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IMPROVED ANIMAL ROTAVIRUS ISOLATION IN MA_{104} CELLS USING DIFFERENT TRYPSIN CONCENTRATIONS

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ABSTRACT

Rotavirus is one of the most important etiological agents in infant gastroenteritis and diarrhea in neonatal animals. The purpose of this study was the optimization of isolation of rotaviruses in MA_{104} (fetal kidney Rhesus monkey) cells by the comparison of culture, taking into account the number of samples, different treatments with trypsin, number of serial passages, and positivity by polyacrylamide gel electrophoresis (PAGE). Twenty diarrheic fecal samples of calves and piglets from São Paulo, State, Brazil, all positive for rotavirus in ELISA and PAGE, were submitted to four isolation protocols: in treatment 1, the inoculum was left on the monolayer and the maintenance medium was trypsin-free; treatment 2 was the same as treatment 1 except that the inoculum was discarded; in treatment 3 the inoculum was left on the monolayer and the maintenance medium had 5 µg/mL of trypsin; treatment 4 was the same as the monolayer treatment 3, except that the maintenance medium had 10 µg/mL of trypsin. Fifteen samples were successfully isolated, representing an efficacy of 75% (15/20). Treatment 3 was the most suitable because the lower number of serial passages and lower cytotoxity to the cells, with statistical significance ($p < 0.001$) using Freedman's and Wilcoxon's tests.

KEY WORDS: Rotavirus, MA_{104} cells, diarrhea, isolation, trypsin.

RESUMO

OTIMIZAÇÃO DO ISOLAMENTO DE ROTAVÍRUS ANIMAIS EM CULTURA DE CÉLULAS DA LINHAGEM MA_{104} USANDO DIFERENTES CONCENTRAÇÕES DE TRIPSINA. Os rotavírus são importantes agentes virais envolvidos nas gastroenterite infantil e da diarréia dos animais neonatos. O objetivo deste trabalho foi otimizar o isolamento de rotavírus levando-se em conta o número de amostras, diferentes tratamentos com tripsina, número de passagens seriadas e monitoramento das mesmas através de eletroforese em gel de poliacrilamida (PAGE). Foram utilizadas 20 amostras fecais diarréicas, positivas para rotavírus por meio de ELISA e PAGE, oriundas de bezerros e leitões, as quais foram submetidas a quatro protocolos usando tripsina. No tratamento 1 o inóculo foi mantido e o meio de manutenção não continha tripsina; no tratamento 2 o inóculo foi dispensado e o meio de manutenção sem tripsina; no tratamento 3 o inóculo foi mantido e o meio de manutenção continha tripsina na concentração de 5µg/mL; no tratamento 4 o inóculo foi mantido e o meio de manutenção continha tripsina na concentração de 10 µg/mL. Todos os inóculos continham tripsina na concentração de 5 mg/mL. Dentre as amostras estudadas, 15 foram isoladas com sucesso, o que representa uma eficiência de 75% (15/20). Dentre os protocolos, o tratamento 3 foi o que apresentou melhor desempenho, considerando-se o número de passagens seriadas, a ausência de citotoxidade e significância estatística ($p < 0,001$), quando se aplicou o teste de Freedman e o teste de Wilcoxon.

PALAVRAS-CHAVE: Rotavírus, células MA_{104}, diarréia, isolamento, tripsina.

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INTRODUCTION

Rotaviruses are the major cause of infant gastroenteritis and diarrhea in neonatal animals of several species, mainly bovine and porcine, with economic losses (Reinhardt et al., 1986; Lucchelli et al., 1992; Liprandi et al., 1987; Snodgrass et al., 1986; Johnson et al., 1992).

The first report of rotavirus isolation in cell culture was made by Mebus et al. (1972), who was also the first to describe the virus (Mebus et al., 1969). From then on, several other attempts to isolate the virus in cell culture have been reported, however delays in the adaptation period and loss of infectivity after serial passages were observed (Wyatt et al., 1974; Banatvala et al., 1975; Albrey & Murphy, 1976; Bryden et al., 1977).

The use of MA_{104} cells, associated to trypsin in the pre-treatment of the inoculums and maintenance medium by Sato et al. (1981), increased rotaviruses isolation from several animal species. After that, different concentrations of tripsin and treatments were described Bachmann et al. (1981), Hoshino et al. (1981), Kutsizawa et al. (1982), Imagawa et al. (1984), Castrucci et al. (1985), Makabe et al. (1985) and Ferrari et al. (1986). The limitation in the understanding of the mechanisms by what means trypsin improved the rotavirus infectivity lasted until Arias et al (1998) demonstrated the specifically cleavage in VP_4 in VP_5 and VP_8 in rotavirus strain SA_{11}.

The present article the reports evaluation of four different approaches to optimize animal rotavirus isolation in MA_{104} cells, taking into to account the number of samples, serial passages and positivity by PAGE.

MATERIALS AND METHODS

Fecal samples

From August 1999 to March 2000, 20 fecal samples, all positive to rotavirus in ELISA (Gregori et al., 2000) and PAGE (Herring et al., 1982) were collected from calves and piglets from several outbreaks of diarrhea in São Paulo State, Brazil.

Rotavirus reference strain

The bovine rotavirus strain NCDV (Nebraska Calf Diarrhea Virus) G[6] P[1] was used as a positive control.

Cell culture

Monolayer cell culture MA_{104} (fetal kidney Rhesus monkey) were used with 48h of growth.

Fecal sample preparation

A 20% fecal suspension in TRIS 0.1 M pH 7.3 buffer was initially prepared for each sample. After vortex homogenization and centrifugation at 10,000 g for 30min, the supernatant was recovered and filtered in Millex-Millipore® units with 0.22 μm pores.

Inoculums

The filtered fecal suspensions were mixed 3:1 (v/v) with V.A.S. (Virus Activation Solution = 5 mg/mL of crystalline trypsin Sigma® in MEM-Eagle Cultilab® for cleavage of VP_4 in VP_5 and VP_8 (Arias et al. 1998) and incubated at 37° C for 30min. Next, the growth medium of the cells was discarded and the monolayers ware washed with PBS 0.1 M pH 7.2. The inoculum was then transferred to the monolayers and virus adsorption was performed at 37° C for 60min.

Treatments

After virus adsorption, the cell monolayers were submitted to four different protocols, in order to attempt rotavirus isolation.

Treatment 1

Addition of the maintenance medium (MEM-Eagle Cultilab®) without discarding the inoculum.

Treatment 2

The inoculum was discarded before the addition of the maintenance medium (MEM-Eagle Cultilab®).

Treatment 3

Addition of maintenance medium (MEM-Eagle Cultilab®) with crystalline tripsin Sigma® at 5μg/mL without discarding the inoculum.

Treatment 4

The same procedure previously described for treatment 3 maintenance medium (MEM-Eagle Cultilab®) but with crystalline trypsin Sigma® at 10 μg/mL.

The cell flasks were incubated at 37° C and the monolayers were observed until the occurrence of the characteristic cytopathic effect. Those that presented the effect up to 96h after inoculation were frozen to be submitted afterwards to at least five serial passages.

Negative control

As negative control, all treatments were repeated, using PBS 0.1 M pH 7.2 instead of the fecal suspension or serial passage inoculum.

Checking of the inoculated cells

Monolayers that presented rotavirus characteristic of cytopathic effect were frozen and checked by PAGE (polyacrylamide gel electrophoresis) according to HERRING et al (1982), to assure that the effect observed was rotavirus-specific instead of a trypsin effect present in V.A.S. or in the maintenance medium. The NCDV strain was used as a standard.

Analysis

Results were compared using Freedman's test for the four treatments and Wilcoxon's test for serial passages in treatment, in groups of two using $p < 0.05$ as the critical value, using SPSS 9.0 software.

RESULTS

The cytopathic effect observed was similar for the majority of the samples isolated in all treatments, with cells becoming round and stretched out (similar to "candle flames") with gradual loosening of the cells. Monolayers were then similar to a "string of pearls" and were totally destroyed in a maximum of 96h.

From the 20 samples, 15 were positive in the isolation, presenting characteristic cytopathic effect, and electrophoretic migration pattern of rotavirus serogroup A in PAGE, when compared to the referent strain NCDV, five from piglets and 10 from calves.

Negative samples were those that did not present characteristic cytopathic effect, those negative by PAGE after the serial passages.

The 15 positive samples isolated showed different pattern results according to each of the four treatments and serial passages (Table 1).

When the treatments were compared by Freedman's test, significative differences ($p < 0.0001$) were observed (Table 2). The number of serial passages in the treatment was then analyzed in groups of two by Wilcoxon's test. They were different from the final result, but treatments 3 and 4 did not present statistical significant differences (Fig. 1).

Table 1 - Number of the serial passage in MA-104 culture for isolation of rotavirus from fecal material of calves and piglets, according the different trypsin treatments.

Sample	Treatment 1	Treatment 2	Treatment 3	Treatment 4
18/00 c	3	4	3	3
01/00 c	5	5	3	3
11/99 c	4	5	3	3
16/99 c	-	-	3	3
09/00 c	4	4	4	3
8189 c	3	4	2	2
8204 c	2	5	2	2
8209 c	2	4	2	2
PB 58 c	1	3	1	1
61/00 c	3	5	2	2
24/00 p	2	2	1	1
29/00 p	3	3	4	4
31/00 p	2	2	2	2
32/00 p	2	3	2	2
FG 72Z p	2	5	2	2
12/99 c	-	-	-	-
09/99 c	-	-	-	-
AB 00M c	-	-	-	-
JJ 114Z p	-	-	-	-
68Z p	-	-	-	-

1- First passage, 2- Second passage, 3- Third passage, 4- Fourth passage, 5- Fifth passage.
c (Calf), p (piglet)
(-) negative until filth passage

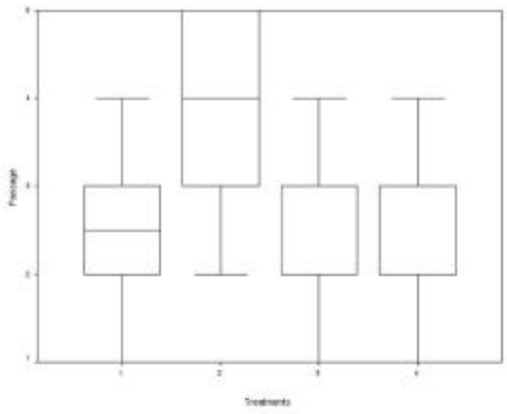

Fig. 1 - Positive samples in isolation according to the treatment with trypsin and number of serial passage in MA_{104} cell culture – São Paulo, 2001. (Boxplot of Wilcoxon's test to the number of serial passages in groups of 2 treatments for isolation in MA_{104} cell)

Table 2 - Freedman's test for comparison among the number of serial passages for the different treatments for rotavirus isolation.

Treatments	1	2	3	4
1	-	-	-	-
2	0.012	-	-	-
3	0.028	0.003	-	-
4	0.018	0.002	0.317	-

Critical value $p < 0.05$ = significant.

DISCUSSION

Rotaviruses have been studied widely due to economic less and serious sanitary problem such as interspecies transmission and zoonotic potential (Lucchelli et al., 1992; Wyatt et al., 1974, Snodgrass et al., 1986).

In this study, 20 samples of rotaviruses were submitted to inoculation in MA_{104} cells monolayers and 15 (15 / 20 = 75%) of them were successfully isolated. In the case of the five samples for which confirmation by PAGE was negative due to the absence of the characteristic eletrophoretic migration pattern of viral RNA – as observed in the NCDV strain – it is possible that changes in the monolayers cells were suggestive of the cytopathic effect caused by rotavirus. In spite of the greater detection threshold PAGE was used in the checking in order to assure that the genetic material would remain intact all over the passages, which contributed to a greater positively throughout the five serial passages.

It was possible to observe differences between treatments and the number of serial passages in MA_{104} cells monolayers, due to the use of trypsin in the inoculum (5 mg / mL) and in the maintenance medium (5 or 10 µg / mL), the number of passages, and the isolation period (Bachmann et al., 1981; Hoshino et al., 1981; Kutsizawa et al., 1982; Imagawa et al., 1984; Castrucci et al., 1985; Makabe et al., 1985; Ferrari et al., 1986). Hoshino et al. (1981) isolated a canine sample of rotavirus using 2.5 µg / mL of trypsin in the inoculum. Kutsizawa et al. 1982 isolated human samples from subgroup I and II using 10 µg / mL and 0.5 µg / mL of trypsin in the inoculum and maintenance medium, respectively, with a longer interval between the first and second serial passage, until cytopathic effect occurred. Imagawa et al. (1982) isolated equine samples of rotavirus using trypsin at 1 µg / mL in the inoculum and 20 µg / mL in the maintenance medium. Castrucci et al. (1985) isolated rabbit samples of rotavirus using 1,000 µg / mL in the inoculum and 5 µg / mL in the maintenance medium. Makabe et al. (1985) isolated ovine samples using 25 µg / mL of pancreatine, both in the inoculum and maintenance medium. Ferrari et al. (1986) isolated swine samples using 10 µg / mL of trypsin in the inoculum, but no trypsin in the maintenance medium, with 7 days between the passages; the isolation was successful in the 7th passage (after 49 days). Ramos et al. (1998) isolated five rotavirus from swines using an average of 3 serial passages and 30 µg / mL in the inoculum and 10 µg / mL in the maintenance medium. Therefore, trypsin concentration in the inoculum ranges from 1 µg / mL to 1,000 mg / mL , and in the maintenance medium from zero up to 25 µg / mL, which is in agreement with the results found in the present study.

Besides the differences among samples, it was also possible to observe differences among treatments and number of serial passages. Freedman's test demonstrated significant differences ($p < 0.0001$) among the four treatments and Wilcoxon's test analysis of serial passages in groups of 2 treatments demonstrated that treatment 3 and treatment 4 were the best ones, when compared to treatment 1 and treatment 2. However, the treatment 4 used a greater trypsin concentration (10 µg / mL in the maintenance medium), which may potentially mix cytopathic effect and toxicity caused by trypsin.

Treatment 1 does not require trypsin in the maintenance medium; this procedure enables a better visualization of the cytopathic effect, without any toxicity interference. However, the disadvantage in this treatment was only efficient in later passages, different from what was observed in treatments 3 and 4.

Treatment 2 was the least efficient. The results led to the hypothesis that residual trypsin in the inoculum would have two advantages. First, it would increase the possibility of VP_4 cleavage of viral particles that had not been cleaved during the incubation with V.A.S.; after that, as it happens with trypsin when it is present in the maintenance medium, it would enable new particles from infected cells to also have their VP_4 cleaved, which would lead to a larger number of infected cells in the monolayer.

Treatment 3 associates the advantages of an earlier isolation with less trypsin (5 µg / mL) preventing toxicity and requiring less checking when a larger number of samples is analyzed, which makes this the most suitable protocol for rotavirus isolation.

Other factors related to the initial viral concentration may have influenced the efficiency of rotavirus isolation: the onset of symptoms of diarrhea and harvest time of the fecal material, the conditions of the harvest and transport to the laboratory, and even the association with other agents.

The protocols here proposed for rotavirus isolation may contribute studies in antigenic vaccinology in regard to the genetic diversity and epidemiology of rotaviruses.

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