# ISOLATION OF ROTAVIRUS STRAINS FROM NATURALLY INFECTED PIGLETS

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**Summary.** – Twenty rotavirus strains were isolated in 1991–92 from 60 faecal samples collected from diarrhoeic piglets in the Czech and Slovak Republics. Three isolates were adapted to the growth in the cell line MA-104 and produced cytopathic effect. Rotavirus was demonstrated by immunofluorescence test, electron microscopy, polyacrylamide gel electrophoresis, immunoperoxidase test and ELISA.

**Key words:** rotavirus; diarrhoea; piglet; MA-104 cell line

## Introduction

Rotaviruses – a genus of the family Reoviridae – are together with *Escherichia coli*, transmissible gastroenteritis virus, *Isospora suis*, and *Clostridium perfringens* the most frequently identified causal agents of diarrhoea in pigs (Fitzgerald, 1983). It has been postulated that probably no pig herd free of rotavirus infection occurrence exists at all (Woode *et al.*, 1976). Mostly affected are suckling and weaned animals. Clinical signs usually develop in piglets up to the age of 4 weeks, but infections and deaths in 8 week-old weaners were also reported (Bohl *et al.*, 1978).

A report on the adaptation of porcine rotaviruses to cell cultures has not been published from the Czech Republic yet. The diagnosis of rotavirus infections is usually based here on ELISA and electron microscopy (EM) (Šmíd *et al.*, 1980). In our work, these methods were complemented by immunofluorescence test (IF), polyacrylamide gel electrophoresis (PAGE) and immunoperoxidase test (IP).

## Materials and Methods

*Reference virus.* The serogroup A porcine rotavirus strain OSU (Ohio State University), propagated in the cell line MA-104 (foetal macaque kidney) with Eagles's minimum essential medium (MEM) supplemented with trypsin (1 μg/ml), was used. The virus was activated by trypsinization (trypsin 10 μg/ml, 37 °C, 30 mins) prior to use.

*Samples.* 60 samples collected from diarrhoeic piglets were examined. Either rectal swabs from live piglets or small and large intestine contents from necropsied piglets were collected. A 30 % suspension was prepared in PBS pH 7.2, centrifuged at 1500 x g for 20 mins and filtered through a 400 nm Millipore filter to remove microbial contamination. The suspension was stored at –20 °C until use.

*Virus isolation.* Both stationary and roller cultures were used. The MA-104 cells were grown in MEM containing 5 % foetal calf serum and antibiotics (penicillin 100 U/ml and streptomycin 200 μg/ml). The cultures were washed prior to inoculation. The filtered 30 % suspension was diluted 1:2 – 1:10 with MEM, treated with trypsin and inoculated onto 24–48 hrs old cultures of MA-104 cells. The inoculum was removed after 1 hr adsorption at 37 °C and the cells were, without washing, overlaid with serum-free MEM supplemented with 1 μg/ml trypsin. Each passage was maintained for at least 8 days. The cultures were then subjected to 2 cycles of freezing and thawing. The attempt was considered as unsuccessful when no cytopathic effect (CPE) was evident up to passage 4 and the presence of the virus was not detected by IF, IP, PAGE, ELISA and EM.

*Direct IF.* A monolayer of 24–48 hrs old MA-104 cells grown on slides was overlaid with the inoculum prepared as described above. The inoculum was removed after 30 mins and the slides were washed with PBS, placed into Petri dishes containing the maintenance medium (MEM with 1 μg/ml trypsin) and kept in atmosphere containing 5 % of carbon dioxide. 18 hrs after inoculation the slides were washed with PBS and dried. After fixation in cold (4 °C) acetone, the cells were overlaid with the rabbit anti-rotavirus immunoglobulin FITC conjugate (VRI, Brno). After 30 mins the slides were washed, dried, mounted into glycerol and examined in fluorescence microscope.

*Indirect IF.* Cryostat sections of the jejunum of diarrhoeic piglets were fixed in cold acetone and overlaid with swine anti-rotavirus serum (PS-2546, VRI, Brno). After 40 mins incubation, the sections were washed with PBS, dried, overlaid with the rabbit anti-swine immunoglobulin FITC conjugate (Institute of Sera and Vaccines, Prague), and incubated for another 40 mins. Then they were again washed, dried, mounted into glycerol and examined.

*EM.* A drop of the specimen was transferred onto a formwar-coated grid, stained with 2 % ammonium molybdenate and viewed in the electron microscope Tesla BS500.

*PAGE.* 1 ml of the sample was mixed with 15 µl of 20 % polyethylene glycol ($M_r$ 6000) in 2.5 mol/l NaCl, left to stand at room temperature for 10 mins and then centrifuged at 15 000 × g for 10 mins. The sediment was resuspended in 100 µl of bidistilled water and RNA was extracted by 100 µl of phenol pH 7.6. Ten µl of 3 mol/l sodium acetate and 300 µl of ethanol were added to the water phase obtained after centrifugation (15 000 × g, 5 mins) and RNA was left to precipitate for 1 hr at –20 °C. The precipitated RNA was recovered by centrifugation (15 000 × g, 10 mins), vacuum-dried and resuspended in TE buffer (10 mmol/l Tris pH 7.6, 1 mmol/l EDTA) or distilled water. The resuspended RNA was applied to 3–10 % polyacrylamide gel gradient. After electrophoresis (Bio-Rad Miniprotean, 200 V, 2 hrs), RNA was stained with ethidium bromide and the gel was viewed and photographed under UV light.

*IP.* The procedure described for direct IF was used up to the fixation of infected cells in acetone. The fixed cells were dried, washed in PBS for 5 mins and incubated for 10 mins with 0.1 % sodium azide containing 0.3 % of hydrogen peroxide. Then the cells were washed (3 × 5 mins) in PBS and incubated for 45 mins with rabbit anti-rotavirus serum (PS-5278, VRI, Brno) conjugated with horseradish peroxidase. The incubation was followed by three 10 mins washings in PBS, 15 mins incubation with 3,3' -diaminobenzidine tetrahydrochloride containing hydrogen peroxide, and one 10 mins washing in PBS. The slides were examined in light microscope.

*ELISA.* The wells of polystyrene microtiter plates were activated with 1 % glutaraldehyde at 4 °C for 1 hr prior to use. The IgG fraction of the bovine anti-rotavirus serum (VRI, Brno) was pipetted into each well and left to adsorb at 4 °C. The tested samples and the positive and negative control antigens were added into the respective wells on the subsequent day and the plates were incubated in a wet chamber for 1 hr at 37 °C. After the addition of the rabbit anti-rotavirus serum conjugated with horseradish peroxidase (VRI, Brno) and 1 hr incubation at 37 °C, the substrate solution (hydrogen peroxide, 5-aminosalicylic acid) was added and the plates were incubated for 1 hr at 20 °C. All the wells were washed with PBS supplemented with 0.2 % Tween after each step. The absorbance was read at 492 nm. The virus titer was defined as the reciprocal value of the highest sample dilution showing $A_{492}$ higher than 0.1 and at least twice as high as that of the negative control.

## Results

Rotavirus was demonstrated by IF, EM, ELISA, PAGE and IP in 20 from the total of 60 faecal samples collected from diarrhoeic piglets in 1991-92. Three of the 20 field strains were successfully adapted to the growth in MA-104 cells. The adapted strains (ZKS, SLS, OVS) produced CPE characterized by vacuolization of cytoplasm, cell degeneration and cell detachment at passage 3 (Fig. 1, Table 1).

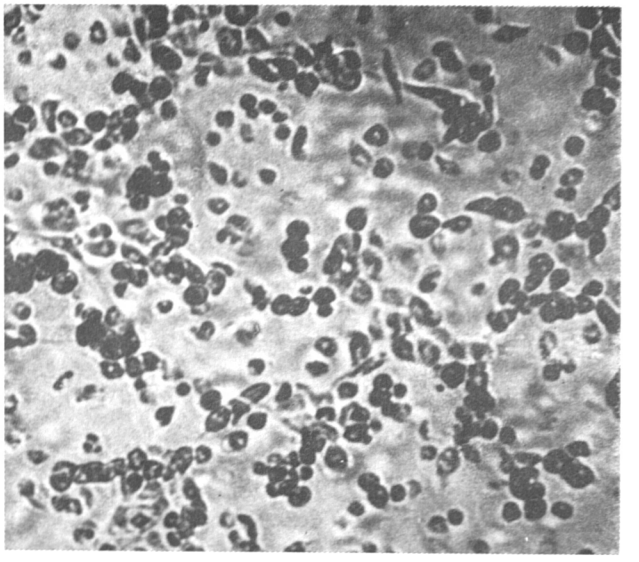

**Fig. 1**
**CPE in MA-104 cells 48 hrs after infection with the strain ZKS**
20th passage (direct magnification 20 ×).

**Table 1. Development of CPE during the first five passages of the porcine rotavirus strains ZKS, SLS and OVS in MA-104 cells**

| Strain | Passage No. | | | | |
|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 |
| ZKS | – | + | + | + | + |
| SLS | – | – | ± | + | + |
| OVS | – | – | ± | + | + |

\+ positive
– negative
± positive or negative

Prior to the passage level where a CPE was evident, serial blind passages in MA-104 cells had to be done. A slight CPE was first noticed in the 2nd passage of the strain ZKS and in the 3rd passage of both SLS and OVS strains. This CPE was observed on day 4 or 5 after inoculation but was not accompanied with monolayer destruction. During subsequent passaging of all these three isolates an increase of CPE intensity was noticed. In the 10th passage a specific rotavirus CPE was visible on the 2nd day after infection and progressed so that the monolayer was completely destroyed within 4 days.

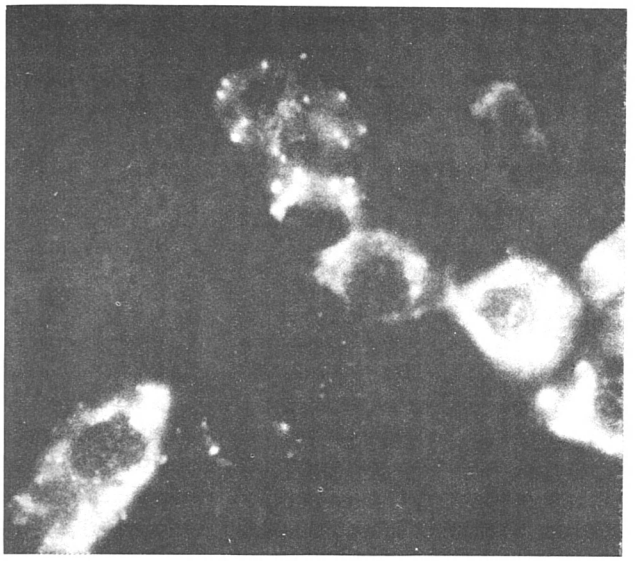

**Fig. 2**
**Direct IF in MA-104 cells 19 hrs p.i. with the 1st passage of the strain ZKS**
Fluorescence in single cells (direct magnification 252 ×).

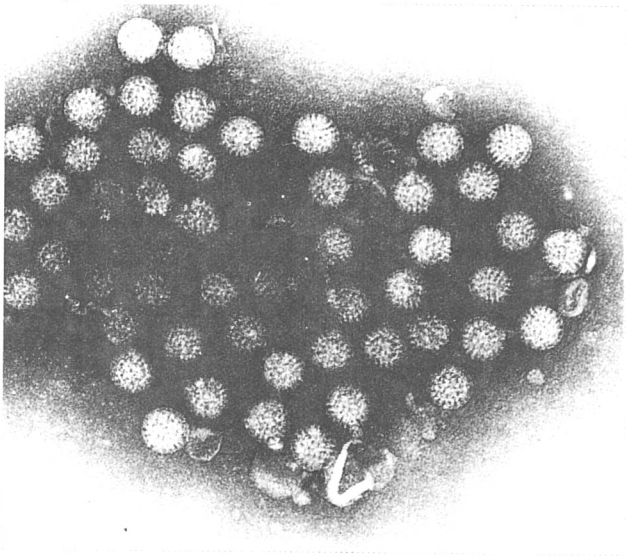

**Fig. 4**
**EM of rotavirus (strain OVS) particles from faeces of a diarrhoeic piglet**
Direct magnification 42 000 ×.

**Fig. 3**
**Indirect IF in jejunum of piglet with rotavirus diarrhoea**
Cryostat section of jejunum of one week-old piglet. Fluorescence in epithelial cells on the tops of the vili (direct magnification 10 ×).

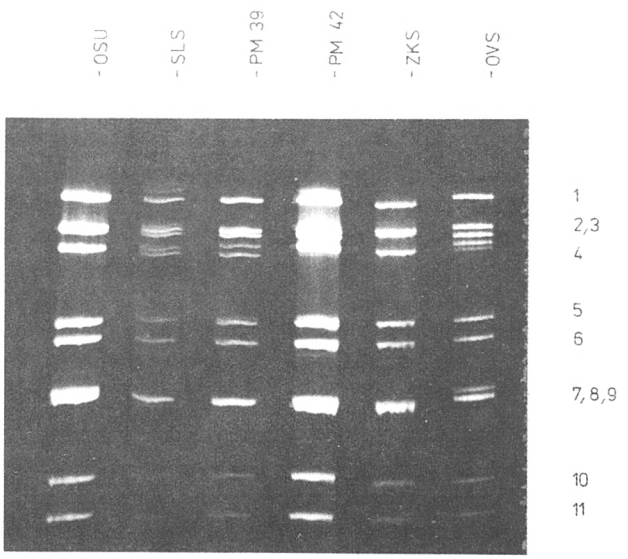


**Fig. 5**
**PAGE of rotavirus RNAs**
RNA samples were isolated from infected MA-104 cell cultures (strains OSU, SLS, ZKS and OVS) or from 30 % faeces suspensions (strains PM-2539 and PM-2542). Numbers 1–11 indicate individual RNA segments.

To show that the isolates replicated in MA-104 cells, endpoint titrations of single passages were performed and investigated by IF. The $TCID_{50}$ titers were $10^2$ and $10^4$–$10^5$ in the 2nd and 10th passages, respectively. Titers of $10^8$–$10^9$ were detected in the 30th passage and regulary thereafter without any further increase (data not shown).

Attempts to propagate the 20 strains of porcine rotavirus serially in MA-104 cells were successful only with 3 of

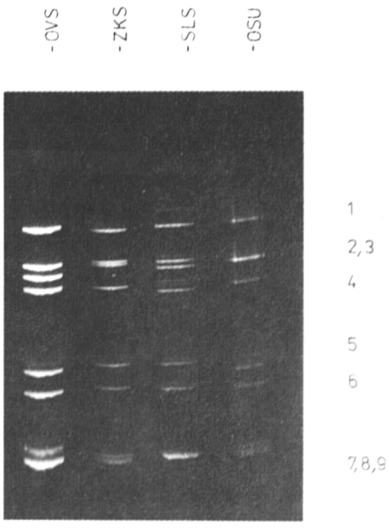


**Fig. 6**
**PAGE of rotavirus RNAs**
For legend see Fig. 5. The 10th and the 11th segments ran out of the gel. This PAGE allows to distinguish the 2nd and the 3rd segments.

them. With the 17 remaining strains CPE was not observed even in the 4th passage. But all these strains proved infectious when inoculated onto cultures of MA-104 cells and investigated by IF. However, the evidence for virus replication as judged by the appearance of immunofluorescence positive cells was found only in the first 2–3 passages. All these 20 rotavirus strains were classified as A serogroup rotaviruses because of their cross reactions with polyclonal anti serogroup A serum in ELISA, IF and IP. Using direct IF and IP, rotavirus antigen was detected in the cytoplasm of MA-104 cells, the highest densities being observed in the perinuclear region (Fig. 2). The rotavirus antigen was demonstrated by indirect IF in epithelial cells of the small intestine of the infected animals (Fig. 3).

When examined by EM (Fig. 4) the supernatants of infected cell cultures and faeces of affected animals contained particles with the typical morphology of both single and double shelled rotavirus.

The arrangement of the 11 RNA segments obtained from the 3 isolates by electrophoretic separation was similar to that found in the reference virus, i.e. 4-2-3-2. Small deviations were found in the positions of the 2nd and the 3rd segments (Figs 5 and 6).

## Discussion

Great effort has been devoted to the adaptation of rotavirus strains to the propagation in cell cultures. The strains SA11 and 0-agens were the first to be adapted successfully, using the monkey kidney cell line VMK (Kurstak *et al.*, 1978). As a rule, more strains must be examined to select one which is adaptable to passaging in cell cultures. Several cell lines were used in the adaptation attempts and various results were obtained (Debouck *et al.*, 1983). Currently the cell line MA-104, derived from foetal macaque kidney cells, is considered to be the most suitable for the adaptation of rotaviruses. The essential prerequisite for a successful infection of a cell culture is trypsinization of the inoculum.

Three of the twenty field strains were isolated in cell cultures in our experiments – a proportion, which is comparable to that reported by other authors (Gouveia *et al.*, 1991). We assume that the concentration and the integrity of virus particles in the sample are the decisive factors for the success of an adaptation attempt.

A polyclonal serum, containing antibodies to serogroup A rotaviruses, was used in our examinations. Further experiments should include also serological typing of the strains, using monoclonal antibodies or methods of molecular biology.

## References

Bohl, E. H., Kohler, E. H., Saif, L. J., Cross, R. F., Agnes, A. G., and Theil, K. W. (1978): Rotaviruses as a cause of diarrhoea in pigs. *J. Amer. vet. Ass.* **172**, 458–463.

Debouck, P., Callebaut, P., and Pensaert, M. (1983): The pattern of rotavirus and pararotavirus excretions in pigs on closed swine herds, pp. 77-89. In *Proceedings of the Fourth International Symposium of Neonaltal Diarrhea*, VIDO, Saskatoon.

Fitzgerald, G. R. (1983): Diarrhea in young pigs: Comparing the incidence of the five most common infections agents. *Vet. Med.* **88**, 80–87.

Gouveia, A. M. G., Nozawa, C. M., and Araújo, H. (1991): Cell culture propagation of rotavirus from feces of diarrheic piglets. *Rev. Microbiol. Sáo Paulo* **22**, 108–111.

Kurstak, E., Kurstak, C., Van den Hurk, J., and Morisset, R. (1978): *Comparative diagnosis of viral diseases.* Vol. 4, Part 2, pp. 105–141.

Šmíd, B., Valíček, L., and Štěpánek, J. (1980): Fast electron microscopic diagnostic of rotavirus diarrhoea in calves and piglets. *Veterinářství* **30**, 399-401 (in Czech).

Woode, G. N., Bridger, J., Hall, G. A., Jones, J. M., and Jackson, G. (1976): The isolation of reovirus-like agents (rotaviruses) from acute gastroenteritis of piglets. *J. med. Microbiol.* **9**, 203–209.