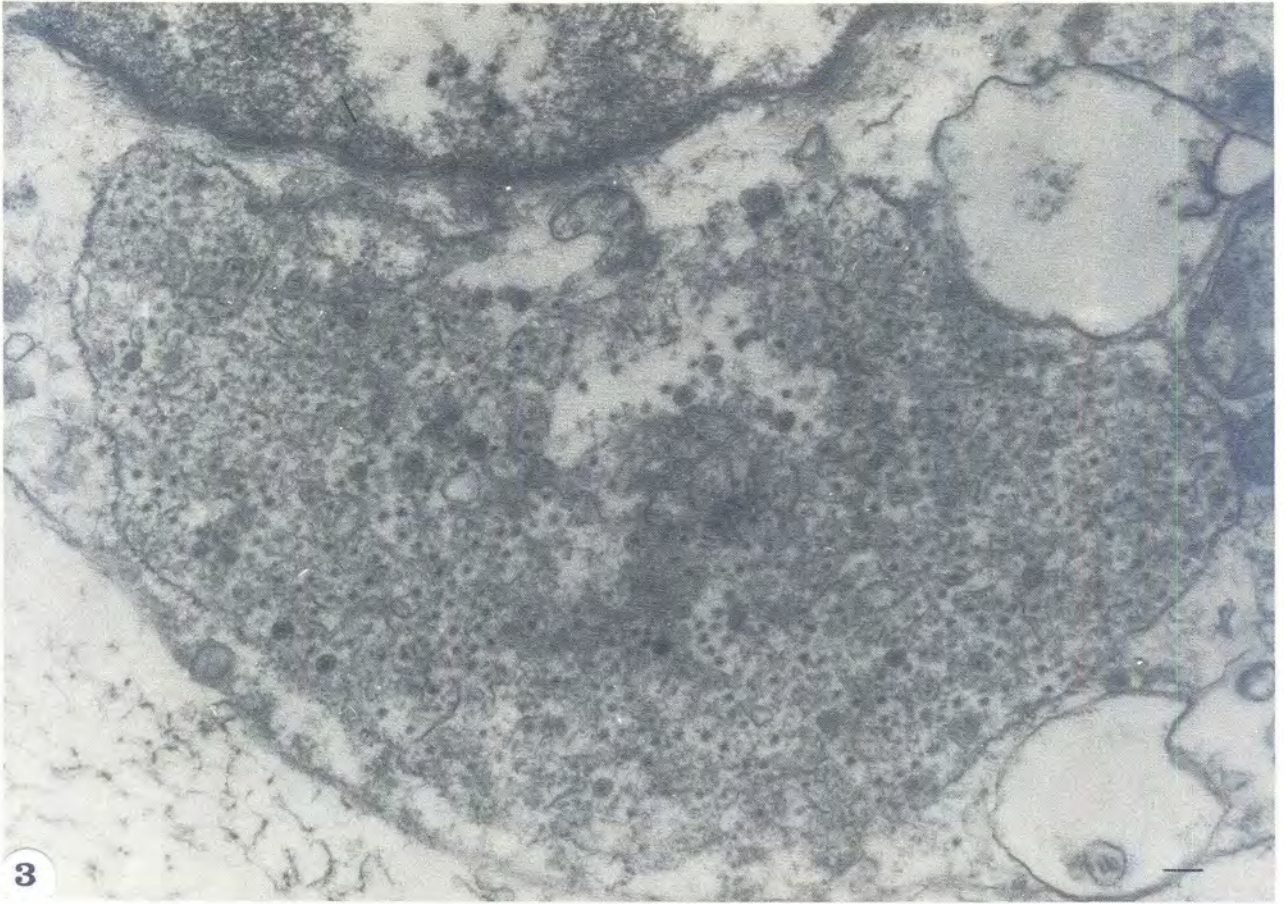


FIG. 3 Aggregate of particles at various developmental stages in a swollen, rough, endoplasmic reticulum  
FIG. 4 Vesicles of swollen endoplasmic reticulum packed with enveloped particles. Bar equal to 100 nm

## DISCUSSION

The growth rate and the type of CPE of the virus isolated in primary FCK cultures from the calves resemble that of rotaviruses described by Fernelius *et al.* (1972). According to the electron micrographs, the particles seen in the infected cells are identical in size and morphology with the rotaviruses described by Fernelius *et al.* (1972), Welch (1971) and Woode *et al.* (1974). The identification of the virus is confirmed by the fact that the infected cells stained positively with a rotavirus conjugate but negatively with the coronavirus conjugate.

The results prove that the calf rotavirus, known world-wide, is present also in the Republic of South Africa and by means of a one-way FA test it was shown that the virus is related to the Nebraska strain of rotavirus earlier known as "reo-like" virus.

This strain of rotavirus was easily adapted to LLC-MK2 cells and the presence of trypsin shortened the incubation period from 5 days to 24 h. The virus concentration in these cells was very high, a fact which is reflected by the electron microscopical and FA results. The serological survey clearly revealed that on a farm where rotavirus could be demonstrated, the majority of cows possessed antibodies, while sera of colostrum-deprived calves and of cows from other farms were negative. The absence of protection by maternal antibodies of the calves during the first week of post-natal life, when the majority were scouring, cannot be explained at present. Presumably a minimal concentration of antibodies in the colostrum is a prerequisite for protection, but these quantitative aspects were not investigated in this study.

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