Isolation, immunochemical demonstration of field strains of porcine group A rotaviruses and electrophoretic analysis of RNA segments of group A and C rotaviruses

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ABSTRACT: Rotaviruses are major cause of acute diarrhea in animals and humans which can result in huge economic losses in farm animals including pigs. We collected 195 samples of feces of diarrhoeic animals. Rotavirus was demonstrated by electron microscopy using the method of negative staining in 27 samples and by ELISA test using monoclonal antibodies to the group antigen VP6 in 44 samples. Nine samples were selected for virus isolation. Three virus isolates (P375/4, P410/4 and P646/1) were successfully adapted to growth in cell line MA-104. These isolates were allocated to group A rotaviruses based on ELISA, immunoperoxidase test and electropherotype analysis. Electropherotype analysis demonstrated changes during passage in cell line in two of the three isolates. The selected sample P543/1 proved negative in ELISA in a fecal sample. Electropherotype analysis of this sample revealed a "longer" electropherotype profile. The profile was suggestive of group C rotavirus. Rotavirus group C was confirmed by RT-PCR and by sequence analysis in this sample.

Keywords: cell line MA-104; electron microscopy; immunoperoxidase test; ELISA; monoclonal antibody; electropherotype; group A and C rotavirus; RT-PCR

Viral infections are frequent causes of gastroenteritis in humans and animals. Infections caused by rotaviruses belonging to RNA viruses from the family Reoviridae are among the most significant as they are very frequent and cause great economic losses. Their genome is formed of double-stranded RNA arranged into 11 segments. Each of the 11 segments codes for at least one protein. The viruses are uncapsulated, 65–75 nm in diameter and possess a triple-layered protein capsid (Estes, 1996). The outer capsid contains two major neutralizing antigens, virus protein 4 (VP4) and virus protein 7 (VP7). Virus protein 6 (VP6) of the inner capsid layer is so called group antigen which is common to all group A rotaviruses.

Rotaviruses causing diarrhea in pigs were demonstrated by Woode et al. (1976). Clinical form of the disease is common in piglets aged 3 to 8 weeks, how-ever, all age categories are susceptible to the infection. Reinfections of lactating sows via piglets have been described (Bohl and Saif, 1981). Antibodies to group A rotavirus, whose distribution is worldwide can be found in 90–100% pigs. Rotaviruses of groups B and C initially called rotavirus-like agents and pararotaviruses (Saif et al., 1988), respectively, are also present in swine herds but more detailed data on the incidence of antibodies to these groups are not available. However, in seronegative herds, group B and C rotaviruses might cause an epizootic infection (Paton and Done, 2002).

Clinical signs of diarrheal diseases of viral etiology in pigs are much alike. In piglets, watery diarrhea of yellow to green colour containing mucus or sometimes blood is typical, followed by dehydration, cyanosis, anorexia, changes in blood circulation. High morbidity and mortality rates till

the age of 2 weeks is usual. In porkers and adult pigs, inappetence and diarrhea lasting for few days can be observed. Diagnosis is based on electron microscopy and ELISA to distinguish rotaviruses from other entheropathogenic viruses, namely two coronavirus species: transmissible gastroenteritis virus (TGEV) and porcine epidemic diarrhea virus (PEDV).

In vitro cultivation of rotaviruses is not easy as they require the presence of proteolytic enzymes both in the sample and maintenance medium. These enzymes are essential for outer capsid VP4 digestion. Usually trypsin is used to digest VP4 to viral protein 5 (VP5) and 8 (VP8), thus enabling virus penetration into a cell (Estes et al., 1981). The first successful cultivation of human rotavirus was described in 1981 in cell line MA-104 and this method is also applicable to cultivation of rotaviruses from fecal samples of other animal species (Sato et al., 1981).

The present study was focused on the isolation of rotaviruses from rotavirus electron positive samples of faeces on a susceptible tissue culture. During the isolation on line MA-104, they were identified by electron microscopy and immunoperoxidase test. Our attention was given to the samples with discrepancies in the results obtained by electron microscopy and ELISA, or samples where a non-typical electrophoretype profile was detected. For identification of nongroup A rotavirus, we applied RT-PCR technique for a specific detection of group C rotavirus.

MATERIAL AND METHODS

Sample preparation. Intestinal contents, obtained from 194 pigs and 1 calf showing clinical signs of diarrhea, were used to prepare 20% suspension in Eagle's medium MEM (E MEM supplemented with antibiotics: penicillin 20 U/ml, streptomycin 20 µg/ml and amphotericin B 0.05 µg/ml of medium). The suspension was then centrifuged for 20 min at 5 000 g. In the obtained supernatant, rotavirus was demonstrated by electron microscopy using the method of negative staining and by competitive blocking ELISA (CB-ELISA) for demonstration of group A rotaviruses (Rodak et al., 2004). The supernatant was used for RNA extraction.

Electron microscopy (EM). Demonstration of the virus in suspensions was carried out by the method of negative staining according to Brenner and Horne (1959) using 2% aqueous solution of

ammonium molybdenate at pH 7.0 and nets with 1 000 meshes. The number viral particles on areas of five meshes at magnitude 18 000x was assessed as follows: + demonstration of 1–2 viral particles, ++ demonstration of 3–5 viral particles, +++ demonstration of 6–10 viral particles and ++++ 11 and more viral particles per 5 meshes. Electron microscope Tesla BS 500 was used for visualization.

Demonstration of rotavirus by CB-ELISA. The presence of group A rotaviruses in fecal samples and culture media was demonstrated by CB-ELISA (Rodak et al., 2004). The wells of microtitre plates coated with swine rotavirus antibodies were filled with a mixture of the tested sample with porcine rotavirus A positive or negative serum. After 1 h incubation at 37°C solution of monoclonal antibodies to VP6 protein of rotavirus A was added to the wells and incubated 1 h at 37°C. After another incubation with a conjugate (porcine antibodies to mice immunoglobulines conjugated with peroxidase), the reaction was visualized by incubation with a solution of chromogene TMB (3,3',5,5'-tetramethyl-benzidine, Sigma) and absorbances were determined spectophotometrically at 450 nm. The tested samples were regarded as positive if the difference of absorbances in wells incubated with porcine positive and negative sera were >0.1, and the reaction in wells with positive sera was blocked by >50%.

Isolation and cultivation of rotaviruses. For virus isolation we selected samples from 6 suckling pigs, 1 weaned pig, 1 sow after farrowing and 1 calf originating from 8 herds. Selection of samples was carried out based on the results of electron microscopy examination (assessment ++ to +++). The selected samples were used as a primary viral suspension after filtration through a disposable filter pore size 450 µm (Minisart, Sartorius AG, Germany).

Isolation of rotaviruses from field samples of feces was carried out in MA-104 cells grown in Eagle's medium MEM supplemented with 5% precolostral calf serum without antibodies to rotavirus. The primary virus suspension was pre-incubated for 45 min at 37°C in Eagle's medium MEM with 10 µg/ml trypsin (Trypsin 1:250, Sigma, USA). Cell line was cultured 24–48 h, washed twice in serum free Eagle's medium MEM, infected with 0.5 ml treated viral suspension and incubated for 90 min at 37°C; then the inoculum was removed without rinsing. Eagle's medium MEM without serum, supplemented with 5 µg/ml of trypsin was used as the maintenance medium. Cytopathic effect (CPE) was

monitored for 7 days. Single passages were frozen following a seven-day- incubation even in case that no CPE was observed. The virus was demonstrated continuously by electron microscopy and immunoperoxidase test.

Rotavirus strain OSU-122 obtained from the Collection of Animal Pathogenic Microorganisms as CAPM V-334[1] was cultured in each passage of virus isolation as a control. This virus is adapted for growth in cell line MA-104 and produces a specific cytopathic effect i.e. cytoplasmatic vacuolization and cell degeneration with total destruction of monolayer within 24–48 h after the infection.

Demonstration of rotavirus by immunoperoxidase test (IP test). The infected cell lines MA-104 on slips of 2 cm^2 were collected 48–72 h post infection and fixed with cool acetone (10 min at 4°C). After inhibition of endogenous cell peroxidase with sodium azide and washing with PBS (phosphate buffered saline), 1 h incubation with monoclonal antibody to VP6 protein of group A rotavirus labeled by peroxidase followed (Rodak et al., 2004). After washing with PBS (5 min 3 times), rotavirus was visualized by incubation in the solution of substrate with chromogene AEC (3-amino 9-ethylcarbazole, Sigma, USA).

RNA isolation and PAGE analysis. Trizol LS reagent was used for RNA isolation according to manufacturer's recommendation (GibcoBRL, Grand Island, N.Y., USA). This method can be used for RNA extraction from both fecal samples and tissue culture. Extraction was performed from supernatant of fecal samples and cell culture in a passage at the beginning of adaptation and at the end of the adaptation in order to monitor the electrophoretype changes. Extracted RNA was resuspended in RNase-free water and was stored at –80°C.

RNA segments of rotaviruses were separated by electrophoresis in 8% polyacrylamide gel (3 h at 80 V) and visualized by staining with silver nitrate (Herring at al., 1982).

RT-PCR for VP6 gene of group C rotavirus. The pair of primers used for production of partial-length of VP6 gene were: 5´-CTC GAT GCT ACT ACA GAA TCA G-3´ (C1, plus sense, nucleotide 997–1018) and 5´-AGC CAC ATA GTT CAC ATT TCA TCC-3´(C4, minus sense, nucleotide 1329–1352). These primers have been described previously (Gouvea et al., 1991).

Extracted dsRNA from the sample P543/1 was denatured with dimethyl-sulphoxide (Sigma-Aldrich, USA) at 97°C for 5 min (Martella et al., 2001). The reverse transcription (RT) of dsRNA was carried out using StrataScript Reverse Transcriptase (StrataScript RT, Stratagene, USA) and PCR amplification was carried out with *Taq* DNA Polymerase (Promega, USA). Final concentrations in RT mixture of 20 µl volume were: StrataScript Buffer (1×), primer C1 (2.5 µM), primer C4 (2.5 µM), dNTP (0.5mM), RNase Block Ribonuclease Inhibitor (40 U) and StrataScript RT (50 U). RT was carried out according to manufacturer's recommendation. After synthesis of cDNA the RT mixture was brought up to a volume of 100 µl of the PCR mixture containing : PCR Buffer (1×), MgCl$_2$ (1.5mM), dNTP mix (200 µM), *Taq* DNA Polymerase (5 U) and both of the primers (250 nM). PCR amplification was accomplished in 30 cycles (94°C for 1 min, 42°C for 2 min, 72°C for 1 min), followed by a final cycle of 7 min at 72°C (Gouvea et al., 1991).

PCR products (8 µl) were analyzed in 1.5% agarose in Tris-borate buffer containing 0.5 µg of ethidium bromide per ml. Gel was electrophoresed at 110 V for 60 min and photographed under UV light.

Sequence analysis. PCR product from the strain P543/1 of 356 bp was sequenced for further identification. After purification by QIAquick PCR Purification Kit (QIAGEN GmbH, Hilden, Germany), the C1 to C4 amplicon underwent sequence analysis with MegaBACE^{TM} DNA Analysis Systems (Amersham Biosciences) in the company Genomac (Prague, Czech Republic). Sequence was assembled and analyzed using BioEdit Sequence Alignment Editor (Department of Microbiology, North Carolina State University, USA) (Hall, 1999) and NCBI's analysis tool and ClustalX program (Thompson et al., 1997). The obtained sequence was compared with that of the VP6 gene of the porcine group C/Cowden strain (accession number M94157).

RESULTS

Identifications of rotaviruses in clinical and laboratory samples

Several methods were used for identification of rotaviruses in clinical and laboratory samples.

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[1]CAPM = Collection of Animal Pathogenic Microorganisms, Veterinary Research Institute, Hudcova 70, 621 32 Brno, Czech Republic

Table 1. Results obtained by the methods used at rotavirus demonstration in clinical and laboratory samples

Sample	Sample origin	From feces			From TC (during adaptation)		
		EM	ELISA	PAGE	EM	IP	CPE/adaptation
P375/4	weaned pig	+++	+	group A	++	+	+(not specific)/+
P410/4	calf	+++	+	group A	++	+	+/+
P531/2	suckling pig	+++	+	group A	–	–	–/–
P543/1	sow after farrowing	+++	–	group C	–	–	–/–
P620/4	suckling pig	++++	+	–	–	–	–/–
P637/1	suckling pig	+++	+	group A	–	–	–/–
P639	suckling pig	+++	+	group A	–	–	–/–
P646/1	suckling pig	+++	+	group A	+++	+	+/+
P646/3	suckling pig	++++	+	group A	–	–	–/–

EM – examination by electron microscopy; ELISA – enzyme-linked immunosorbent assay; PAGE – polyacrylamide gel analysis; IP – immunoperoxidase test; TC – tissue culture; CPE – cytopathic effect; + to ++++ – quantity assessment of physical particles with typical rotavirus morphology (see. Material and methods)
– = negative; + = positive

Using *electron microscopy,* rotavirus was demonstrated in 27 of 195 samples. We selected 9 samples for virus isolation experiments on tissue cultures based on quantity assessment of physical particles with typical rotavirus morphology.

In addition, *CB-ELISA,* based on monoclonal antibodies, was used for demonstration of group A rotaviruses. Rotavirus antigens were detected in 44 of 195 samples. Of nine samples selected for isolation, eight were positive by CB-ELISA and the sample P543/1 was negative (Table 1).

Rotavirus isolates P646/1, P375/4 and P410/4 were identified using *immunoperoxidase test* since passage 4 of their adaptation (Figure 1). This test employs monoclonal antibody to VP6 protein of group A rotaviruses.

Virus isolation*.* Of the total of 9 selected rotavirus samples, two isolates from pigs (P646/1, P375/4) and one from calf (P410/4) were adapted to growth in cell line MA-104. CPE was produced after 7 passages with 4-day-incubation period (sample P646/1) or after 5 passages with 5-day-
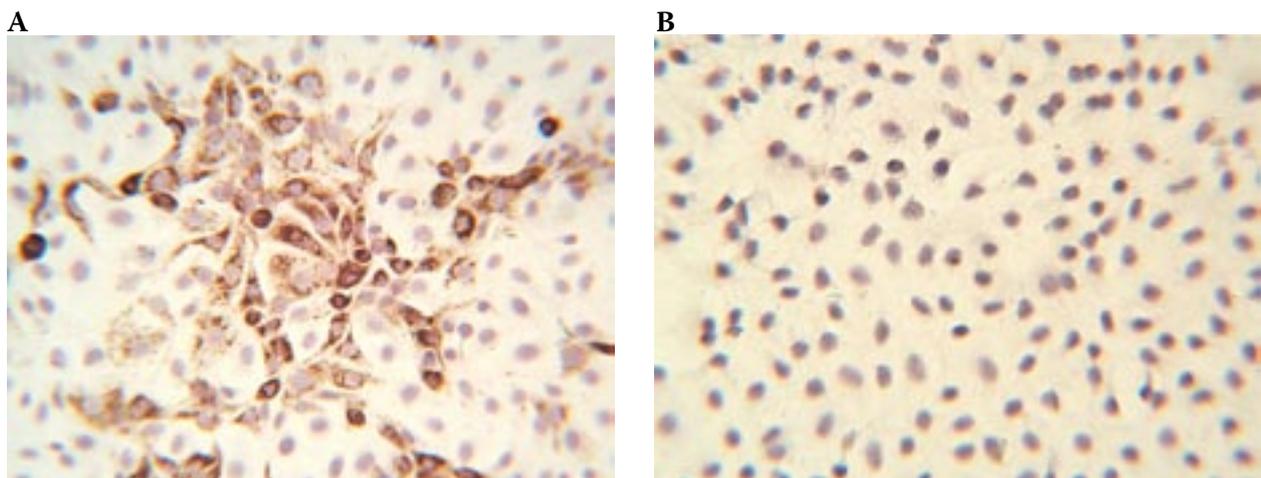
A

B

Figure 1. **A**. Detection of P375/4 strain rotavirus in infected cells by immunoperoxidase test. **B**. Negative control. Direct detection of rotavirus in cell line MA-104 using monoclonal antibody to VP6 protein of group A rotavirus labeled by peroxidase. Intense colour can be seen in cytoplasm of cells in which rotavirus propagation occurred. Cell nuclei were counterstained with hematoxylin
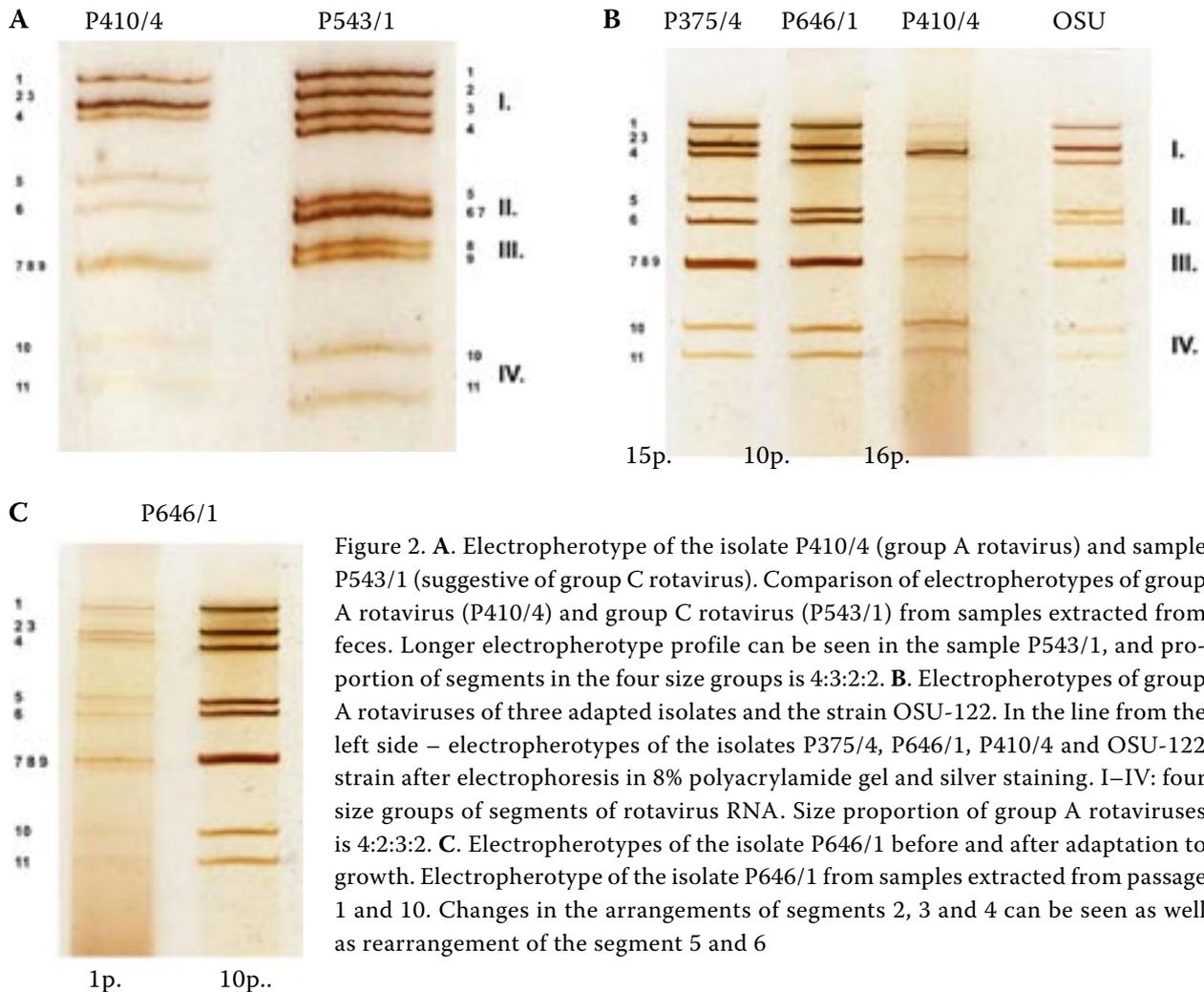
A   P410/4   P543/1

B   P375/4   P646/1   P410/4   OSU

15p.   10p.   16p.

C   P646/1

Figure 2. **A**. Electropherotype of the isolate P410/4 (group A rotavirus) and sample P543/1 (suggestive of group C rotavirus). Comparison of electropherotypes of group A rotavirus (P410/4) and group C rotavirus (P543/1) from samples extracted from feces. Longer electropherotype profile can be seen in the sample P543/1, and proportion of segments in the four size groups is 4:3:2:2. **B**. Electropherotypes of group A rotaviruses of three adapted isolates and the strain OSU-122. In the line from the left side – electropherotypes of the isolates P375/4, P646/1, P410/4 and OSU-122 strain after electrophoresis in 8% polyacrylamide gel and silver staining. I–IV: four size groups of segments of rotavirus RNA. Size proportion of group A rotaviruses is 4:2:3:2. **C**. Electropherotypes of the isolate P646/1 before and after adaptation to growth. Electropherotype of the isolate P646/1 from samples extracted from passage 1 and 10. Changes in the arrangements of segments 2, 3 and 4 can be seen as well as rearrangement of the segment 5 and 6

1p.   10p..

incubation period (sample P410/4). Inoculation of P375/4 sample did not produce any specific CPE even after 16 passages.

Electropherotyping

We extracted RNA from supernatant of nine selected fecal samples. In 7 samples the distribution of RNA segments was typical for group A rotaviruses. In isolate P543/1, a longer electrophoretic profile (Figure 2A) suggesting of different rotavirus could be seen. The segmented RNA was not demonstrated in the strain P620/4. The results are summarized in Table 1.

Figure 2B shows electropherotypes of the adapted rotavirus isolates P646/1, P375/4, P410/4 and the strain OSU-122. Comparison of electropherotypes revealed changes in the isolates P646/1 and P410/4. Migration patterns of RNA segments of the isolate P646/1 obtained in passage 1 and 10 are not identical

(Figure 2C). Changes in the arrangement of the segments 2, 3 and 4 and rearrangement of the segments 5 and 6 were apparent. Changes in arrangement of the segments 2, 3 and 4 were found in the isolate P410/4, when RNA extracted from original feces and passage 16 was compared (Figure 2A and 2B).

RT-PCR detection of group C rotavirus and sequence analysis

The product obtained after RT-PCR using primers C1 and C4 was of the expected length 356 bp. The result is shown in Figure 3. Partial sequences of 323 nucleotides obtained by sequence analysis of C1–C4 product were compared to the corresponding part of VP6 gene of the Cowden strain, representing porcine group C rotaviruses (Figure 4). The sequence of the strain P543/1 showed more than 92% similarity with the Cowden strain at the nucleotide level.
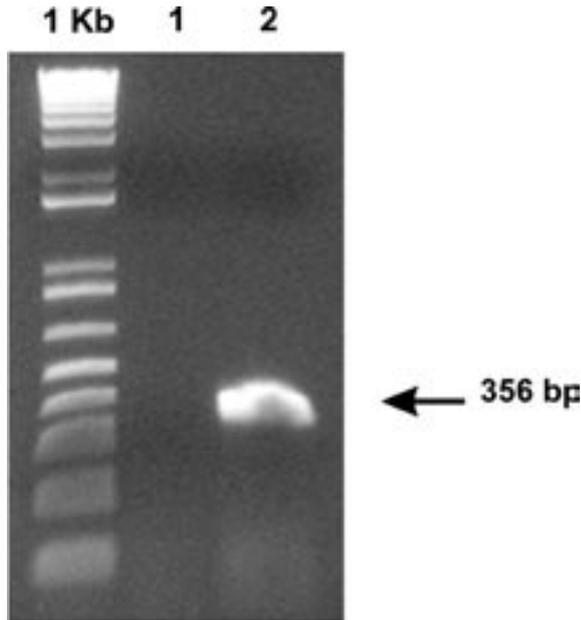
Figure 3. The RT-PCR product of field sample P543/1 detected with primers for the partial VP6 gene of group C rotavirus. 1 Kb – molecular size marker, 1 – negative control, 2 – field fecal sample P543/1

Figure 4. Comparison of partial sequence of P543/1 sample, obtained by sequence analysis of C1–C4 product, with the corresponding part of the VP6 gene of Cowden strain, representing porcine group C of rotaviruses (accession number M94157). The numbers show nucleotide succession of the corresponding part of VP6 gene of the Cowden strain

DISCUSSION

We adapted three field isolates of group A rotavirus, causative agents of swine diarrhoea, to growth on cell line MA-104. Cell line MA-104 is suitable for rotavirus isolation, and in combination with trypsin treatment meets the requirements for virus culture *in vitro*. Trypsin concentration in the maintenance medium was in our experiments increased to 5 µg/ml, compared to 1 µg/ml as mentioned in the literature (Albert and Bishop, 1984). Fecal samples of diarrheal animals, in which high

number of physical particles with typical rotavirus morphology was demonstrated by EM examination, were used for virus isolation.

Sestak and Musilova (1994) demonstrated rotaviruses by immunofluorescence test in all cases in 2–3 passage in cell line MA-104, but not all strains were adapted. We used IP test since passage 4 when we supposed that positivity was evidence of adaptation of a particular rotavirus sample in cell line Ma-104. Samples where the virus was demonstrated since passage 4 were at the end successfully adapted in cell line MA-104. Successfulness of group A rotaviruses adaptation was higher (37.5%) than that mentioned by Sestak and Musilova, (1994). We suppose that higher effectiveness of the isolations can be associated with functional maintenance of VP4 and VP7 in outer capsid, which are important for penetration and adhesion of the virus in cell cytoplasm (Estes et al., 1981).

It is known that group C rotaviruses are causal agents of gastroenteritides in pigs of all age categories: piglets prior to weaning (Paton and Done, 2002; Janke et al., 1990; Magar et al., 1991), weaned pigs (Janke et al., 1990, Magar et al., 1991), and for the first time were detected in older pigs in fattening by Kim et al. (1999). The investigated positive sample of rotavirus group C originated from a sow after farrowing with clinical signs of diarrhoea from a location Nikolcice, thus rotavirus group C was also demonstrated in older age category of pigs.

Of a group of 195 samples, 44 were positive at examination by CB-ELISA and 27 samples were positive at electron microscopy. The comparison showed higher sensitivity of CB-ELISA. Only 19 EM positive samples were also positive in CB-ELISA. This discrepancy can be explained by the fact that EM can detect rotaviruses of all groups in contrast to CB-ELISA which can only detect group A rotaviruses.

Extraction of viral RNA and polyacrylamide gel electrophoresis were successfully used for nucleic acid demonstration in both feces and infected tissue culture samples. Electropherotypes of group A rotavirus isolates corresponded to the basic electrophoretic profile typical of this rotavirus. Differences in electropherotypes of the isolates P646/1 and P410/4 before and after adaptations are most probably due to rearrangements within the segments 2, 3, 4, 5, 6 and 2, 3, 4 in these isolates (Figure 2). A similar electropherotype changes after adaptation of rotavirus in cell culture were also described in bovine rotavirus (Hundley et al., 1985) and in human rotaviruses isolated from im-

munodefficient patients, or chronically and asymptomatically infected immunocompetent children (Estes, 1996).

Differences between group A and C of rotaviruses were determined by electropherotypes of viral RNA (Paul and Stevenson, 1999). Our experiments also confirmed the occurrence of rotavirus group C by electrophoretyping of fecal sample (Figure 2A). RT-PCR and sequencing of PCR product were further used for demonstration of group C rotavirus. We used the primers C1 and C4 designed from and available sequence of VP6 gene by Gouvea et. al. (1991). The results of the above authors showed that 356-bp segment was only produced in the samples rotavirus group C positive but not in the samples positive for group A and B rotaviruses. Using an inner primer C3 for reamplification, they found all human and porcine group C rota. As rotavirus group C has not been so far isolated in our country, genetic and comparative analyses with the published sequence of Cowden strain have been used for identification of our sample.

RT-PCR and the sequencing methods will be used in other experiments for detection and comparison of rotaviruses of group C and B, and possibly of other groups occurring in our country. The results of rotavirus group A positive samples will be used for further research, their G and P typing, to illustrate the differences between recent isolates and the strains described earlier.

Acknowledgement

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