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PLAQUE ASSAY OF BOVINE ROTAVIRUS

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BUTCHAIAH, G. and E. LUND: *Plaque assay of bovine rotavirus.* Acta vet. scand. 1983, *24*, 362—373. — Incorporation of trypsin and diethylaminoethyl-dextran in the overlay was found to be necessary for infectivity assay of the UK strain of bovine rotavirus by plaque assays. Small plaques of about 1 mm in radius were formed in BGM cells. Large plaques of about 3—4 mm in radius were consistently produced in monolayers of secondary calf kidney cultures.

bovine rotavirus; plaque assay; trypsin; diethylaminoethyl-dextran.

Rotaviruses are now considered to be a major cause of diarrhoea in animals and humans (*Flewett & Woode* 1978, *McNulty* 1978, *Wyatt et al.* 1978 b). They are excreted in very large numbers in the faeces of infected individuals, but most rotaviruses cannot yet be readily propagated in vitro. However, success in the vitro cultivation of bovine (*Mebus et al.* 1971, *McNulty* 1978) and simian (*Malherbe & Strickland-Cholmey* 1967) rotaviruses has been achieved. It has been reported that trypsin facilitates the cultivation of bovine, ovine and porcine rotaviruses (*McNulty et al.* 1976, *Babiuk et al.* 1977, *Theil et al.* 1977, *Almeida et al.* 1978, *Clark et al.* 1979). *Matsuno et al.* (1977) reported that trypsin was required also for plaque formation by the Lincoln strain of neonatal calf diarrhoea virus (NCDV). On the other hand, *Wyatt et al.* (978) found that neither the UK strain of bovine rotavirus nor the simian rotavirus SA 11 required the addition of proteases or other enzymes for plaque production.

A reliable quantitative plaque assay procedure has not yet been described for rotaviruses. Therefore, immunofluorescent cell counting procedure is adapted currently for infectivity assay of

rotaviruses. The investigation described below was aimed mainly at developing a plaque method to assay the infectivity of bovine rotavirus.

MATERIALS AND METHODS

Cells

BGM cells, a continuous cell line derived from primary African green monkey kidney cells were obtained from the State Serum Institute, Copenhagen. Stock cultures were maintained in prescription bottles by weekly passage of cells using a split ratio of 1:15. Cultures for production of stock virus and plaque assays were prepared in 25 cm^2 plastic tissue culture flasks (Nunc, Denmark) using initial concentrations of $2—4 \times 10^6$ cells.

Primary cells of calf kidney (CK) were received weekly from State Veterinary Institute for Virus Research, Lindholm, Denmark. The cells were initially cultivated in prescription bottles. Secondary cultures for plaque assays were prepared in 25 cm^2 plastic flasks using a split ratio of 1:3. The cells were screened for bovine virus diarrhoea virus infection before use.

The growth medium used for cultivation of cells was Eagle's minimum essential medium (MEM) in Eagle's base (Autopow; Flow Laboratories) supplemented with 2 mM L-glutamine (Wellcome), 20 mM sodium bicarbonate and 10 % virus and mycoplasma tested new-born calf serum (Gibco Biocult) besides 100 U of penicillin and 100 µg of streptomycin per ml. Monolayer cultures were trypsinized by treatment with a solution of trypsin (0.25 %) and versene (0.5 %) in cation-free phosphate buffered saline.

Virus

The virus pool used in this study was prepared in BGM cells*. After the growth medium was removed, the cell monolayers were washed at least thrice with MEM. Washed monolayers were then infected with virus that had been treated with trypsin at 10 µg per ml. After virus absorption for 1 h at 37°C, MEM containing 0.5 % bovine serum albumin and 2µg trypsin per ml was added to the cultures and incubated at 37°C. When extensive cyto-

* Cell culture adapted UK strain of bovine rotavirus was kindly supplied by A. Meyling of State Veterinary Serum Laboratory, Copenhagen.

pathic effect was observed, the cells were frozen and thawed 3 times. After low speed centrifugation at 1000 g for 20 min the supernatant fluid was dispensed in 0.5 ml volumes and frozen at —80°C. Electron microscopic examination of the pool confirmed the presence of rotavirus particles.

Solidifying agents

Bacto agar (Difco), Noble agar (Difco), Purified agar (Oxoid), Purified agar (Behring werke AG, Germany), Indubiose (L'industrie Biologique Francaise, France) and Agarose type no HSIF (Litex, Denmark) were tested for their suitability as solidifying agents in the overlay medium. The appropriate amount of the solidifying agent was suspended in a measured volume of glass distilled water and sterilized by autoclaving.

Additives for overlay medium

Diethylaminoethyl (DEAE) -dextran (Pharmacia) was prepared as 1 % stock solution in glass distilled water. Bovine serum albumin fraction V (Sigma Chemical Co.) was prepared as 20 % stock solution in Earle's balanced salt solution (EBSS). Both solutions were sterilized by filtration before storage at 4°C. A 0.25 % stock solution of trypsin (1:250; Difco) prepared in EBSS was sterilized by filtration and frozen in 1 ml volumes at —20°C.

Plaque test procedure

After the growth medium was removed, cell monolayers were washed at least thrice with MEM. Serial 10-fold dilutions of virus prepared in MEM containing 0.5 % bovine serum albumin were inoculated in 0.5 ml amounts per each culture using 3 cultures per dilution. After 1 h of absorption at 37°C, excess inoculum was removed and 5 ml of the agar overlay medium under test was added to each flask. After the overlay medium solidified the flasks were incubated in an inverted opsition at 37°C for 5 days. The monolayers were then fixed for at least 1 h in a 10 % solution of formalin in normal saline. After the removal of the fixative and overlay, the cultures were washed in tap water and stained with crystal violet solution (*Preston & Morrel* 1962). After 5 min exposure to the stain, the cultures were washed finally in tap water and dried.

RESULTS

Plaque assay of bovine rotavirus in BGM cells

Our preliminary work on in vitro cultivation of UK strain of bovine rotavirus showed that BGM cells are highly sensitive. We therefore, initially attempted plaquing of the virus in BGM monolayers. We used agar overlay containing MEM with 2 μg DEAE-dextran per ml and 0.5 % bovine serum albumin. We also tested the suitability of different types of agar for plaque assay. As shown in Table 1, when an overlay consisting of 0.7 % Bacto agar (Difco) or Noble agar (Difco) was used, the virus failed to form detectable plaques after 5 days of incubation at 37°C. However, with overlay consisting of 0.7 % purified agar (Oxoid) or purified agar (Behring werke AG, Germany) or Indubiose (L'industrie Biologique Francaise, France) or 0.4 % Agarose type no HSIF (Litex, Denmark), small plaques of about < 1 mm radius could be seen after 5 days at 37°C (Fig. 1). Longer incubation or increase in the concentration of trypsin and DEAE-dextran resulted in cell degeneration.

Table 1. Effect of the type of agar on UK strain of bovine rotavirus plaque formation in BGM cells.

Type of agar[a]	PFU/ml[b]	Plaque size (radius in mm)
Bacto agar (Difco)	0	—
Noble agar (Difco)	0	—
Purified agar (Oxoid)	4.9×10^6	< 1
Purified agar (Behring werke AG, Germany)	4.7×10^6	< 1
Indubiose (L'industrie Biologique Francaise, France)	4.9×10^6	< 1
Agarose type $\neq\neq$ HSIF[c] (Litex, Denmark)	4.9×10^6	< 1

[a] Overlay consisted of Eagle's MEM, 2 μg trypsin and 100 μg DEAE-dextran per ml, 0.5 % bovine serum albumin and 0.7 % agar under test.

[b] Numbers represent the mean of plaque forming units obtained in separate experiments.

[c] Agarose type $\neq\neq$ HSIF (Litex, Denmark) is a highly purified preparation of agar and used as 0.4 % in the overlay.

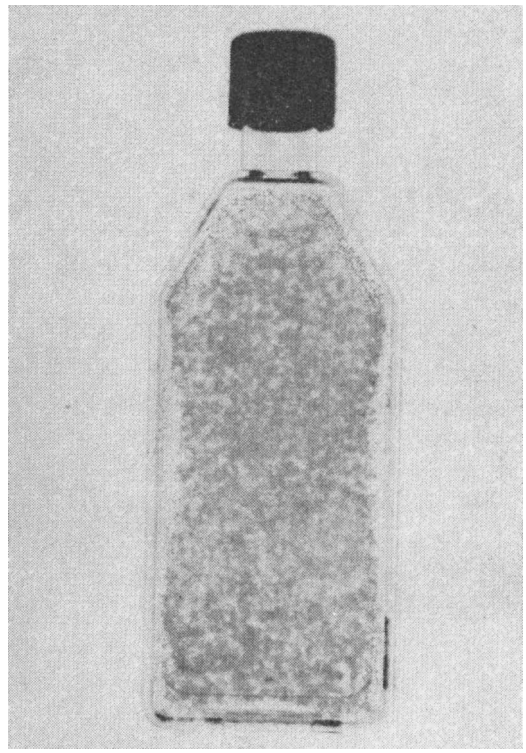

Figure 1. Bovine rotavirus plaques in monolayers of BGM cells.

Plaque assay of bovine rotavirus in calf kidney cells

When our attempts to increase the size of plaques in BGM monolayers failed, we initiated the work on the use of CK cells for plaque assay of bovine rotavirus. Since primary cultures often do not produce sufficiently uniform and satisfactory monolayers for virus plaquing, the CK cells were used as secondary cultures in all the experiments. For each set of experiment, cells of the same batch were used throughout.

As seen in Table 2, the overlay consisting of 0.7 % purified agar (Oxoid) gave large plaques (3 to 4 mm in radius). There was no further improvement with regard to either plaque size or plaque numbers when overlay consisting of Purified agar (Behring werke AG, Germany) or Indubiose (L'industrie Biologique Francaise, France) or Agarose type no. HSIF (Litex, Denmark) was used. The plaques were smaller under overlays containing Bacto agar (Difco) or Noble agar (Difco). Fig. 2 shows

Table 2. Effect of the type of agar on plaque formation by UK strain of bovine rotavirus in CK cells.

Type of agar[a]	PFU/ml[b]	Plaque size (radius in mm)
Bacto agar (Difco)	4.7×10^7	2
Noble agar (Difco)	4.9×10^7	2
Purified agar (Oxoid)	5.9×10^7	3—4
Purified agar (Behring werke AG, Germany)	5.9×10^7	3—4
Indubiose (L'industrie Biologique Francaise, France)	5.8×10^7	3—4
Agarose type $\neq\neq$ HSIF[c] (Litex, Denmark)	5.9×10^7	3—4

[a] Overlay consisted of Eagle's MEM, 2 μg trypsin and 100 μg DEAE-dextran per ml, 0.5 % bovine serum albumin and 0.7 % agar under test.

[b] Numbers represent the mean of plaque forming units obtained in separate experiments.

[c] Agarose type $\neq\neq$ HSIF (Litex, Denmark) is a highly purified preparation of agar and used as 0.4 % in the overlay.

the appearance of UK strain of bovine rotavirus plaques in monolayers of CK cells. Plaques were consistently formed in CK cells. On the basis of these observations, CK cells were chosen for plaque assay of UK strain of bovine rotavirus with purified agar (Oxoid) as the solidifying agent for the overlay in subsequent experiments.

Need for trypsin and DEAE-dextran in the overlay

Wyatt et al. (1978 a) reported that trypsin or other proteolytic enzymes were not needed for plaque formation by UK strain of bovine rotavirus. Based on this finding, we tested whether the incorporation of trypsin and DEAE-dextran in the overlay was necessary for plaque formation by UK strain of bovine rotavirus in CK cells. Without trypsin and DEAE-dextran although plaques formed, they were small in size, much less in numbers and indistinct (Table 3). With an overlay containing DEAE-dextran but not trypsin, there was no appreciable improvement. When an overlay consisting of trypsin but not DEAE-dextran was used, plaques were larger in size (2—3 mm in radius) and numerous.

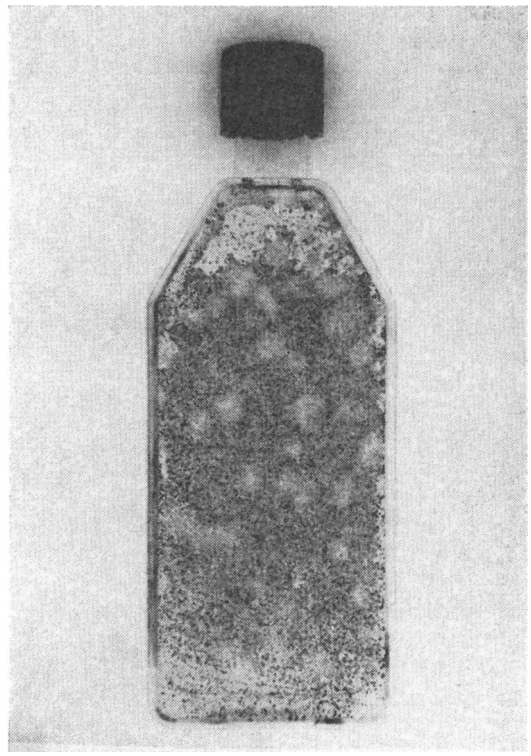

Figure 2. Bovine rotavirus plaques in monolayers of calf kidney cells.

Table 3. Effect of trypsin and DEAE-dextran on plaque formation by UK strain of bovine rotavirus in CK cells.

Additive in overlay[a]		PFU/ml[b]	Plaque size
Trypsin 2 μg/ml	DEAE-dextran 100 μg/ml		(radius in mm)
—	—	2.5×10^2	1
—	+	2.9×10^3	1
+	—	3.1×10^7	2—3
+	+	5.9×10^7	3—4

[a] Overlay consisted of Eagle's MEM, 0.7 % purified agar (Oxoid), 0.5 % bovine serum albumin and the indicated concentration of additive under test.

[b] Numbers represent the mean of plaque forming units obtained in 3 separate experiments.

+ = Presence of additive in overlay.
— = Absence of additive in overlay.

The addition of DEAE-dextran to the overlay containing trypsin increased the plaque numbers about two-fold with a slightly further increase in plaque size (3—4 mm in radius) beyond that achieved by trypsin alone (Table 3). Even after the treatment of virus with 10 μg trypsin per ml for 30 min prior to inoculation, there was no improvement in plaque formation in cultures which received overlay consisting of DEAE-dextran but not trypsin. It was thus evident that the incorporation of trypsin and DEAE-dextran was essential for infectivity assay of the UK strain of bovine rotavirus in CK cells by the plaque method.

Effect of overlay containing varying concentrations of trypsin and DEAE-dextran

Trypsin at a concentration of higher than 3 μg per ml of overlay impaired the integrity of the monolayer. The presence of DEAE-dextran in concentrations higher than 100 μg per ml of overlay did not contribute to further improvevment of plaque size or numbers. Addition of 200 μg or more of this cationic polymer made the overlay cytotoxic.

Effect of trypsin treatment of virus prior to inoculation in cell cultures

The plaque numbers obtained with virus that had been treated with 10 μg trypsin per ml for 30 min at 37°C prior to inoculation in monolayers of CK cells which later received overlay containing MEM, 2 μg trypsin and 100 μg DEAE-dextran per ml, 0.5 % bovine serum albumin and 0.7 % purified agar (Oxoid) were 5-fold higher than those obtained with virus not treated with trypsin prior to inoculation in cell cultures which later received the same overlay medium.

Plaque characteristics

The passage history of the UK strain of bovine rotavirus used in the present study was unknown, but it had probably undergone a large number of passages in CK cells before we received it. In our laboratory, the virus was passaged 10 times in CK cells and 8 times in BGM cells. When the stock virus grown in BGM cells was plaqued in monolayers of BGM cells, the plaques were smaller in size (< 1 mm in radius) with clear centres and sharp boundaries. In monolayers of CK cells the plaques formed by the

same stock virus were large in size (3—4 mm in radius) with sharp boundaries and opaque centres. Thus it was evident that the plaque characteristics of the virus were influenced by the type of cells used for plaquing. Further, the characteristics of the virus passaged eight times in BGM cells did not change when tested in CK cells.

DISCUSSION AND CONCLUSIONS

Trypsin has been shown to enhance the infectivity of reoviruses (*Spendlove et al.* 1970) and influenza viruses (*Klenk et al.* 1975, *Lazarowitz & Choppin* 1975) in vitro, to permit plaque formation by influenza viruses (*Appleyard & Maber* 1974, *Tobita & Kilbourne* 1974, *Huprikar & Robinowitz* 1980) and to enhance the size and number of vaccinia virus plaques (*Gifford & Klapper* 1967, *Valle* 1971). A similar potentiating effect of trypsin on rotavirus infectivity in cell cultures has been demonstrated (*Babiuk et al.* 1977, *Almeida et al.* 1978, *Barnett et al.* 1979, *Clark et al.* 1979). It has also been reported that trypsin was necessary for plaque formation by the Lincoln strain of NCDV (*Matsuno et al.* 1977) and simian rotavirus SA 11 (*Ramia & Sattar* 1979, *Smith et al.* 1979). But there have also been reports of the lack of enhancement of infectivity of NCDV and SA 11 in cell cultures in the presence of trypsin (*Schoub & Bertran* 1978). Only slight increase in infectivity titre of UK strain of bovine rotavirus in the presence of trypsin and α-chymotrypsin has been reported by *Wyatt et al.* 1978 a).

The present study showed that trypsin was necessary for efficient plaque formation by UK strain of bovine rotavirus in CK cells. This is in contrast to an earlier report by *Wyatt et al.* (1978 a) where trypsin or other proteolytic enzymes were found to be unnecessary for plaque formation by the UK strain of bovine ratavirus and simian rotavirus SA 11. Our findings are however, in agreement with the observations of *Matsuno et al.* (1977), who reported efficient plaque formation by Lincoln strain of NCDV in MA-104 cells only in the presence of trypsin. They also observed an additive effect of DEAE-dextran in increasing plaque numbers by about 6-fold beyond that achieved by trypsin alone. In our experiments with UK strain of bovine rotavirus and CK cells, the addition of DEAE-dextran to the overlay increased plaque numbers by about 2-fold with a slight in-

crease in plaque size beyond that achieved by trypsin alone. Further, the plaques were very distinct and easily countable when overlay consisting of both trypsin and DEAE-dextran was used. Thus it was evident that both trypsin and DEAE-dextran were necessary for infectivity assay of UK strain of bovine rotavirus by plaque method. A similar potentiating effect of both trypsin and DEAE-dextran on plaque formation by simian rotavirus SA 11 has been reported (*Smith et al.* 1979). Whereas, *Ramia & Sattar* (1979) reported that the need for trypsin but not for DEAE-dextran in the overlay was indicated for plaque formation by simian rotavirus SA 11 in MA-104 cells. They also found that DEAE-dextran in amounts greater than 100 µg ml of overlay resulted in a slight reduction in size and numbers of plaques. In the present study, we did not observe any adverse effect of DEAE-dextran in concentrations up to 200 µg per ml of overlay on plaque formation by UK strain of bovine rotavirus in CK cells. However, DEAE-dextran in concentrations above 200 µg per ml made the overlay cytotoxic.

It had been reported by *Huprikar & Robinowitz* (1980) that influenza virus absorbed to cell cultures in the presence of trypsin did not require trypsin in the overlay for plaque formation. We acted on this observation and tested in the present study whether the UK strain of bovine rotavirus absorbed to CK cells in the presence of trypsin or treated with trypsin prior to inoculation would need trypsin in the overlay for optimal plaqe formation. When the overlay did not contain trypsin we found the titres much less and plaques small in size, thus further confirming the need for continued presence of trypsin in the overlay for infectivity assay of bovine rotavirus. However, trypsin treatment of virus prior to inoculation in cell cultures has an additive effect in increasing plaque numbers by about 5-fold. A similar increase in plaque forming units of rotavirus treated with trypsin before inoculation in cell cultures has also been reported by *Graham & Estes* (1980).

Wyatt et al. 1978 a) while reporting that the UK strain of bovine rotavirus produced plaques in primary African green monkey kidney (AGMK) cells, primary calf kidney cells and 2 continuous cell lines CV 1 and MA-104, observed consistency in plaque production only in AGMK cells. In the present study, we have tested 25 batches of CK cells for their ability to support plaque formation by UK strain of bovine rotavirus. Plaques were

formed consistently in all batches of CK cells tested. As the CK cells are sensitive to bovine rotavirus and our finding that these cells permitted plaque formation consistently by UK strain of bovine rotavirus would help in using these cells for infectivity assay of bovine rotaviruses by plaque method with certain limitations concerning primary cells.

In conclusion, the plaque test for infectivity assay of bovine rotavirus should include the use of i) sensitive CK cells; ii) trypsin treatment of virus before inoculation; and iii) an overlay containing purified agar (0.7 %), MEM, trypsin (2 μg/ml), DEAE-dextran (100 μg/ml) and bovine serum albumin (0.5 %).

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SAMMENDRAG

Plaque titrering af bovin rotavirus

Ved at lade trypsin og diethylaminoethyl-dextran indgå i det faste substrat var det muligt at påvise bovin rotavirus (UK stammen) gennem plaque teknik. I kulturer af abeceller (BGM celler) blev der opnået 1 mm store plaques, mens der i sekundære kulturer af kalvenyreceller regelmæssigt sås plaque af 3—4 mm størrelse.

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