Rev. Elev. Méd. vét. Pays trop., 1984, 37 (4) : 400-405.

Isolation and propagation of bovine rotavirus in cell culture

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RÉSUMÉ

OJEH (C. K.). — Isolement et propagation des rotavirus bovins en cultures cellulaires. *Rev. Elev. Méd. vét. Pays trop.*, 1984, 37 (4) : 400-405.

Seize isolements de rotavirus bovins ont été réalisés à partir de prélèvements diarrhéiques de veaux sur cellules MA 104, mais non en cultures primaires de cellules bovines (EBK), ovines (EOK) ou en cultures de lignées continues LLC-MK_2, Vero et MDBK.

Le pré-traitement de l'inoculum du virus fécal par la trypsine (5 à 10 µg/ml), complété par 1,0 µg/ml de trypsine dans le milieu nutritif et l'incubation de cellules MA 104 sur flacons roulants sont des facteurs essentiels pour le développement en continu des rotavirus bovins isolés à partir de prélèvements effectués sur le terrain. Des souches de rotavirus bovins adaptés à la culture cellulaire ont acquis la faculté de réinfecter des cellules jusqu'alors non permissives.

Mots-clés : Rotavirus bovins - Isolement - Culture cellulaire.

SUMMARY

OJEH (C. K.). — Isolation and propagation of bovine rotavirus in cell culture. *Rev. Elev. Méd. vét. Pays trop.*, 1984, 37 (4) : 400-405.

Sixteen isolates of bovine rotavirus from diarrhoeic calves were made in MA 104 cells, but not in primary bovine (EBK), ovine (EOK), nor in continuous cell lines of LLC-MK_2, Vero and MDBK monolayers. It was found that pre-treatment of faecal virus inoculum with trypsin (5-10 µg/ml), and 1.0 µg/ml trypsin in the maintenance medium and incubation of cultures on roller drums of MA 104 cells were essential for the continuous propagation of field isolates of bovine rotaviruses. Established tissue culture adapted strains of bovine rotavirus acquired the ability to reinfect erstwhile refractory cells.

Key words : Bovine rotavirus - Isolation - Cell culture.

INTRODUCTION

Rotavirus were first reported in neonatal calf diarrhoea in the USA (19). Since, then, the viruses have been shown to be very important causative agents of acute gastroenteritis in both infants and neonatal animals (10, 14). After the first report, many attempts have been made to isolate the virus in tissue culture (9, 27). Although four bovine strains, namely Lincoln, Cody (18), UK Compton (4) and Northern Ireland (15) were initially isolated and propagated in different tissues of bovine origin, detailed virological and serological studies (e.g. serotyping) were hampered for a long time because of the difficulties encountered in routinely isolating and propagating other rotaviruses in tissue culture. However, these difficulties have now been largely overcome by incubating rotavirus inoculum with trypsin, by incorporating trypsin in the maintenance medium and by using roller drum cultures (1, 2, 24, 26).

In this study, the susceptibility of six different cell types (2 primary cell cultures and $\frac{3}{4}$ continuous cell lines) to bovine rotavirus isolates and the ability of trypsin to enhance

their infectivity in cell culture were investigated.

MATERIAL AND METHODS

Cells and Cell Culture

Two primary cell cultures, embryonic bovine (EBK) and ovine (EOK) kidney cells were prepared as described by PAUL (21) and used in this investigation. In addition, 4 continuous cell lines comprising of Vero (African green monkey kidney cells), LLC-MK_2 (Adult rhesus monkey kidney cells), MDBK (Adult bovine steer kidney cells) and MA104 (Embryonic rhesus monkey kidney cells) were also used.

Cells were grown and maintained either in medium 199 or E'59 (MA104 cells), containing 10 p. 100 foetal bovine serum, 2 p. 100 sodium carbonate, 1 p. 100 lactalbumin hydrolysate, 0.025 M HEPES, 1 p. 100 yeast extract, 100 IU/ml penicillin, 200 µg/ml streptomycin, 1 µg/ml fungizone and 5-10 µg/ml trypsin (Sigma Chemicals, U.K.). Cells were seeded at a concentration of 3×10^5/ml (primary cell cultures) and 2×10^5/ml (continuous cell lines), dispensed in 1.0 ml amount in test tubes containing flying cover slips and allowed to monolayer over night at 37 °C.

VIRUSES

Preparation of faecal samples

Sixteen calf faecal samples, shown to contain rotavirus by electron microscopy EM (22) and ELISA (8) were used. All were from outbreaks of diarrhoea in calves aged between 8 days and 4 weeks. In addition, the well characterised tissue culture strain of bovine rotavirus, the U.K. (Compton) strain (4) was used as standard reference.

Faecal samples were homogenised to form 20 p. 100 suspension in serum-free maintenance medium, centrifuged at 2,100 x g for 30 min and the aequous phase collected and treated with 10 µg/ml tyrpsin (Sigma Chemicals, U.K.) at 37 °C (water bath) for 1 h.

Isolation procedure

Two hundred µl of the trypsin-treated supernatant was used to infect monolayers of the cells (3 tubes per isolate). Virus was allowed to adsorb for 90 min at 37 °C, after which the inoculum was washed. The culture was fed with serum-free maintenance medium, containing 1.0 µg/ml trypsin and then incubated in roller drums at 37 °C. Monolayers were observed daily for cytopathic effects (cpe) and viruses were subcultured blindly every three days from pooled materials after being subjected to 3 cycles of freezing and thawing. Cover slips were examined by immunofluorescence (IF) for evidence of virus replication at each passage level, with antiserum to the U.K. (Compton) strain of bovine rotavirus prepared in rabbits known to be free of rotavirus antibodies (20). Infected cultures were centrifuged at 154,400 x g for 1 h and the pellets examined under EM for rotavirus particles.

Studies with trypsin

Two representative isolates (639 and 678) were used at their 7th - 10th passage levels in MA104 cells. The effect of three concentrations of trypsin (10 µg/ml, 5 µg/ml and 0 µg/ml) on the infectivity of these isolates on MA104 cells at different passage levels were investigated.

Studies with erstwhile refractory cells

Based on the results obtained (see below), isolates 639 and 678 at the 12th and 13th passage levels respectively in MA104 cells were used to infect previously refractory cells : EBK and EOK (Primary cell cultures), MDBK and Vero (continuous cultures). Isolates 639 and 678 were later adapted and passaged 3 times in these various cells with the aid of trypsin and their infectivity in these cells compared with that in MA104 cells.

RESULTS

Infectivity in cell types

All 16 faecal samples containing rotavirus replicated in MA104 cells but not in any other cell type. On the other hand, the U.K. (Compton) strain replicated in all the cell types including MA104 cells, as judged by IF staining. Based in this results, two representative isolates 639 and 678 were further studied. By the 4th or 5th passages in MA104 cells cpe began to appear as an initial rounding of cells

with indistinct outline, followed by 'heaping' or aggregation of dead cells. Some of the cells assumed spindle shapes with one end attached to the glass surface while the other end floated in the medium. By the 6th or 7th passage levels, as the cpe progressed, the cell sheet became completely destroyed after 2 or 3 days incubation (Photos 1a, b, c).

Examination of pelleted virus from tissue culture by EM revealed typical rotavirus particles.

Effect of trypsin on 639 and 678

No appreciable change in the virus titre was observed when the concentration of trypsin was varied from 10 µg/ml to 5 µg/ml. On the contrary, when trypsin was omitted completely in the treatment of the virus inoculum, there was a considerable drop in the virus infectivity (Table I).

TABLE N°I-Effect of varying concentrations of trypsin on infectivity of 639 and 678

Virus	Passage level in MA104 cells	Concentrations of Trypsin in µg/ml (*)	Titre in MA104 cells $\log_{10}TCID_{50}$/ml
639	8	10	6.2
678	7	10	5.9
639	10	5	5.9
678	9	5	5.9
639	9	0	4.5
678	8	0	4.8

(*) Concentration of trypsin in maintenance medium remained at 1.0 µg/ml throughout the test while concentration of trypsin on the virus inoculum was varied as shown.

Infectivity of 639 and 678 on previously refractory cells

Both isolates 639 and 678 now infected and replicated in all cell types as judged by IF staining. However, infectivity in the various cell types was lower than that obtained in MA104 cells. Adaptation of the isolates by passaging them 3 times in the various cell types did not seem to enhance their infectivity for these cells as the titres before and after adaptation did not vary significantly. In some of the cell types e.g. EBK and EOK, the virus titre of 639 approached that of the faecal virus in MA104 cells. By contrast, the titres obtained with 678 in these various cells were considerably lower than that of the faecal virus in MA104 cells. However, best results were consistently obtained with MA104 cells adapted 639 and 678 in MA104 cell cultures (Table II).

TABLE N°II-Infectivity of previously insusceptible cells with tissue culture strains 639 and 678

Virus	Passage level in MA104 cells (Cell Type)	Cell Type	IF	Titre $\log_{10}TCID_{50}$/ml in MA104 cells (Cell type)
639	12	MA104	+	6.3
678	13	MA104	+	5.8
639	12 (3)a	EBK	+	4.9 (4.6)b
678	13 (3)a	EBK	+	3.8 (4.9)b
639	12 (3)a	EOK	+	4.8 (4.8)b
678	13 (3)a	EOK	+	3.8 (2.8)b
639	12 (3)a	MDBK	+	3.1 (3.8)b
678	13 (3)a	MDBK	+	3.8 (3.8)b
639	12 (3)a	Vero	+	3.8 (4.3)b
678	13 (3)a	Vero	+	2.8 (2.8)b
639(*)	0	MA104	+	4.9
678(*)	0	MA104	+	5.0

a :Passage level of virus in the cell type concerned after a previous 12 or 13 serial transfers in MA104 cells ;
b :Virus titre in the cells concerned after 3 passages for adaptation to cell type ;
+ :Presence of fluorescing cells - evidence of replication in cell type.
(*) : faecal filtrate virus.

Photo 1. — Propagation of isolates of rotavirus in MA104 cells.

1a. Uninfected monolayers of MA 104 cells. Control × 360.

1b. Early cpe, showing focus of cpe, 24 h p.i. Note : rounding of cells. × 360.

1c. Advanced cpe, showing destruction of more cells 48-72 h p.i.

Note : many cells have detached, while other cells are spindle shaped × 360.

DISCUSSION

This investigation demonstrates the isolation and propagation of 16 strains of bovine rotavirus from diarhoeic calves in cell culture. However for primary isolation, only MA104 cells, an embryonic rhesus monkey kidney cell line was susceptible to the isolates. The earlier reports of isolation of rotavirus on primary calf kidney cells (4, 15, 18), and the fact that rotaviruses from different species have been used to infect LLC-MK_2 cells (5, 25) were shown not to be sufficient basis for attempting to isolate bovine rotavirus on primary bovine (EBK) and ovine (EOK) as well as continuous cell lines of Vero, LLC-MK_2 and MDBK as these cell types did not support the growth of field isolates of bovine rotavirus. Other workers have also found that EBK, BHK-21 and Vero cells were resistant to infection with calf, lamb and pig rotaviruses (6, 16).

On the contrary, the already established tissue culture U.K. (Compton) strain of bovine rotavirus readily replicated in all the cell types. As a result, isolates 639 and 678 which have been adapted and propagated in MA104 cells were used to infect cell cultures that were previously insusceptible to infection with freshly isolated strains of bovine rotaviruses. Interestingly, it was found that all the culture systems were now susceptible to both strains (Table II). Although, the reasons for this are not clearly established, it would appear that tissue culture adapted viruses (both of which had undergone at least 12 serial passages in MA104 cells and a further 3 serial transfers in the different cell systems under study (Table II), have acquired a much wider range of susceptible host cells. It was also possible that during the process of these serial transfers, a population of virus specially adapted to *in vitro* cultivation have been selected.

The beneficial effects of trypsin for the isolation and propagation of rotaviruses have been exploited by many workers (2, 16, 26), all of whom employed trypsin routinely for the isolation and propagation of different strains of rotaviruses from calves, chicken and humans. Nevertheless, there are a few reports of the successful isolation and growth of rotaviruses from calves, dogs, chicken and infants without the use of trypsin (3, 12, 13, 15, 17). There are also reports that some strains of rotavirus are less dependent on trypsin for subsequent passages, following the initial trypsin-associated isolation (12, 13). The reason for this would appear to be that the need for trypsin as an aid in the isolation and replication of rotavirus from field specimen depend to some extent on the strain of rotavirus. In this present investigation, the results indicated that pre-treatment of virus inocula with trypsin, incubation of culture in roller drum and the use of MA104 cells were essential for the continuous isolation, propagation and high titre yields of field isolates of bovine rotavirus.

The mechanism of the action of trypsin (7, 11) on the infectivity of rotavirus, whereby non-infectious virus was converted to infectious forms, might explain why there was over a 10-fold-drop in virus infectivity when trypsin was withdrawn (Table 1). GRAHAM and ESTES (11) noted that when trypsin was present in the culture medium during multiple cycles of virus replication, the infectivity of rotavirus was enhanced between 10- and 1 000-fold.

RESUMEN

OJEH (C. K.). — Aislamiento y propagación de los rotavirus bovinos en cultivos celulares. *Rev. Elev. Méd. vét. Pays trop.*, 1984, **37** (4) : 400-405.

Se realizaron 16 aislamientos de rotavirus bovinos a partir de muestras diarreicas de terneros sobre células MA 104, pero no en cultivos primarios de células bovinas (EBK), ovinas (EOK) o en cultivos de líneas continuas LLC-MK_2, Vero y MDBK.

El pre-tratamiento del inoculo del virus fecal por la tripsina (5 a 10 µg/ml), completado por 1,0 µg/ml de tripsina en el medio nutritivo y la incubación de células MA 104 sobre frascos roleando son factores esenciales para el desarrollo continuo de los rotavirus bovinos aislados a partir de muestras efectuadas en el campo. Cepas de rotavirus bovinos adaptados al cultivo celular tuvieron la propiedad de reinfectar células hasta ahora no permisivas.

Palabras claves : Rotavirus bovinos - Aislamiento - Cultivos celulares.

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