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FIVE YEAR STUDY OF ROTAVIRUS GASTROENTERITIS IN BULGARIA

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Summary. — We tested by electron microscopy 7 530 samples from children admitted to different hospitals in Sofia between February 1981 and February 1986; from these, rotaviruses were found in 725 (9.6 %) faecal samples. Electron microscopic analysis of 264 samples from 181 children admitted to the hospital of infectious diseases in Shumen between December 1984 to February 1986 revealed rotaviruses in 120 (66.6 %) of the tested children. A part of the samples positive by electron microscopy was tested by ELISA and polyacrylamide gel electrophoresis for rotavirus RNA segment patterns. Rotaviruses with eight different electropherotypes were found in Bulgaria. The seasonal culmination of the rotavirus gastroenteritis in the winter months has been confirmed. Rotavirus antibodies were found in 73 % of the sera from 152 children tested.

Key words: rotaviruses; RNA migration profiles; concurrent respiratory symptoms; electron microscopy; ELISA

Introduction

Rotaviruses are the major aetiological agents of diarrhoea in infants in the age group from 6 months to 3 years. Intensive studies of rotaviruses established their worldwide distribution as well as their biological properties and antigenic chracteristics. Summarized results from these studies were reviewed elsewhere (Estes *et al.*, 1983; 1984). The hardships connected with isolation of rotaviruses on cell cultures have compeled researchers to use alternative methods for their detection — electron microscopy (EM), ELISA, etc.

In addition to standard techniques, the methods of molecular epidemiology have been introduced for follow-up of rotavirus-induced disease and characterization of rotaviruses, i.e. the analysis of RNA migration profiles (electropherotypes) of the segmented genome of rotaviruses by electrophoresis in 10 % polyacrylamide gels. Previously we have presented data concerning hospitalised children with rotavirus infection on yearly basis (Shin-

darov *et al.*, 1982; 1983). The present study summarizes the results of a five year study of rotavirus gastroenteritis in Bulgaria.

Materials and Methods

Virus specimens. A 20 % suspension with PBS, pH 7.3 was prepared from faeces of hospitalised children; the samples were homogenised and centrifuged at 3 000 g for 15 min. Aliquots from the supernatant of this material were used for EM, ELISA and nucleic acid analysis.

Electron microscopy. Faecal samples were examined by negative contrast with 5 % ammonium molibdenate and sodium phosphotungstate, pH 7.3.

Enzyme immunoassay. We used a diagnostic kit for detection of rotavirus antigen produced by the WHO rotavirus reference laboratory in Birmingham, England.

Nucleic acid analysis. For RNA extraction rotaviruses in 20 % suspension were disrupted with SDS, extracted with phenol and precipitated overnight with ethanol at −20 °C. The precipitate was collected by centrifugation at 10 000 g for 15 min. The RNA was resuspended in electrophoresis sample buffer containing 0.05 mol/l Tris-HCl, pH 6.6, 5 % glycerol, 1 % SDS, 5 % B-mercaptoethanol and 0.00015 % phenol red.

Electrophoresis. Electrophoresis was performed in 10 % polyacrylamide slab gels using the Laemmli's discontinuous buffer system, as described (Dimitrov *et al.*, 1984).

Staining of RNA. In part of the cases RNA in gels was stained with ethidium bromide at a concentration of 4 μg/ml and after that the gels were photographed. For detection of RNA we also used silver staining by the method of Herring *et al.* (1982) modified from us (Dimitrov, 1986).

Virus neutralization test. For serological proof of rotavirus infection simian rotavirus SA11 was used. Paired sera were coming from hospitalised children. The test was performed according to standard procedures (Shindarov *et al.*, 1983).

Results

In the course of five years, between February 1981 and February 1986 a total of 7 530 samples was tested by electron microscopy. The samples were coming from 7 530 children admitted to different hospitals in Sofia. Among these 725 (9.6 %) were positive for rotaviruses. On the other hand, 222 control samples were tested in 1981 from children of the same age group coming from two nurseries and one orphanage in Sofia. All children from the control group were negative for rotaviruses.

Table 1 presents the number of children tested and the number of positives, divided according to the age groups by years. The percentage of positives ranged from 11.3 in 1981 to 7.1 in 1983. The total percentage of positives in the age group from 1 to 3 years was 9.9 and the percentage of positives in the age group 0—1 year was quite similar, 9.6, i.e. there was no significant difference between the two age groups. Out of 725 children positive by EM for rotaviruses 70.2 % fall in the age group of 0—1 year, 26.8 % were in the age of 2—3 years and 3 % were in the age between 3 to 7 years.

The monthly distribution of the samples positive for rotaviruses according to age groups is presented in Table 2. It shows that the rate of rotaviruses was the highest during the cold months. Higher percentage positive for rotavirus infection is registered between November and January. The average percentage for the three cold months (November—January) was 15.9 (1 852 samples), the highest percentage 18.3 was found in January. The positive rate during the three cold months ranged from 13 to18.3 %. On the

Table 1. Age distribution on yearly base of rotaviruses in the faecal samples of children tested by electron microscopy from February 1981 to February 1986

Age	Year																	
	1981			1982			1983			1984			1985—1986			Total		
	No. test.	No. posit.	%	No. test.	No posit.	%	No test.	No posit.	%	No. test.	No. posit.	%	No. trst.	No. posit.	%	No. test.	No. posit.	%
0—1	1255	134	10.68	962	107	11.12	1078	72	6.67	1374	148	10.77	634	48	7.57	5303	509	9.60
2—3	525	66	12.57	452	37	8.19	385	31	8.05	395	41	10.38	199	19	9.55	1956	194	9.90
4—7	100	13	13.00	96	1	1.04	23	3	13.04	47	5	10.64	5	—	—	27	22	8.10
Total	1880	213	11.33	1510	145	9.60	1486	106	7.13	1816	194	10.68	838	67	8.00	7530	725	9.63

Table 2. Age distribution on a monthly base of rotaviruses among children tested by electron microscopy for the presence of rotaviruses in the faecal samples in the period from February 1981 to February 1986

Age	January			February			March			April			May			June		
	No. test.	No. posit.	%	No. test.	No. posit.	%	No. test.	No. posit.	%	No. test.	No. posit.	%	No. test.	No. posit.	%	No. test.	No. posit.	%
0—1	433	74	17.09	596	71	11.89	743	58	7.81	391	40	10.23	402	40	9.55	388	31	7.99
2—3	136	27	19.85	143	16	11.19	282	33	11.70	160	12	7.50	167	13	7.78	140	9	6.43
4—7	15	6	40.00	8	1	12.50	18	1	5.56	24	3	12.50	21	1	4.76	26	—	—
Total	584	107	18 32	748	88	11.76	1043	92	8.82	575	55	9.57	590	54	9.15	554	40	7.22

Table 2. continued

Age	July			August			September			October			November			December		
	No. test.	No. posit.	%	No. test.	No. posit.	%	No. test.	No. posit.	%	No. test.	No. posit.	%	No. test.	No. posit.	%	No. test.	No. posit.	%
0—1	355	11	3.10	369	14	3.79	347	15	4.32	410	35	8.54	418	66	15.79	450	54	12.00
2—3	117	5	4.27	142	5	3.52	143	6	4.20	181	11	6.08	167	29	17.37	178	28	15.73
4—7	22	—	—	56	—	—	9	—	—	17	—	—	30	7	23.34	25	3	12.00
Total	494	16	3.24	567	19	3.35	499	21	4.21	608	46	7.57	615	102	16.59	653	85	13.02

other hand the average positive rate during the three warm months (July—September) was between 3.2 and 4.2 %, the lowest positive rate found was 3.2 in July.

According to monthly distribution of the different age groups it is evident from Table 2 that the highest positive rate in the age goups of 0—1 year and 2—3 years was in January, 17.0 % and 19.8 %, respectively. The lowest percentage for the age group of 0—1 year was in July — 3.1 and the lowest percentage for the age group 1—3 years was in August — 3.5. The data also demonstrate that there was no significant difference in the monthly distribution in the different age groups.

From 1984 to 1986, 264 samples were tested by electron microscopy from 181 children admitted to a hospital in Shumen; from these 120 (66.6 %) were positive for rotaviruses.

All EM positive samples were stored frozen. Fourhundred and eighty EM positive samples were tested by ELISA; rotavirus antigen was found in 353 samples, i.e. in 73.5 % of the EM positive samples were also positive by ELISA. This may be due to the fact that some of the samples before testing by ELISA were kept frozen for 3 years.

Between 1981 and 1985 we tested 235 EM positive faecal samples by electrophoresis in polyacrylamide gels from children hospitalised in Sofia. Typical RNA migration profiles were found in 130 (55.5 %) of these samples. During the given period we found rotaviruses with eight different electropherotypes; five of the most frequent electropherotypes are shown in Fig. 1.

Since 1985 by electrophoresis were tested only the EM-positive faecal samples. We have demonstrated the good efficiency of electrophoresis in the aetiological diagnosis of rotavirus infections. In the winter season of 1986—87 we used only the electrophoresis method for screening of faecal samples. We tested 235 samples; typical RNA migration profiles were found in 52 (21.12 %) of the tested samples. The method was used for decoding of epidemic outbreaks in the towns Lovetch, Blagoevgrad, Pernik and Shumen. Rotavirus aetiology of the diseases was established in Shumen, but no rotaviruses were detected as aetiological agents in the outbreaks in Lovetch, Blagoevgrad and Pernik.

For serological proof of rotavirus infection we tested paired sera from 152 hospitalized children by virus neutralization test. A fourfold and higher increase of antibodies was proven in 24.3 % of children. A twofold increase was found in 13.1 %. In 35.5 % we found antibodies without seroconversion, but in high and equal titres in the first as well as second serum sample (above 1 : 16). Only 26.9 % children revealed low titres (below 1 : 16) in both serum samples. As a whole 73 % of children had titres above 1 : 16.

Table 3 shows the clinical course of disease in 551 hospitalised children, which were EM-positive for rotavirus infection. Among these children 96 revealed mixed rotavirus infection with enteropathogenic bacteria. As shown in Table 3, the most frequent symptoms in the cases with sole rotavirus infection were — diarrhoea (65.0 %), fever (58 %) and vomiting (41.3 %). Quite often we diagnosed pharyngitis (28.7 %) and concurrent

Table 3. Clinical course of the disease in 551 hospitalized children with electron microscopic confirmation of rotavirus infection for the period February 1981—February 1986

	Rotavirus infection	Mixed rotavirus and bacterial infection
Diarrhoea	296 (65.05%)	82 (85.42%)
Fever	264 (58.02%)	61 (63.54%)
Vomiting	188 (41.32%)	44 (45.83%)
Intoxication	30 (6.59%)	17 (17.71%)
Convulsions	8 (1.76%)	2 (2.08%)
Pharyngitis	131 (28.79%)	29 (30.21%)
Acute respiratory disease	85 (18.68%)	10 (10.42%)
Total	455	96

acute respiratory diseases, catarrh of the upper respiratory tract, tracheobronchitis, and in separate cases pneumonia (18.6 %).

In the cases with mixed viral and bacterial infection the frequency of the clinical symptoms was similar: the most frequent was diarrhoea (85.4 %) followed by fever and vomiting. In the cases with mixed infection the percentage of children with diarrhoea and intoxication was higher. In one case, a 1-month-old hypotrophic baby, which was hospitalized in Sofia in 1984 a long term excretion of rotavirus was noticed. During the three months when the child was in the hospital, the faecal samples were EM-positive for rotaviruses and the electrophoretic analysis showed that rotaviruses from different samples had the same electropherotype.

From epidemiological point of view from particular interest are the epidemic outbreaks of rotavirus gastroenteritis. In the period 1984—1987 we studied the outbreaks in Shumen and Shumen district. During the first outbreak which occured in the winter season of 1984—85, the faecal samples of 47 hospitalized children contained rotaviruses with identical "short" electropherotypes such as shown on Fig. 1-1. During the second outbreak in the winter season of 1985—86 the rotaviruses with "short" electropherotypes were still prevalent and were found in the faecal samples from 31 hospitalized children, but in two children we found rotaviruses with "long" eledtropherotypes (Fig. 1-4). In one child a mixed rotavirus infection was

found caused by rotaviruses with "short" and "long" electropherotypes. In the winter season of 1986—87 already prevalent were rotaviruses with "long" electropherotypes which were found in 13 children while rotaviruses with „short" electropherotypes were detected in 3 children.

Discussion

We report of winter seasonality of the disease, which affects mainly children up to 3 years old. Similar data have been reported by other countries with temperate climate (Brandt *et al.*, 1983; Gurwith *et al.*, 1981). Concerning the clinical course of disease, other authors also reported that the most frequent symptoms were diarrhoea, fever and vomiting (Carr *et al.*, 1976).

The role of rotaviruses in other diseases is still unclear: there is a report of isolation of rotaviruses from the respiratory tract of children with pneumonia (Santosham *et al.*, 1983). In this respect from interest is the fact that concurrent respiratory symptoms — pharyngitis, acute respiratory diseases and in some cases pneumonia were noticed in rotavirus gastroenteritis in our country. On the other hand, from special interest are our results on the presence of respiratory diseases in all cases in which antigenically distinct rotaviruses also called atypical rotaviruses or pararotaviruses were found (Dimitrov *et al.*, 1983; Dimitrov *et al.*, 1986*c*).

During the five year study in Bulgaria we found rotaviruses with eight different electropherotypes. In a similar study in Australia (Rodger *et al.*, 1981) in 188 faecal samples rotaviruses with 19 different electropherotypes were found. The most frequent electropherotypes were prevalent for a period of 2—3 years. These results as well as the data from Houston, U.S.A. (Dimitrov *et al.*, 1984) allowed to expect a shift from the predominant rotaviruses with "short" electropherotypes such as found in Shumen, Bulgaria (Dimitrov *et al.*, 1987); the expected shift has occured in the winter of 1986—87.

With the help of electrophoresis we found mixed infections caused by rotaviruses of different electropherotypes.

The electrophoresis technique combined with silver staining of the RNA demonstrated typical rotavirus electropherotypes in the faecal samples of 22.12 % of the children tested in the winter season of 1986—1987. By electron microscopy, the average positive rate among samples during the winter seasons was 15.9 %. We consider that the electrophoretic analysis of the genome of rotaviruses is a reasonable test and it can be used independently for the clarification of the aetiology of rotaviruses. In contrast to EM and standard ELISA, the electrophoretic analysis could present information for the number, variability and persistance of rotavirus strains as well as the establishment of dual infections and other more detailed studies. On the other hand, one should not forget that the electrophoretic analysis is aimed at the detection of viruses only from the family *Reoviridae* while electron microscopy demonstrates all viruses with a characteristic morphology.

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Legend to the Figure (Plate XLI):

Fig. 1. Electropherotypes of the five most frequent rotaviruses found in Bulgaria (1—7) and eletropherotype of the standard simian rotavirus SA11.
The figure is a composite of photographs taken of several 10 % polyacrylamide gels stained with ethidium bromide, each of which contained SA11 as an internal marker.