Braz. J. vet. Res. anim. Sci.,
São Paulo, v. 35, n. 2, p. 84-87, 1998.

The infectivity of pig rotavirus in stools

Infecciosidade de rotavírus suíno em fezes

CORRESPONDENCE TO:
Carlos Mitihiko Nozawa
Departamento de Patologia Geral – CCB
Universidade Estadual de Londrina
Caixa Postal 6001
86057-970 – Londrina – PR
e-mail: nozawa@sercomtel.com.br

1- Departamento de Patologia Geral do CCB da UEL – PR

Ana Paula Dores RAMOS[1]; Carla Cristina STEFANELLI[1]; Rosa Elisa Carvalho LINHARES[1]; Benito Guimarães de BRITO[1]; Carlos Mitihiko NOZAWA[1]

SUMMARY

Rotaviruses are the major pathogen for both human and animal. They affect young animals in intensive rearing and cause great economic losses. This study intended to evaluate the infectivity of porcine rotavirus maintained for 32 months at approximately 10ºC. in the original stools specimens. Thirty stools specimens of 1-4-week-old piglets originating from breeding farms located in the southwest region of the Paraná State were selected for this study. They were randomly chosen from stools samples positive for rotavirus by polyacrylamide gel electrophoresis (PAGE) at the time of collection. The thirty stools samples maintained for 32 months at approximately 10ºC were re-tested by PAGE and 11 out of 30 were still positive showing the integrity of the eleven bands of viral RNA. To demonstrate the maintenance of viral infectivity, clarified and trypsin-treated stools specimens homogenates were inoculated in MA-104 cell cultures. After an average of 3 blind passages 5 out of 11 samples demonstrated cytophathic effect similar to that of standard simian rotavirus (SA-11). To confirm these findings, a immunofluorescent test was used and demonstrated typical cytoplasmic granular fluorescence. Electron microscopy of stools samples showed that most of the virus particles were single- shelled and some were found to be in an advanced state of degradation. Therefore the conclusion was that porcine rotavirus infectivity is maintained for a long period of time in stool specimens at low temperature. This certainly is an important aspect for the maintenance of viable virus in natural condition as well as for the transmission of the disease.

UNITERMS: Rotavirus; Rotavirus Infection; Diarrhea; Swine.

INTRODUCTION

Rotaviruses are major human and animal pathogens[5,8,14]. Neonatal diarrhea of pigs is known as a disease of significative economic impact in intensive pig rearing due to animal death, growth delay and involved costs.

Outbreaks of rotavirus infection in pigs are observed in weaning piglets with 2-4 weeks of age[9].

The study of simian rotavirus (SA-11) in cell culture has demonstrated that its infectivity is relatively stable upon treatment with organic solvents (ether, chloroform), repeated cycles of freezing and thawing and sonication. It resists for 1 hour at 37ºC, 24-48 hours at 25ºC or 5 min. at 50ºC[7]. It was also demonstrated that human rotavirus is stable even after milk pasteurization (15 sec. at 80ºC)[4]. Treatment with proteolytic enzymes such as trypsin, pancreatin or elastin increases its infectivity in cell culture. At pH 3.5-10.0 it maintains its infectivity[14]. Chelant agents such as EDTA turn complete particles into incomplete ones devoided of infectious capacity[6]. Nonionic disinfectant, sodium hypochloride and formaldehyde act with low efficiency on rotavirus present in faeces, being ethanol at 95% the most effective[14].

The main route of virus transmission is fecal-oral and virus excretion in faeces can happen over a long period of time[1].

Due to the via of virus transmission, virus excretion by pregnant animals[20], lack of an efficient vaccine and the resistance of the virus, as well as the control of the infection becomes a difficult task.

In the present work we demonstrated the stability of porcine rotavirus nucleic acid in faeces and the infectivity of the virus maintained for 32 months at a temperature of approximately 10ºC.

MATERIAL AND METHOD

Cell culture

MA-104 cell cultures were prepared in medium 199 (Sigma Chem. Co., USA), supplemented with 10% foetal calf serum (FCS), antibiotics and fungizon.

Virus

Simian rotavirus (SA-11) was grown in MA-104 cell culture in FCS free medium added of 10 µg/ml of crystalline trypsin (Merck, Germany). Virus inocula were also treated with crystalline trypsin at a final concentration of 30 µg/ml during 50 min. at 37ºC before inoculation.

Stool samples

Stool samples of 1-4-week-old piglets with acute diarrhea originated from farms located at the southwest region of the Parana State were collected from March to October 1991. The samples were

submitted to polyacrylamide gel electrophoresis (PAGE) for viral RNA soon after the collection. Thirty PAGE-positive stool samples randomly chosen were maintained at approximately 10ºC for 32 months. Before any procedure, stool samples were homogenized at 10-20% (v/v) in phosphate-buffered saline pH 7.3 with the aid of glass beads and clarified at 2,000 x g for 20 min. at 4ºC. For cell culture inoculation clarified homogenates were treated with antibiotics and fungizon followed by treatment with 30 µg/ml of crystalline trypsin for 50 min. at 37ºC.

Polyacrylamide gel electrophoresis

Two volumes of the clarified stool homogenates without any RNA extraction were treated with one volume of dissociating buffer (0.0625 M Tris/HCl pH 6.8; 5 M urea; 5% 2-mercaptoethanol; 3% SDS; 0.01% bromophenol blue and 10% glycerin) and incubated in a water bath at 60ºC for 50 min. The samples were submitted to slab gel PAGE (7%) at 25 mA for 2 hours[12]. After PAGE viral RNA bands were observed with the use of silver staining. Simian rotavirus (SA-11) submitted to the same treatment was used as control.

Cell culture infection

Seventy percent confluent MA-104 cell cultures grown in 13 x 100 mm glass tubes were used for virus inoculation. One tenth of ml of the faecal suspension previously treated was inoculated in quadruplicate. After 1-hour-adsorption cultures were refed with 0.9 ml of fresh medium free of FCS and added of 10 µg/ml of crystalline trypsin[10]. Inoculated cultures and controls were kept at 37ºC and daily observed for cythopathic effect (CPE) for at least 7 days. Simian rotavirus was used as positive control.

Indirect immunofluorescence

Forthy-eight-hour MA-104 cell cultures grown in Leighton tubes were inoculated with 0.1 ml of virus suspension adaptated in cell culture and previously treated with 10 µg/ml crystalline trypsin and refed with fresh medium free of FCS. Seventy-two hours after inoculation the coverslips were removed and washed with PBS pH 7.3. Cells were fixed with cold acetone (-20ºC) for 20 min. and incubated with 1:20 dilution of guinea pig anti-simian rotavirus serum for 30 min. at 37ºC. After three washes with fresh PBS the cultures were incubated with 1:20 dilution of FITC conjugate goat anti-guinea pig IgG (Sigma Chem. Co., USA) for 30 min. at 37ºC. After three washes with PBS, coverslips were mounted in slides with 50% glycerin-PBS and observed in an UV microscope[16].

Electron microscopy

A drop of clarified faecal homogenates was placed on a 300 mesh copper grid previously covered with formvar-carbon. The excess of the material was removed, the grid was allowed to dry and negatively stained with 2% sodium phosphotugstate pH 6.3[18]. The observation was carried out in a transmission electron microscope.

RESULTS

From the samples of the collected faeces, 30 of them positive by PAGE at the time of collection were randomly chosen for the study. The stools samples were kept at approximately 10ºC for 32 months without any additive. After this period of time, faeces samples were re-tested for PAGE and it was demonstrated that 36% (11/30) were still positive showing the integrity of the 11 bands of RNA (Fig. 1) with profile of group A as they were when firstly tested. When these samples were submitted to cell culture inoculation it was shown that 45% (5/11) of them developed CPE, some of them after two or three blind passages. In general CPE was observed 48-72 hours after infection. The CPE was characterized by foci of round and refringent cells in the monolayer. Foci of cells presenting spindle-like cytoplasm were also observed as well as cells detachment from the monolayer (Fig. 2). The CPE was similar to that of the simian rotavirus used as positive control.

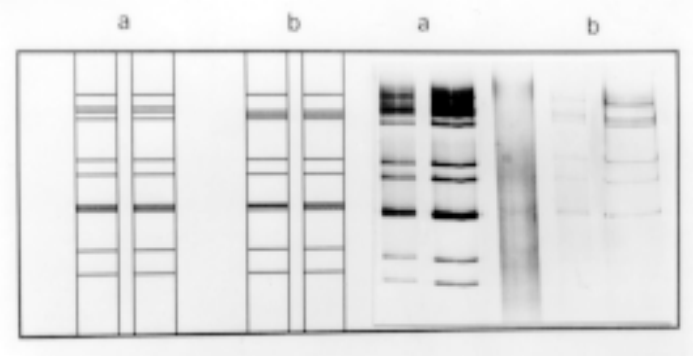

Figure 1
Polyacrylamide gel electrophoresis (PAGE) of porcine rotavirus RNA (sample 451) maintained for 32 months at approximately 10ºC (column A, duplicate) and simian rotavirus (column B, duplicate), also presented as drawing. PAGE was carried out with a current of 25 mA over 2 hours, and RNA bands were revealed with silver staining[12].

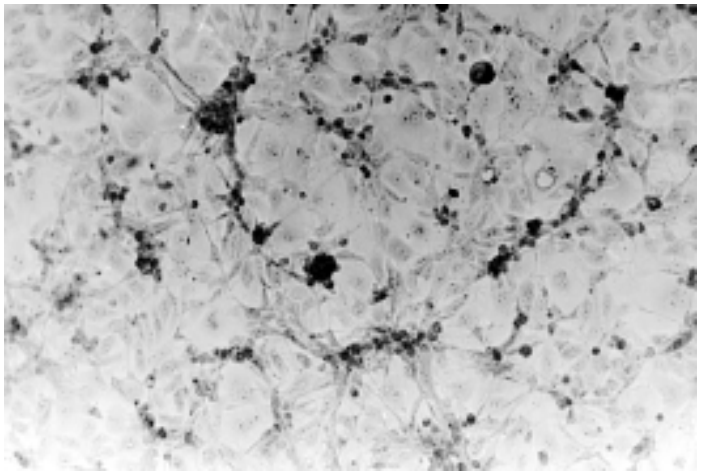

Figure 2
Giemsa stained MA-104 cell culture infected with porcine rotavirus (sample 1154), 48-72 hours post infection. (100X).

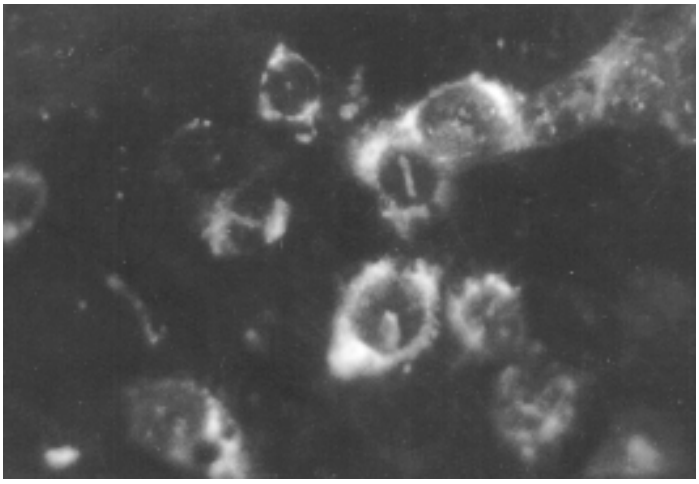

Figure 3
Indirect immunofluorescence of MA-104 cell culture infected with porcine rotavirus (sample 1154), 48 hours post infection. (400X).

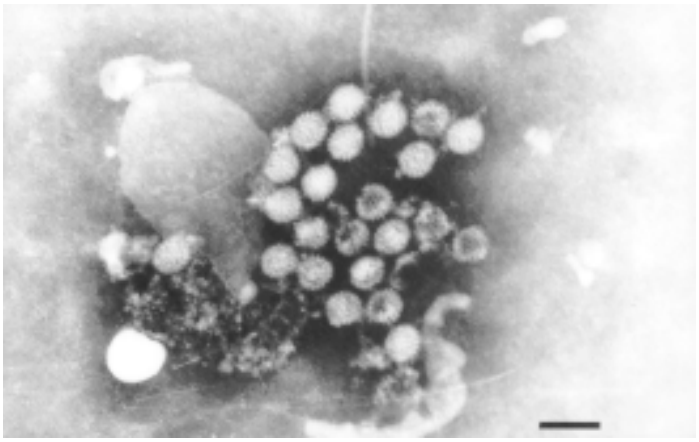

Figure 4
Negative staining electron micrography of porcine rotavirus (sample 962) maintained for 32 months at approximately 10ºC. Bar: 100nm.

Porcine rotavirus CPE was confirmed by indirect immunofluorescence. It was shown that within 48-72 hours post infection, foci of cells presenting specific granular cytoplasmatic fluorescence typical of rotavirus could be observed (Fig. 3).

Faeces processed by negative staining demonstrated that most of the viral particles were destitute of the outer capsid and some of the particles were in an advanced stage of degradation (Fig. 4).

DISCUSSION

The maintenance and the dissemination of rotavirus are associated to the nutritional and immunological states of the young hosts and occurrence of sub-clinical infections in adult and environmental factors such as humidity and temperature. Although the environmental factors such as temperature are considered for the maintenance and dissemination of infectious rotavirus, little has been done about the period of time in which the virus keeps its infectivity in faeces. Most of the studies of human or animal rotavirus detections in faeces, collected over periods of several months or years, do not mention the conditions in which faeces were kept, whether as original faecal specimens or clarified faecal homogenate[2,3,22] and presumably kept below 0ºC, normally -20ºC or -70ºC. It has been demonstrated that rotavirus was capable to be infectious, after 7 and 9 months kept at room temperature, probably at 18-20ºC, in faeces[23,24]. It was also shown that porcine rotavirus, adapted in cell culture, deliberately added to a porcine faeces sample and kept at room temperature (average 20-25ºC) for 4 months, maintained its infectivity. However, the viral titer decreased by 2 log[9]. In a different situation rotavirus deliberately added to several sources of water lost infectivity in two log after 40 days in river water and 64 days in tap water at 20ºC. At the same temperature no decrease in viral titer was observed after 64 days, when river water was filtered through a 0.22 µm membrane[19]. These results demonstrated that in spite of the treatment carried out in these sources of water, waterborne rotavirus infection is possible. In fact, waterborne rotavirus outbreak that originated from well water contaminated with the virus was confirmed[17], probably by the low efficiency of virus adsorption to ground particles[13], maintenance of virus infectivity on the ground[11] and consequently the possibility of ground water contamination[15]. Human rotavirus was shown to be resistant for 6 days at 4ºC and 20ºC, and for 6 months at -30ºC in pasteurized milk[4].

It was shown that bovine rotavirus RNA decreased in intensity in electrophoresis, when bovine clarified faecal homogenate was maintained at -20ºC for 6 months[21]. Differently, simian rotavirus (SA-11) adapted in cell culture, turned out to be infectious for 24 hours at 25-39ºC, however at 50ºC for 30 min. its infectivity was reduced in 99 %[7].

Therefore most of the data concerned to the maintenance of rotavirus infectivity were obtained in situations that: a) faeces samples were kept at sub-zero temperature to preserve the infectious virus, b) virus particles were extracted from faeces, c) utilization of cell culture adapted virus, d) temperature over 25ºC to demonstrate the resistance of the virus and e) studies carried out for a short period of time.

Presently it was demonstrated that porcine rotavirus kept in the original faeces samples for 32 months at approximately 10ºC still maintained the infectivity. Although electron microscopy revealed that most of the observed virus particles were in a varied state of degradation it suggests that some virus particles were functionally maintained. It is important to consider the protective effect of faeces and/or its components in the infectivity of the virus and on the stability of rotavirus RNA even at -20ºC, such as suggested elsewhere[9,21].

In conclusion it is suggested that porcine rotavirus may well be maintained infectious for a long time in faeces at low temperature and this may be an important factor to be considered for the control of the disease.

ACKNOWLEDGEMENTS

The authors thank Dr. Ortrud Monica Barth Schatzmayr of Fundação FIOCRUZ (Rio de Janeiro) for the collaboration in electron microscopy and also thank CAPES, CNPq (grant 302772/82) and CPG-UEL (279249/93) for the financial support. This work is part of APDR M.Sc. thesis. We also thank Mrs. S.M.A.Vieira for typing the manuscript.

RESUMO

Os rotavírus constituem-se nos principais patógenos da diarréia em humanos e animais. Afetam os animais jovens em criações intensivas e causam grandes perdas econômicas. Este estudo avaliou a infecciosidade do rotavírus suíno mantido por 32 meses a aproximadamente 10ºC nas amostras originais de fezes. Trinta amostras de fezes de leitões de 1-4 semanas de idade, provenientes de granjas da região sudoeste do Paraná, foram selecionadas para o estudo. As amostras foram colhidas no período de março a outubro de 1991 e selecionadas ao acaso dentre as positivas para rotavírus pela eletroforese em gel de poliacrilamida (EGPA), à época da colheita. Estas foram retestadas por EGPA 32 meses após manutenção à temperatura de aproximadamente 10ºC. Onze das 30 amostras ainda foram positivas, mostrando a integridade das 11 bandas de RNA viral. Com o intuito de demonstrar a manutenção da infecciosidade viral, os homogenatos fecais clarificados, previamente tratados com tripsina, foram inoculados em culturas de células MA-104. Das 11 amostras, 5 demonstraram efeito citopático semelhante ao do rotavírus símio (SA-11), após em média 3 passagens cegas e confirmado pelo teste de imunofluorescência indireta, demonstrado pela fluorescência específica citoplasmática típicamente granular. A microscopia eletrônica das amostras fecais mostrou que a maioria das partículas virais apresentavam-se sem capsídio externo e outras encontravam-se em adiantado estado de degradação. Concluiu-se, portanto, que a infecciosidade do rotavírus suíno é mantida por longo período em amostras fecais em baixa temperatura. Este certamente é um aspecto importante para a manutenção do vírus viável em condição natural assim como para a transmissão da doença.

UNITERMOS: Rotavírus; Infecções por Rotavírus; Diarréia; Suínos.

REFERENCES

1- AHMED, R.; STEVENS, I.G. Viral persistence. *In*: FIELDS, B.N. *et al.* (2.ed.) **Virology**. New York : Raven Press, 1990. V.1. p.241-65.

2- BABIUK, L.A.; MOHAMMED, K.; SPENCE, L.; FAUVEL, M.; PETRO, R. Rotavirus isolation and cultivation in the presence of trypsin. **Journal of Clinical Microbiology**, v.6, n.6, p.610-7, 1979.

3- BACHMANN, P.A.; HESS, R.G. Routine isolation and cultivation of bovine rotavirus in cell culture. **American Journal of Veterinary Research**, v.42, n.12, p.2149-50, 1981.

4- BENKADDOUR, M.; TACHE, S.; LABIE, C.; BODIN, G.; EECKHOUTTE, M. Influence of temperature, acidity and lactic acid bacteria on the stability of rotavirus and coronavirus in milk. **Review de Medicine Veterinaire**, Toulousse, v.144, n.1, p.709-16, 1993.

5- BRANDT, C.D.; KIM, H.W.; RODRIGUEZ, W.J.; ARROBIO, J.O.; JEFFRIES, B.C.; STALLINGS, E.P.; LEWIS, C.; MILLES, A.J.; CHANOCK, R.M.; KAPIKIAN, A.Z.; PARROT, R.H. Pediatric viral gastroenteritis during 8 years of study. **Journal of Clinical Microbiology**, v.18, n.1, p.71-8, 1983.

6- COHEN, J.; LAPORTE, J.; CHARPILIENNE, A.; SCHERER, R. Activation of rotavirus RNA polymerase by calcium chelation. **Archives of Virology**, v.60, n.3-4, p.177-86, 1979.

7- ESTES, M.K.; GRAHAM, D.Y.; SMITH, E.M.; GERBA, C.P. Rotavirus stability and inactivation. **Journal of General Virology**, v.43, n.2, p.403-9, 1979.

8- FLEWETT, T.H.; WOODE, G.N. The rotavirus. Brief review. **Archives of Virology**, v.57, n.1, 1-23, 1978.

9- FU, Z.P.; HAMPSON, D.J.; BLACKMORE, D.K. Detection and survival of group A rotavirus in a piggery. **Veterinary Record**, v.125, n.23, p.576-8, 1989.

10- GOUVEIA, A.M.G.; NOZAWA, C.M.; ARAUJO, H. Cell culture propagation of rotavirus from feces of diarrheic piglets. **Revista de Microbiologia**, v.22, n.2, p.108-11, 1991.

11- GOYAL, S.M.; GERBA, C.P. Comparative adsorption of human enterovirus, simian rotavirus and selected bacteriophage to soils. **Applied Environmental Microbiology**, v.38, n.2, p.241-50, 1979.

12- HERRING, A.J.; INGLIS, N.F.; OJEH, C.K.; SNODGRASS, D.R.; MENZIES, J.D. Rapid diagnosis of rotavirus infection by direct detection of viral nucleic acid in silver stained polyacrylamide gels. **Journal of Clinical Microbiology**, v.16, n.3, p.473-7, 1982.

13- HURST, C.J.; GERBA, C.P.; CECH, I. Effects of environmental variables and soil characteristics on virus survival in soil. **Applied Environmental Microbiology**, v.40, n.6, p.1076-9, 1980.

14- KAPIKIAN, A.Z.; CHANOCK, R.M. Rotaviruses. *In*: FIELDS, B.N. *et al.* (2.ed.) **Virology**. New York : Raven Press, 1990. V.2. p.1353-87.

15- KESWICK, B.H.; GERBA, C.P. Viruses in groundwater. **Environmental Science Technology**, v.14, n.1, p.1290-7, 1980.

16- LINHARES, R.E.C.; WIGG, M.D.; LAGROTA, M.H.C.; NOZAWA, C.M. The in vitro antiviral activity of isoprinosine on simian rotavirus (SA-11). **Brazilian Journal of Medical and Biological Research**, v.22, n.9, p.1095-103, 1989.

17- MURPH, A.M.; GROHMAN, G.S.; SEXTON, M.F. Infectious gastroenteritis in Norfolk Island and recovery of viruses from drink water. **Journal of Hygiene**, v.91, n.2, p.139-46, 1983.

18- NOZAWA, C.M.; VAZ, M.G.S.; GUIMARÃES, M.A.A.M. Detection of astrovirus-like in diarrheic stool and its coexistence with rotavirus. **Revista do Instituto de Medicina Tropical**, São Paulo, v.27, n.5, p.238-41, 1985.

19- RAPHAEL, R.A.; SATTAR, S.A.; SPRINGTHORPE, V.S. Long-term survival of human rotavirus in raw and treated river water. **Canadian Journal of Microbiology**, v.31, n.2, p.124-8, 1985.

20- RUBIO, P.; AAVAREZ, M.; CARMENES, P. Relacion entre la eliminacion de rotavirus por cerdos madres y sus camadas en condiciones de exploracion convencionales. *In*: THE INTERNATIONAL CONGRESS OF PIG VETERINARY SOCIETY, Rio de Janeiro, 1998. **Anais**. Rio de Janeiro : Pig Veterinary Society, 1988. p.215.

21- SAN JUAN, C.S.; BELLINZONI, R.C.; MATTION, N.; LATORRE, J.; SCODELLER, E.A. Incidence of group A and atypical rotaviruses in Brazilian pig herds. **Research in Veterinary Science**, v.41, n.2, p.270-2, 1986.

22- WARD, R.L.; KNOWLTON, D.R.; PIERCE, M.J. Efficiency of human rotavirus propagation in cell culture. **Journal of Clinical Microbiology**, v.19, n.6, p.748-53, 1984.

23- WOODE, G.N. Epizootiology of bovine rotavirus infection. **Veterinary Record**, v.103, n.3, p.44-6, 1978.

24- WOODE, G.N.; BRIDGER, J.C. Viral enteritis of calves. **Veterinary Record**, v.96, n.4, p.85-8, 1975.

Received: 04/12/1996
Accepted: 30/07/1997