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ELECTRON MICROSCOPY, IMMUNE ELECTRON MICROSCOPY, ENZYME IMMUNOASSAY AND IMMUNOFLUORESCENT EVALUATION OF ROTAVIRUSES ISOLATED FROM INDIVIDUAL CALVES AND PIGLETS

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Summary. — Rotaviruses were isolated in primary bovine foetal kidney cells from 3 of 13 (23.1%) tested suckling calves and from 3 of 14 (21.4%) suckling piglets both suffering of acute diarrhoea, as confirmed by serological tests. The presence of rotaviral antigen in culture supernatant was revealed by double sandwich enzyme immunoassay (EIA) and direct immunofluorescence (IF). Transmission electron microscopy (TEM) and immune electron microscopy (IEM) showed particles with an average diameter of 70 nm. The strains adapted to continuous pig kidney cells PK-15 have lost their reactivity in EIA, although TEM revealed the presence of rotavirions.

Key words: *rotaviruses, electron microscopy, immune electron microscopy, enzyme immunoassay, immunofluorescence*

Introduction

Various enteropathogenic agents, including rotaviruses, may be found in the alimentary tract of man, animals, and birds (Flewett and Woode, 1978; 1978; McNulty *et al.*, 1980; Estes *et al.*, 1983). Since 1980, neonatal calf and pig diarrhoea has been intensively studied in a breeding farm in Poland (Malicki *et al.*, 1981 and 1988; Samól, 1983; Zmudziński *et al.*, 1984). They found that 40—80% of piglets and calves suffering from and succumbed to acute diarrhoea shed rotaviruses in their faeces. It has also been noted that coronaviruses accompanied rotaviral infection (Malicki *et al.*, 1981).

This paper describes the rotavirus isolation from individual calves and piglets in Poland as compared with the efficacy of transmission electron

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microscopy (TEM), immune electron microscopy (IEM), double sandwich enzyme immunoassay (EIA) and direct immunofluorescence (IF) when used as diagnostic tools.

Materials and Methods

Viruses and cell cultures. The 13 tested suckling calves and the 14 suckling piglets with symptoms of acute diarrhoea were obtained from 9 different sources. Rotaviruses (field strains) were isolated from 3 of 13 tested suckling calves (named C3, C5 and C8) and 3 of 14 suckling piglets (named P2, P3 and P4). Standard simian SA-11 and calf RFC-17 rotavirus strains were kindly supplied by the University of Veterinary Sciences, Budapest (Hungary) and Station de la Recherche Agronomique, Thierval-Grignon (France), respectively.

Primary bovine foetal kidney (PBFK) cells were used for isolation of the field strains and for propagation of the standard strains. A trial was also made to adapt them to a continuous PK-15 cell line derived from a pig kidney (Malicki *et al.*, 1981).

Detection of viral antigen in cell cultures. The presence of rotaviral antigen was checked by (i) double sandwich enzyme immunoassay (EIA) (Hovi *et al.*, 1982), (ii) direct immunofluorescence (IF) test with self-made FITC-conjugate prepared from rabbit polyclonal antibodies (r-pAb) to the SA-11 strain (Coons and Kaplan, 1950; Fauvel *et al.*, 1978). Specificity of the IF reaction was demonstrated by a blocking test whereby preincubation of infected cells with r-pAb to SA-11 blocked the reaction. Normal rabbit serum did not prevent fluorescence. As additional controls served uninfected cultures and infected cultures stained nonspecifically with (a) FITC-labelled conjugate directed against equine herpesvirus type 1 (EHV-1) (Gamakon, Bioveta, Nitra, Czechoslovakia), and (b) self-made FITC-conjugate against swine vesicular disease virus (SVDV) (Niemiałtowski, 1983*a*, *b*).

Transmission electron microscopy (TEM) and immune electron microscopy (IEM). The size and morphology of the viral isolates were determined by TEM as described elsewhere (Malicki *et al.*, 1981; Pyndiah *et al.*, 1988). A sample was considered positive if one or more rotavirus-like particle was observed. The IEM technique was carried out using the method previously described by Kapikian *et al.* (1975). Hyperimmune rabbit anti-SA-11 serum has been used for virion aggregation. Preimmune rabbit serum was used as negative control.

Results

Three bovine strains (designated C3, C5, and C8) and three swine strains (named P2, P3, and P4) were isolated from 13 intestinal filtrates of 13 suckling calves and 14 of suckling piglets (21.1% and 21.4%, respectively). Single rounded refractive cells were observed after 48 hr in PBFK cells

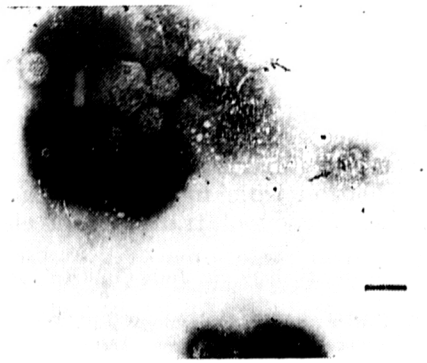

Fig. 1
Rotavirus isolate C3. Arrows indicate the 70 nm particles. ×100 000. Bar represents 100 nm.

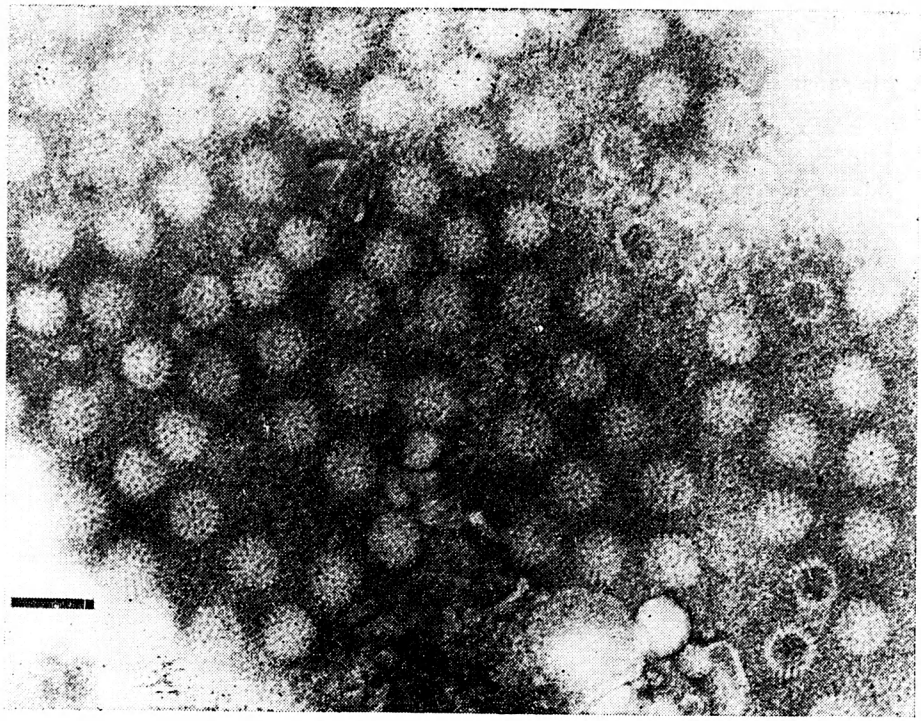

Fig. 2
Rotavirus (isolate C8) particles precipitated by antiserum. × 1000 000. Bar represents 100 nm.

infected with intestinal filtrates of calves which formed small foci within the next 24 hr. Some of the cells became necrotic. The intensity of cytopathic effect (CPE) increased in the 4th—5th passage and remained later on unchanged.

Negative-stained electron microscopy specimens revealed a virus with icosahedral symmetry and a diameter of 70 nm, typical for rotaviruses (Fig. 1). The viruses and isolates have been identified as bovine and swine rotaviruses according to the origin, size, symmetry, growth properties and specific immunofluorescence. All virions were aggregated by anti-SA-11 serum (Fig. 2). Examination of positive intestinal samples revealed virus particles sometimes even 40 or more per cluster. In all positive specimens rotavirus particles were readily recognizable by their characteristic structure. In some preparations material suggestive of antibody was seen among the particles, but often invisible linkages were seen due to the background density. The presence of rotaviral antigen was detected in successive passage by the EIA test. Similar results were obtained when passaging standard rotavirus strains, but CPE was much more pronounced.

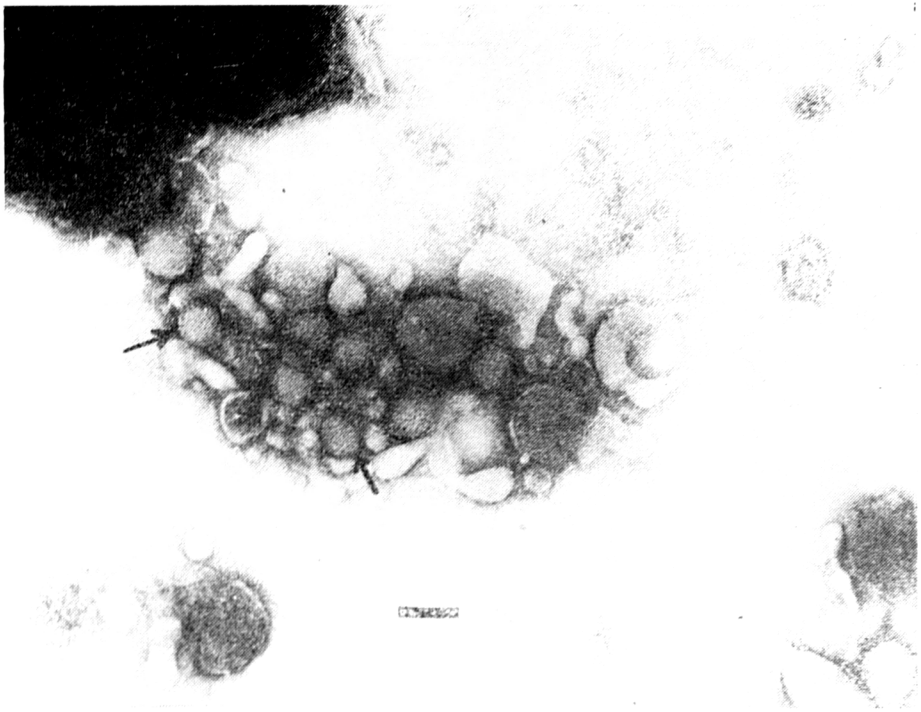

Fig. 3

Particles of the rotavirus isolate C3 adapted to PK-15 cells (EIA negative). Arrows indicate the 70 nm particle s. ×66 000. Bar represents 100 nm.

Isolation of rotaviruses from piglets was more difficult. During the first two passages no CPE has been observed in primary cell culture. They remained negative. Slight CPE appeared in passages 3—4 and became distinct in passage 6.

In PK-15 cells infected with field strains adapted to PBFK cells a distinct CPE with up to 80% of disrupted cells was observed; IF examination allowed detection of antigen presence only in the first 2—3 passages. In further passages negative results were obtained, although CPE was extensive. This was confirmed by EIA test in which rotaviral antigen has not been demonstrated before 3—4 passages. On the electronmicrographs, however, particles characteristic for rotaviruses were found (Fig. 3) regardless whether standard strains or field isolates had been adapted.

Discussion

Our results suggest that rotaviruses were isolated from newborn calves and piglets from different farms in Poland suffering from acute diarrhoea; rotaviruses were isolated from 23.1% of calves and 21.4% of piglets. In our

evaluation TEM, IEM, EIA and IF tests were employed for detection of rotaviruses. Our results were in agreement with conclusions of Pyndiah *et al.* (1988) who compared the value of TEM versus PAGE for diagnosis of rotaviral infections, and found TEM very sensitive and valuable. Pyndiah *et al.* (1988) in their comparative study stressed the high agreement of PAGE with TEM (97.4%) and the slightly higher sensitivity of PAGE (99.6% vs 95.0% for TEM). In our electronmicrographs we have seen virions of 70 nm in size and of morphology typical for rotaviruses (Flewett and Woode, 1978; Nicolaieff *et al.*, 1980) which could not be confused with other viruses or cell debris.

Studies of Cooner *et al.* (1984) and Gillespie *et al.* (1984) in the faeces of horses have shown that the correlation of TEM and ELISA was in very good agreement although ELISA was slightly more sensitive. Data obtained by Gillespie *et al.* (1984) suggested that complement-fixation (CF) test was the least sensitive when comparing with TEM and ELISA. Our observations confirmed that the correlation between TEM and EIA for the detection of rotaviruses in the intestinal content of calves and piglets was also high.

An intriguing phenomenon was the disappearance of the antigen reacting in the EIA test during adaptation of field and standard strains to the PK-15 cells, especially when the serum was prepared against SA-11 strain multiplied in primary cell culture preserved its neutralizing properties towards the virus adapted to PK-15 cells. It is possible that this may be connected with the occurrence of atypical rotaviruses deprived of a common group-specific antigen. Intestinal samples used as a source of virus could have been contaminated with another, unrelated passenger virus, which might possess a selection preference in the new host. However, this has been excluded by IF. Likewise, no other viruses were detected in the concentrated cell culture supernatant by TEM.

We would like to point out that (i) methods (TEM, IEM, EIA, and IF) used for identification of isolated rotaviruses proved themselves as very sensitive, (ii) according to methods used the rotaviruses isolated from calves and piglets in Poland were undistinguishable from the standard SA-11 and RFC-17 strains, (iii) the question why samples could be EIA negative, whereas by TEM virions could be detected in the same material, needs further investigations, and finally (iv) the open question remains, which serotypes represent rotaviruses isolated from calves and piglets in Poland.

Moreover, we would like to stress, that (i) rotaviruses could be isolated from up to 80% of faecal and intestinal contents from calves and piglets in Poland, (ii) rotaviral infection in calves and piglets is very often accompanied by stimultaneous infection with *Escherichia coli*, coronaviruses and cryptosporidia, and in piglets and swine, also with *Treponema hyodysenteriae* (Niemialtowski *et al.*, 1983; Binek and Szynkiewicz, 1985; Niemialtowski *et al.*, 1986; Szynkiewicz and Binek, 1986; T. Jakubowski, personal communication).

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References

Binek, M., and Szynkiewicz, Z. (1985): Multiplication in liquid medium of *Treponema* sp. isolated from intestinal contents of swine. *Acta Microbiol. Polon.* **34**, 167—176.

Cooner, M., Kita, J., Kalica, A., Quick, S., Schiff, E., Joubert, J., and Gillespie, J. (1984): Isolation and characteristics of an equine reovirus type 3 and an antibody prevalence survey to reovirus in horses located in New York State. *Vet. Microbiol.* **9**, 15—25.

Coons, A. H., and Kaplan, M. H. (1950): Localization of antigen in tissue cells. II. Improvements in a method for the detection of antigens by means of fluorescent antibody. *J. exp. Med.* **9**, 1—13.

Estes, M. K., Palmer, E. L., and Obijeski, J. F. (1983): Rotaviruses: a review. *Curr. Top. Microbiol. Immunol.* **105**, 123—184.

Fauvel, M., Spence, L., Babiuk, L. A., Petro, R., and Bloch, S. (1978): Haemagglutination and haemagglutination-inhibition studies with a strain of Nebraska calf diarrhoea virus (bovine rotavirus). *Intervirology* **9**, 95—105.

Flewett, T. H., and Woode, G. N. (1978): The rotaviruses. *Arch. Virol.* **57**, 1—23.

Gillespie, J., Kalica, A., Cooner, M., Schiff, E., Barr, M., Holmes, D., and Frey, M. (1984): The isolation, propagation and characterization of tissue-cultured equine herpesviruses. *Vet. Microbiol.* **9**, 1—14.

Hovi, T., Väisäinen, V., Ukkonen, P., and Von Bonsdorff, C. H. (1982): Solid-phase enzyme immunoassay for rotavirus antigen: faecal protease activity as a reason of false negative results. *J. virol. Meth.* **5**, 45—53.

Kapikian, A. Z., Feinstone, S. M., Purcel, R. H., Wyatt, R. G., Thornhill, T. S., Kalica, A. R., and Chanock, R. M. (1975): Detection and identification by immune electron microscopy of fastidious agents associated with respiratory illness, acute gastroenteritis and hepatitis A, pp. 9—47. In *Perspectives in Virology. Antiviral Mechanisms*, Academic Press New York, San Francisco, London.

Malicki, K., Malicka, E., and Bańbura, M. W. (1981): The use of electron microscopy in the diagnosis of rotaviral diarrhoea in piglets and calves (in Polish). *Med. Wet.* **12**, 736—739.

Malicki, K., Bańbura, M. W., Ładyńska, Q., Spohr de Faundez, I., Niemiałtowski, M., Malicka, E., and Kubrat, Z. (1988): Experimental inoculation by different routes of colostrum deprived (CD), 1 day old and SPF-CD piglets with pig and calf rotaviruses. *Ann. Warsaw Agricult. Univ.* **14**, 63—74.

McNulty, M. S., Allan, G. M., Todd, D., McFerran, J. B., McKillop, E. R., Collins, D. S., and McCracken, R. M. (1980): Isolation of rotaviruses from turkeys and chickens: demonstration of distinct serotypes and RNA electrophoretypes. *Avian Pathol.* **9**, 363—375.

Nicolaieff, A., Obert, G., and Van Regenmortel, M. H. V. (1980): Detection of rotavirus by serological trapping on antibody-coated electron microscope grids. *J. clin. Microbiol.* **12**, 101—104.

Niemiałtowski, M. (1983*a*): Plaque morphology and pathogenicity for newborn mice of swine vesicular disease virus. I. Wild strains and their clones. *Acta virol.* **27**, 217—222.

Niemiałtowski, M. (1983*b*): Plaque morphology and pathogenicity for newborn mice of swine vesicular disease virus. II. Temperature-dependent mutants and their clones. *Acta virol.* **27**, 223—227.

Niemiałtowski, M., Szynkiewicz, Z., Binek, M., Klimuszko, D., and Krzyżanowska, L. (1983): Evaluation of different cell cultures for the detection of heat-labile enterotoxins of *Escherichia coli* strains isolated from pigs. *Vet. Quart.* **5**, 49—57.

Niemiałtowski, Szynkiewicz, Z., Binek, M., Klimuszko, D., Ciosek, D., and Jakubowski, T. (1986): Production of enterotoxins by strains of *Escherichia coli* isolated from pigs. *Acta Microbiol. Polon.* **35**, 267—280.

Pyndiah, N., Beguin, R., Richard, J., Charles, M., Rey, A., and Bonifas, V. (1988): Accuracy of rotavirus diagnosis: modified genome electrophoresis versus electron microscopy. *J. virol. Meth.* **20**, 39—44.

Samól, S. (1983): Identification of rotaviruses in calves and piglets faeces using enzyme labelled antibodies (in Polish). *Życie Wet.* **58**, 1—3.

Szynkiewicz, Z., and Binek, M. (1986): Evaluation of selective media for primary isolation of *Treponema hyodysenteriae* and *Treponema innocens*. *Comp. Immunol. Microbiol. Infect. Dis.* **9**, 71—77.

Żmudziński, J., Baczyński, Z., and Majewska, H. (1984): Studies on etiology of viral gastrointestinal infections in calves (in Polish). *Bull. Vet. Inst. (Pulawy)* **27**, 46—54.