CHARACTERISATION OF AN ECHOVIRUS TYPE 11' (PRIME) EPIDEMIC STRAIN CAUSING HAEMORRHAGIC SYNDROME IN NEWBORN BABIES IN HUNGARY

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Summary. – Echovirus 11' (prime) isolates from an epidemic of haemorrhagic syndrome in departments of obstetrics in Hungary have been characterised. The leading component of the clinical disease was carditis and its lethal outcome occurred in 13 newborn babies. Maternal immunity was found to be absent even in women of 41 years of age. The application of monovalent oral poliovirus type 1 vaccine prevented the progress of the epidemic within two weeks. Nevertheless, a serological survey among primovacinees of 3 – 15 months of age revealed that 20% of the babies seroconverted without clinical symptoms during the epidemic. Serological evidence showed that the echovirus 11' infection was unable to interfere with the efficacy of oral poliovirus vaccine (OPV), since seroconversion rates of primovaccinees did not differ significantly from those in the group seroconverted also to echovirus 11' during the vaccination campaign. A 440 nucleotide (nt) fragment of the 5'-non-translated region of 12 epidemic echovirus 11' isolates and 26 echovirus prototype strains was amplified by a nested reverse transcription-polymerase chain reaction (RT-PCR) and analysed using three different restriction endonucleases. The 5'-regions of the echovirus 11' isolates were found to be identical to each other but different from that of the prototype echovirus 11 (Gregory) strain. The results indicate that echovirus 11' isolates underwent genetic changes in the 5'-end and P1 region of the genome before the onset of the epidemic.

Key words: echovirus 11'; haemorhagic syndrome; newborn babies; epidemic; Hungary

Introduction

Non-polio enteroviruses may be responsible for several well-known clinical diseases (Brown, 1973; Lake *et al.*, 1976; Morens, 1978; Pruekprasert *et al.*, 1995; Hill, 1996). Even the diarrhoea may be associated with enteroviral infections in the case of newborn babies or immunosuppressed patients (Brown, 1973; Townsend *et al.*, 1982; Hill, 1996).

The appearence of "prime" enterovirus strains, echoviruses 6', 6' (Committee on Enteroviruses, 1957), 9' (Rosenwirth and Eggers, 1982) and 11' (Schmidt and Lennette, 1970), and coxsackievirus 24' (Schmidt and Lennette, 1970), has been observed in the fifties and sixties in several countries. These virus strains were shown to be non-neutralisable with antisera prepared against the prototype strains of the viruses. Their antigenic structure possessed epitopes absent from the prototype viruses. The "prime" strains, however, possessed all epitopes present on

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Abbreviations: EDTA = ethylene diamine tetraacetate; nt = nucleotide; OPV = oral poliovirus vaccine; RT-PCR = reverse transcription-polymerase chain reaction; SDS = sodium dodecyl sulphate

the surface of the prototypes; therefore, antisera prepared against the "prime" strains neutralised readily the prototype viruses (Schmidt and Lennette, 1970; Rosenwirth and Eggers, 1982; Gjoen *et al.*, 1996).

In Hungary, the last of the three country-wide epidemics was caused by a "prime" strain of echovirus 11 (echovirus 11') in 1989 (Dömök and Molnár, 1960; Dömök, 1981; Nagy *et al.*, 1982; Szirmai *et al.*, 1982; Dömök, 1985).

In 1988, an epidemic of uveitis caused by echovirus 11' has been reported from Siberia (Russian Federation), which had rather unusual virological properties (Lashkevich *et al.*, 1990). A comparison of echovirus isolates obtained from different sources indicated (M. Marennikova, personal communication) that the Siberian virus arrived to Hungary in 1989, but the molecular examination of the different Hungarian isolates showed that the virus has not been suffering major genetic changes after the onset of the epidemic.

The aim of this paper is the evaluation of epidemiological, virological, and molecular biological characterisation of the epidemic of haemorrhagic syndrome in Hungary in 1989 and its etiological agent. It was found that all isolates of the echovirus 11 strain were antigenically different from the prototype Gregory strain. The 5'-non-translated end of the viral genome was found to be also different in each isolate. The mass vaccination with monovalent OPV type 1 stoped the epidemic within one week.

Materials and Methods

Collection of epidemiological data. Hygiene network members of hospitals have been informed about the onset of the epidemic in July 1989. The infectologists of hospital epidemiology departments visited the foci of the epidemic, registered clinical and epidemiological data collected from the medical staff, nurses and patients, and occasionally participated at autopsies.

Collection of diagnostic samples. The majority of stool samples were taken by the personnel of departments of obstetrics and gynecology of hospitals in various regions in Hungary, and were transported to Budapest. Echovirus 11' isolates have been obtained in regional virology laboratories, and forwarded subsequently to the National Reference Laboratory, Budapest, for serotyping.

Stool and occasionally blood samples were taken from sick newborn babies and their mothers and sometimes from contact patients and nursing personnel. Autopsy samples were taken from the gut, lung and heart muscle. Stool samples and throat washings were taken from the mothers or contact persons, symptomless babies and their mothers, and hospital personnel.

Serum samples were not available from babies that died. Nevertheless, in a separate surveillance programme, the efficacy of the OPV campaign (Dömök, 1981, 1985) has been tested with the permission of the Chief Medical Officer of State the next year (1990). Paired serum samples were available for antibody testing from 297 children. The pre-vaccination sera were taken after September 11, 1989. The post-vaccination samples were taken after March 1, 1990.

Sample preparation. Stool samples, throat washings and pathological samples of gut, lung, and heart-muscle were homogenised (using sterilised sea sand in the case of autopsy material), and 10% suspensions were prepared using Medium 199. The suspensions were centrifuged at 15,000 x g for 15 mins in order to eliminate bacterial contamination. In case of residual contamination, the samples were treated with chloroform at 4°C according to the WHO guidelines (WHO/EPI/CDS/POLIO/90.1; Muir *et al.*, 1998). Serum samples were stored at -20°C until titration.

Media and tissue cultures. Primary to tertiary monkey kidney (MK) cells were prepared from *Cercopythecus aethiops* by standard procedure. MK and Vero cells were grown in Medium 199 supplemented with 10% foetal calf serum, penicillin (100 U/ml) and streptomycin (200 µg/ml). The isolation experiments were done on cultures in tubes or disposable 25 cm^2 flasks .

Virus typing and serum neutralisation tests were performed on disposable microplates (Greiner, Nunc or Flow). Twenty-five µl of serum dilution and 25 µl of virus suspension containing 100 –1,000 $TCID_{50}$ per well were used. Following 2 hrs of incubation at 37°C or overnight incubation at 4°C, 100 µl of Vero cell suspension (1 – 2 x 10^4 cells) was added. The plates were covered with adhesive tape so that the incubation could be performed accidentally without CO_2 incubator. The reading of all experiments was performed following 7 days of incubation at 37°C. Differences between serum neutralisation antibody titres were statistically analysed using the Student's *t*-test.

Immunisation of rabbits. Virus inocula were produced on monolayers of Vero cells in Medium 199. When the cytopathic effect of the virus became complete, the culture flasks were frozen and thawed thrice, the cell suspensions were collected and clarified by low speed centrifugation.

Groups of 3 – 4 rabbits were inoculated first intravenously then three times intramuscularly in weekly intervals. The animals were sacrificed 8 – 12 weeks after the beginning of immunisation. The sera obtained from the rabbits were pooled and freeze-dried or stored at -20°C (Berencsi and Nagy, 1973).

The rules issued by the Hungarian Academy of Sciences concerning the performance of animal experiments were followed throughout.

Viruses selected for molecular examination consisted of 12 isolates from the epidemic: 6 isolates from lethal diseases (1108, 1129, 1203, 1237, 1335, and 1337), and 6 isolates from clinically healthy children. Isolates 1135 and 1137 were obtained from the stool and lung tissue of the same child.

The following echovirus prototype strains (Melnick, 1996) were tested: E1 (pX+1/Farouk – no disease), E2 (p40/Cornelis – meningitis), E3 (pX+1/Morrisey – meningitis), E4 (p47/Pesascek –meningitis), E5 (p45/Noyce – meningitis), E6 (pX+2/D'Amori – meningitis), E7 (p46/Wallace – no disease), E8 (pX+3/Hall – unknown disease), E9 (p50/Hill – no disease), E11 (p50/Gregory – no disease), E12 (pX+2/Travis – no disease), E13DC (pX+4/Del Carmen – no disease), E14 (p57/Tow – meningitis), E15 (p77/Charleston – unknown), E16 (pX+8/Harrington – meningitis), E17 (pX+2/CHHE-29 – no disease), E18 (pX+3/Metcalf – diarrhoea), E19 (p78/Burke –diarrhoea), E20 (pX+3/JV-1 – fever), E21 (pX+2/Farina – meningitis), E24 (p8/DeCamp –diarrhoea), E25 (p8/Rosen JV-4 – diarrhoea), E26 (p6/

Coronel – no disease), E27 (p10/Bacon – no disease), E29 (pX+4/JV-10 – no disease), E31 (p5/Caldwell – meningitis), E33 (pX+1/Toluca-3 – no disease), where p indicates the number of passages following isolation or the receipt of the isolate (X + (1–8)). The coxsackie B5 prototype used for comparison was pX+5/Faulkner (mild paralysis). The original strains were obtained between 1958 and 1962 from Drs. M.K. Voroshilova (Moscow), J.L. Melnick (Houston), B. Labzoffsky (Toronto) and V. Vonka (Prague).

Extraction of viral RNA A hundred and sixty µl of serum or cerebrospinal fluid was mixed with 395 µl of lysis buffer (50 mmol/l Tris, 0.5% sodium dodecyl sulphate (SDS), 10 mmol/l ethylenediamine tetraacetate (EDTA), 50 mmol/l NaCl, pH 7.5) and 44 µl of proteinase K (10 mg/ml) (a modification of the techniques of Chapman *et al.* (1990) and Nicholson *et al.* (1994)). The mixture was then vortexed for 30 secs and incubated at 37°C for 1 hr, and 395 ml of water-saturated phenol and 160 µl of chloroform was added. The mixture was then again vortexed for 30 secs and centrifuged at 14,000 rpm for 15 mins in a microcentrifuge.The aqueous phase was collected and 480 µl of isopropanol was added to precipitate the RNA overnight at -20°C. The precipitate was pelleted at 14,000 rpm for 15 mins, washed with 70% ethanol by repeated centrifugation, vacuum dried, and finally dissolved in 8 µl of sterile distilled water just before RT-PCR.

RT-PCR was performed according to Diedrich *et al.* (1995). Primers for the nested PCR were derived from the sequences of the 5'-non-coding region which has been found to be highly conserved in all enteroviruses. The outer sense primer E1 (Chapman *et al.*, 1990) was 5'-CAC CGG ATG GCC AAT CCA-3', the outer antisense primer No. 3 (Diedrich *et al.*, 1995) was 5'-ATT GTC ACC ATA AGC AGC CA-3', the inner sense primer No. 1 (Diedrich *et al.*, 1995) was 5'-CAA GCA CTT CTG TTT CCC CGG-3', and the inner antisense primer E2 (Chapman *et al.*, 1990) was 5'-TCC GGC CCC TGA ATG-3'. The primers were obtained from Pharmacia or Promega Biotec.

Restriction fragment length polymorphism (RFLP) analysis. Five µl of the PCR products was digested at 37°C with 5 – 10 U of *Hpa*II, *Hae*III and *Alu*I restriction endonucleases (Sigma-Aldrich) using the buffers delivered by the manufacturer in a total volume of 20 µl at 37°C for 30 mins. The fragments were electrophoresed in 2% agarose (Pharmacia) gels, stained with ethidium bromide and photographed under UV light.

Results

Clinical and epidemiological data

The first case of lethal haemorrhagic syndrome occurred in eastern Hungary in the first week of July 1989. The clinical symptoms were fever (about 38°C, but hyperpyrexy in some patients), vomiting, diarrhoea and accidentally pneumonia. In some cases, the clinical symptoms developed after the discharge from the hospital. Altogether 87 clinical cases were registered in the first locality of the epidemic, and 5 of these newborns died (Fig. 1).

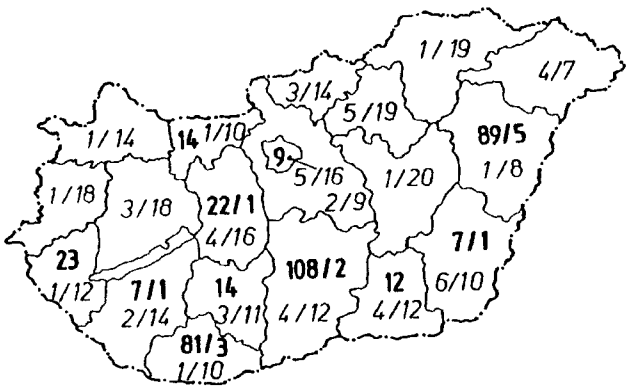

Fig. 1
Geographical distribution of echovirus 11' infections and clinical cases in Hungary in 1989

Numbers of clinical cases are indicated in bold. Nominators indicate numbers of total clinical cases, while denominators indicate numbers of cases with lethal outcome. All of the clinical cases occurred before the beginning of the serological survey of the primovaccinees. Numbers of seroconversion cases are indicated in italics. Denominators indicate numbers of children tested for seroconversion to echovirus 11' between September 1989 and March 1990. Nominators indicate numbers of seroconverted primovaccinees 3 – 15 months of age

The lethal infections were characterised by circulation disturbances and rapid progression. Liver function was severely affected. The haemorrhagic syndrome was observed in the majority of cases and led to the lethal outcome within 3 to 5 days.

The epidemic spread rapidly through the southern regions of the country within 3 weeks. The geographical distribution of the diseases is shown in Fig. 1.

Altogether 378 newborn babies and 8 infants suffered from the disease in 10 counties of Hungary. Lethal outcome was registered in the case of 13 patients in 6 regions. Twenty-nine of the newborns were premature and two of them died. Only 8 infants over four weeks of age became ill, and one of them died, too.

The epidemiological examination showed that no new clinical cases appeared after September 26, 1989, i.e. 2 weeks after the country-wide start of OVP among the children of 3 – 39 months of age. The total of children vaccinated between the 11th and 15th September was about 370 thousand (3.6% of the total population).

Virological examination of patients and contact persons

A total of 243 virus isolates were obtained from sick babies. Only one of them was typed as echovirus 11 using the standard enterovirus typing serum mixtures and procedures (Dömök and Molnár, 1960; Nagy and Takátsy, 1980; Nagy *et al.*, 1982).

Reovirus type 1 and coxsackievirus B3 were obtained in low number only. The enterovirus "nature" of the majority

Table 1. Reciprocals of neutralisation titres and neutralisation indices of various echovirus antisera

Rabbit antisera	Echovirus 11' isolates Nos.[a] TCID$_{50}$						Prototype echovirus 11 (Gregory) strain TCID$_{50}$
	1,203	1,237	1,108	1,129	1,337	1,335	
	30	100	500	3,000	10,000	10,000	300
	Reciprocals of neutralisation titres						
Anti-Gregory (No 1)	2,400	512	98	98	1,240	ND	6,360
Anti-Gregory (No 2)	2,400	780	256	128	186	ND	6,360
Anti-1108 (No.3)	16,384	32,768	65,536	65,536	1,240	ND	6,360
Anti-1337 (No.4)	65,536	65,536	16,384	8,096	16,384	16,384	1,240
	Neutralisation indices[b]						
	13.7	73.3	207	205.7	9.4	ND	0.44
	Reciprocals of neutralisation titres of heterologous unadsorbed rabbit antisera to echoviruses 1-33[c]						
	48-128	12-128	48-128	12-48	12-128	12-64	12-128

[a]Six epidemic isolates were compared in different doses. Comparable antibody titres 1:16,384 – 1:65,536) were obtained with homologous antisera (Nos. 3 and 4) with exception of the isolates 1129 (1:8,096) and 1337 (1:1,240). Therefore, the tests were not performed with different virus doses All virus-antibody mixtures were inoculated into duplicate wells of microtitre plates.

[b]Ratios of geometrical means of antibody titres of antisera Nos. 3 and 4 to geometrical means of antisera Nos. 1 and 2 Differences in neutralisation indices indicate the presence of antibodies directed to cellular receptors.

[c]Rabbit antisera Nos. 1-4 were tested against the prototype echovirus strains. The non-specific neutralisation titres obtained were comparable to those for the prototype (Gregory) strain of echovirus 11.

of isolates could be verified using the chloroform treatment at 4°C (WHO/EPI/CDS/POLIO, 1990). The presence of adeno- and rotaviruses could be excluded by agarose gel electrophoresis of the samples. (Dömök and Molnár, 1960; Berencsi *et al.*, 1978; Nagy *et al.*, 1980). The cytopathic effect of the isolates was characteristic with unusual, reticular character and some cells resistant to the viruses (data not shown). Twenty-six virus isolates originated from counties, where no one characteristic clinical case was registered.

The identification of the isolates was possible by use of rabbit antisera (Table 1) (Dömök *et al.*, 1960; Berencsi and Nagy, 1973). The two isolates selected for immunization (1108 and 1337) originated from the very first lethal cases. The two antisera (Nos. 3 and 4) produced after immunisation of groups of 2–3 rabbits were freeze-dried and tested in virus neutralisation test for the presence of antibodies to echovirus 11 prototype (Gregory) strain and to isolates from the beginning and end of the epidemic. These isolates originated from lethal or symptomless cases. Another two antisera (Nos. 1 and 2) produced against the prototype (Gregory) strain of echovirus 11 were tested also with the six different isolates from the epidemic.

Only the antisera to the patients' isolates gave antibody titres higher than 1:1,000 with the "prime" epidemic strains. No one of the prototype-specific echovirus antisera neutralised the isolates to titres higher than 1:128, except those specific for the prototype strain of echovirus 11, which gave neutralisation titres between 1:98 and 1:2,400.

On the other hand, both antisera prepared against the epidemic isolates 1108 and 1337 neutralised readily the prototype echovirus 11 strain (titres 1:1,240 – 1:6,360) . The results shown in Table 1 proved that the etiologic agent was a "prime" strain of echovirus 11 (Melnick, 1996). When the neutralisation titres were compared at different infection doses of different isolates, the neutralisation indices for prime-strain-specific and prototype-strain-specific antisera were 13.7 – 207.

When the isolate 1108 was tested against the four antisera shown in Table 1 with virus amounts of 30 to 300,000 TCID$_{50}$, the neutralisation indices were 3–28,000 (data not shown).

The cross-neutralisation antibody titres of rabbit antisera prepared to prototype echoviruses 1–33 were tested too. The six isolates from the epidemic showed cross-neutralisation titres of 1:12 to 1:128. These values were found to be identical to those obtained with the prototype echovirus 11 strain, indicating great antigenic differences between echoviruses 11 and 11'.

The weekly distribution of virus-positive samples is shown in Fig. 2. The number of virus-positive samples is also shown in a restricted area of the county. The epidemic was rapidly over (two weeks) affecting 7 hospitals in five cities. However,

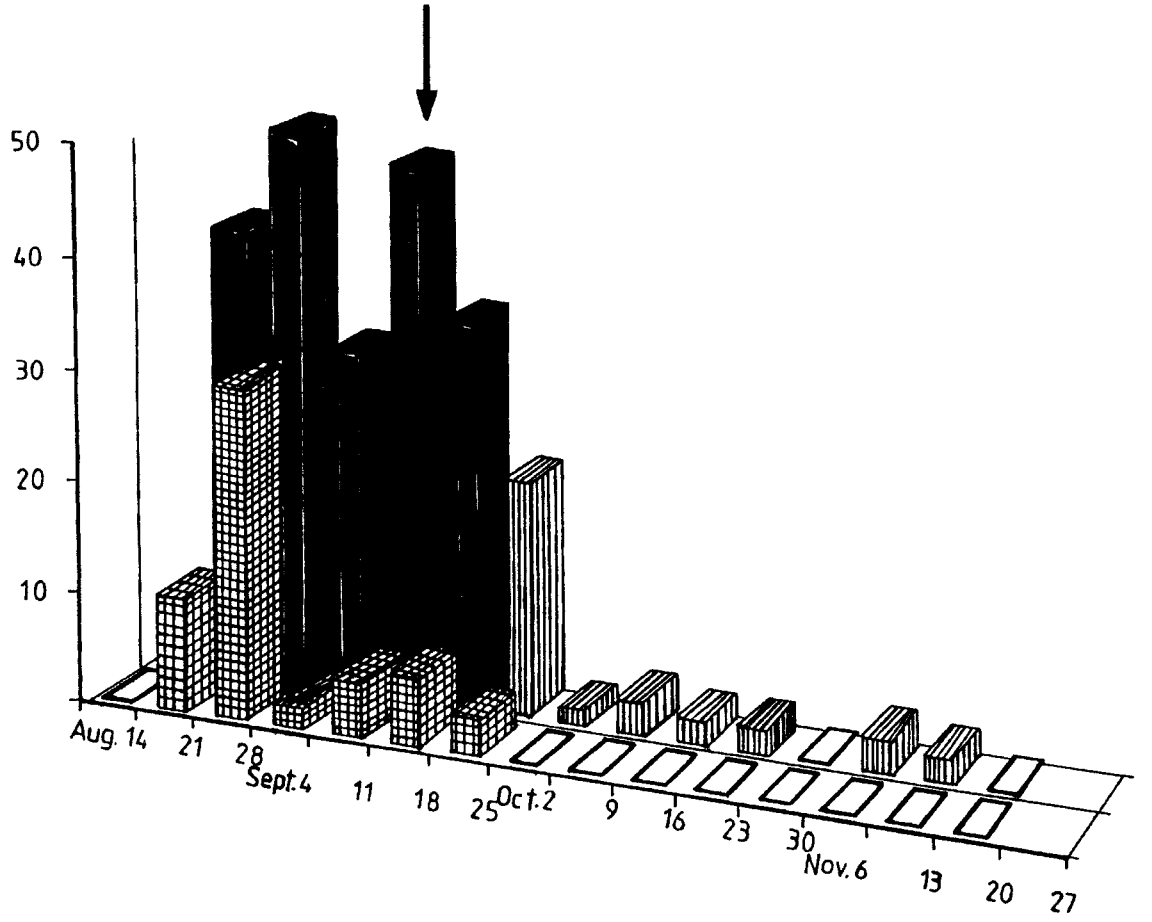

Fig. 2

Time course of the outbreak of echovirus 11' epidemic in Hungary between August 7 and November 27, 1989

Abscissa: date. Ordinate: number of echovirus 11' isolates per week. First row of columns: echovirus 11' isolates from the Baranya county, the most southern county of Hungary. Second row, black columns: total number of echovirus 11' isolates in Hungary. Second row, vertically striated columns: poliovirus type 1 isolates from healthy newborns and pregnant women. Vertical arrow date of the administration of OPV type 1 to age groups of 3 – 38 months

a protracted "tail" of sporadic cases can be seen. The total of virus-positive samples shows that the epidemic continued in other parts of the country.

Fig. 2 indicates also the beginning of the nation-wide vaccination with monovalent OPV type 1 after September 11, 1989. The latest echovirus 11'-associated clinical cases were observed in the third week of September 1989. The figure indicates also that exclusively poliovirus type 1 was isolated after September 18, 1989, except four isolates.

Seroconversion of clinically healthy children to echovirus 11' and poliovirus type 1

Altogether 557 serum samples were collected from healthy children of 3 – 15 months of age to determine the efficacy of the monovalent Sabin OPV about 6 weeks after the distribution of the third dose of the vaccine (February and March 1990). The number of seroconversions to echovirus 11' is shown in Fig. 1. Altogether 53 of 297 children seroconverted to echovirus 11' during the test period. Seven to 20 blood samples were tested from each county of Hungary. The figure indicates that at least every 6th child became seropositive to echovirus 11'. No region of the country remained free of the virus.

Both pre- and postvaccination samples from 260 children were available. As shown in Table 2, 79 –72% of the children seroconverted to the different poliovirus serotypes and 16.2% of them seroconverted to echovirus 11', too. For 11 children, there were no prevaccination sera available. The geometric means of echovirus 11 antibody titres were found to be comparable to those of poliovirus 1 antibodies (1:1,180 vs. 1:1,720). The geometric mean titres of poliovirus type 2

Table 2. Seroconversion of 260 primovaccinees vaccinated during and after the echovirus 11' epidemic in Hungary in 1989–1990

	Antibodies to			
	Poliovirus 1	Poliovirus 2	Poliovirus 3	Echovirus 11'
No. of seropositive children (% of 260)	196(75.4)	205(78.8)	188(72.3)	42(16 2)
Geometric mean titres	1:1720	1:430	1:183	1:1180
No. of echovirus 11' seropositive children with antibodies to poliovirus (% of 42)	29(69.0)	32(76.2)	29(69.0)	–
Geometric mean titres of echovirus 11' seropositives	1:388[a]	1:512[a]	1:152[a]	–
No. of seronegatives from echovirus 11' seropositive children (% of 42)	13(31)	10(23.8)	13(31)	–
Seronegative children to both poliovirus and echovirus 11' (% of 218 children [b])	51(23.4)	45(20.6)	59(27.1)	–

[a]Differences in seroconversion rates are not significant. The geometric mean titre of poliovirus type 1 group is more than fourfold lower than that of the control.
[b]None of the differences is significant (P >0.05)

and 3 antibodies were found to be lower. Seroconversion rates of echovirus 11' seropositive children were not found to be different from those for poliovirus serotypes (76.2 vs. 69.0%). However, the geometric mean titre for poliovirus type 1 was found to be more than fourfold lower (1:388) than that in the echovirus-unaffected babies (1:1,720). This difference, however, was not found to be significant either (P >0.05). No significant difference was observed between the rates of poliovirus type 1 seroconverted children with or without echovirus 11'- seroconversion (20.6 vs. 31.0% in Table 2).

Neutralisation index of an antiserum to the Siera strain of echovirus 11' adsorbed with human placental acetone powder was found to be 20,000 for the isolate 1108 as compared to the echovirus 11 prototype (Gregory) strain (A. van Loon, personal communication). The antiserum to the isolate 1108 gave a neutralisation index of 3,000 for the Russian echovirus isolates from an uveitis epidemic in Siberia as compared to the Gregory strain (M. Marennikova, personal communication; Lashkevich *et al.*, 1990, 1996). These data indicate that only a few neutralisation epitopes of the earlier echovirus 11 isolates were shared by the epidemic echovirus 11' strains. The lower neutralisation indices in the experiments presented here were caused probably by the presence of antibodies directed against tissue culture cell receptor proteins.

RFLP analysis of the 5'-untranslated region of echovirus 11' isolates

Nested RT-PCR was used to amplify a 440 nt (nt 163–603) sequence of the genome of the echovirus 11' isolates and the prototype (Gregory) strain of echovirus 11. The echovirus 11 sequence was obtained from the GenBank (Dahllund *et al.*, 1995). The RT-PCR products (amplicons) were subjected to RFLP analysis using *Hpa*II, *Hae*III and *Alu*I restriction endonucleses.

The results of the analysis indicated that all amplicons of the echovirus 11' isolates had identical restriction endonuclease patterns (Table 3): two *Hpa*II sites, two *Hae*III sites and one *Alu*I site. RFLP analysis of various enteroviruses is shown in Fig. 3.

Sixty different isolates of coxsackieviruses B2, B3, B4 and B5 obtained during 1995 and 1996 from patients suffering from different clinical diseases in Hungary and Bulgaria, and some poliovirus isolates obtained earlier in Bulgaria were also tested using *Hpa*II. All non-polio enterovirus isolates except 2 Bulgarian isolates of coxsackievirus B3 from the recent years had the same pattern as that of the echovirus 11' epidemic strains (data not shown).

The epidemic echovirus 11' strains unlike the prototype echovirus 11 (Gregory) strain contain at least two point mutations in the 5'-untranslated region of the genome. We

Table 3. RFLP patterns of RT-PCR amplicons (nt 163–603) of various echovirus serotypes

RFLP group	Number of sites			Echovirus serotype
	*Hpa*II	*Hae*III	*Alu*I	
1	2	1	1	1,2,4,5,13,16,17,18,20
2	1	1	1	3,26,31
3	2	1	2	7,21,11'
4	2	2	1	8,9,19,24,27,29 (and recent coxsackievirus B5 isolates)
5	2	2[a]	1	6 (D'Amori)
6	1	2	1	11 (prototype)
7	1	2	2	12,14,15
8	2	2	2	25

[a]The second site was located elsewhere in comparison to that present in RFLP groups 4, 6, 7, and 8.

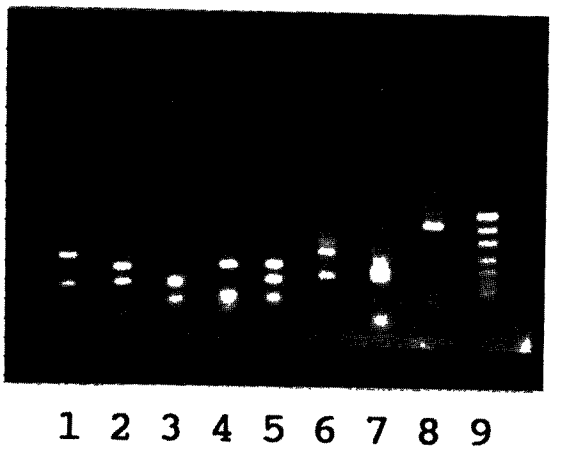

Fig. 3
RFLP analysis of products of products of nested PCR of various enteroviruses
Prototype coxsackievirus B5 (Faulkner), *Hpa*II (lane 1); echovirus 11' isolate 1108, *Hpa*II (lane 2); echovirus 11' isolate 1108 *Hae*III (lane 3); prototype echovirus 6 (D'Amori), *Hae*III (lane 4); prototype coxsackievirus B5 (Faulkner), *Hae*III (lane 5); coxsackievirus B5 isolate from 1995, *Alu*I (lane 6); prototype echovirus 12 (Travis), *Alu*I (lane 7); prototype echovirus 12 (Travis), undigested (lane 8); DNA size marker, pUC18 digested with *Hpa*II (633, 501, 404, 353, 242, 190, 147, 110, 89, 67, 34 (2x), and 26 bp) (lane 9).

included the epidemic echovirus 11' strains together with echoviruses 7 and 21 into one group (RFLP group 3) (Table 3). Recent coxsackievirus B5 isolates were found to be similar to echoviruses 8, 9, 19, 24, 27 and 29 (RFLP group 4.).

Discussion

Different enteroviruses have been shown to cause haemorrhagic syndrome with virus-induced necrosis of the liver in newborn babies and infants (Morens, 1978; Modlin, 1980; Modlin *et al.*, 1981; Mertens *et al.*, 1982; Reyes *et al.*, 1983; Pruekprasert *et al.*,1995; Lashkevich *et al.*, 1996). Peri- and myocarditis caused by the enterovirus may also contribute to the pathomechanism of the syndrome (Bell and Grist, 1970; Grist, 1972; Wenner, 1972; Rose, 1973; Mertens *et al.*, 1982; Szirmai *et al.*, 1982). In addition to direct viral damage, the impaired heart function results in the insufficiency of the blood and oxygen supply to the liver, which prevents the production of fibrinogen and other plasma proteins required for normal blood coagulation (Ohler, 1977; Mertens *et al.*, 1982). Both of them are thought to be responsible for the capillary leakage and haemmorrhagic symptoms preceding the lethal outcome of the disease (Blyuger *et al.*, 1971; Modlin, 1980; Mertens *et al.*, 1982).

Echovirus 11 has been isolated first by Ramos-Alvarez and Sabin (1954). Accumulation of haemorrhagic syndrome with lethal outcome due to a local epidemic caused by an antigenic variant of echovirus 11 has been observed first by Mertens *et al.* (1982) in a maternity unit in Germany.

We have examined epidemiological and virological data in the case of a country-wide epidemic caused by a "prime" strain of echovirus 11 (echovirus 11'), which resulted in the lethal outcome in 13 newborn babies. In spite of the fact that the circulation of echovirus 11' was shown to occur regularly in the country (I. Dömök and G. Berencsi, unpublished results), the population was found to be susceptible to the echovirus 11' antigenic variant. The absence of maternal immunity resulted in the disease in premature and newborn babies. Only 8 infants, more than one month of age, were found to be suffering from clinical symptoms indicating that the age of the patients contributed to the development of clinical symptoms. It has to be mentioned, however, that no intrauterine death has been registered in the epidemic (Johansson *et al.*, 1992).

In contrast to previous enterovirus epidemics (Dömök and Molnár 1960; Dömök 1985; Thorén and Widell, 1994; Sewyer *et al.*, 1994; Romero and Rotbart, 1995, 1997; Kim *et al.*, 1997), no accumulation of serous meningitis cases was observed.

Unfortunately, no specific therapy was available a decade before (Chuang *et al.*, 1993; Jantausch *et al.*, 1995). Antisera specific for antigenic variants of echovirus 11 were not available (Auvinen and Hyypiä, 1989, 1990; Miwa and Sawatari, 1994; Samuelson *et al.*, 1995; Lashkevich *et al.*, 1996). Therefore we can only suggest, that the antigenic differences found in our cross-neutralisation experiments in comparison to the Gregory strain of echovirus 11 were produced by genetic recombinations and/or mutations.

Nucleic acid detection techniques have been used earlier for diagnostic purposes in numerous studies (Abebe *et al.*, 1992; Glimaker *et al.*, 1992; Abraham *et al.*, 1993; Martino *et al.*, 1993; Muir *et al.*, 1993; Nicholson *et al.*, 1994; Romero and Rotbart, 1995; Arola *et al.*, 1996; Kim *et al.*, 1997).

A majority of the previous comparative molecular works have been performed with prototype strains of non-polio enteroviruses (Chatterjee *et al.*, 1988; Auvinen *et al.*, 1989; Auvinen and Hyypiä, 1990; Chapman *et al.*, 1990; Hyppia *et al.*, 1992; Zoll *et al.*, 1994; Pulli *et al.*, 1995; Samuelson *et al.*, 1995; Huttunen *et al.*, 1996; Romero *et al.*, 1997) . Comparative analysis of natural isolates is very rare (Sergeev *et al.*, 1994; Schweiger *et al.*, 1994; Romero *et al.*, 1995; Arola *et al.*, 1996; Gjoen *et al.*, 1996; Hovi *et al.*, 1996; Currey and Shapiro, 1997; Kim *et al.*, 1997; Knowles and McCauley, 1997; Romero *et al.*, 1997; Muir *et al.*, 1998).

Here the experimental evidence is presented that the 5'-non-translated region of the genome of the epidemic echovirus strains is different from that of the corresponding prototype strain. This difference and other minor biological differences such as the absence of meningitis, intrauterine death and uveitis might be explained by mutations or recombinations in other regions of the genome.

The nation-wide campaign with poliovirus type 1 monovalent OPV has prevented the circulation of the pathogenic echovirus 11'. No new isolates of echovirus 11' were found 14 days after the distribution of more than 370,000 doses of Sabin's type 1 vaccine to children between 3 to 39 months of age (Dömök, 1985). Similar interference has been described earlier, too (Shindarov *et al.*, 1979; Nagy *et al.*, 1982).

The seroconversion was tested in 260 primovaccinees (of 3 to 15 months of age in September 1989). The second blood sample has been taken in March 1990. Between these dates, 42 children (16.2%) seroconverted to the echovirus 11'.

The seroconversion rates to poliovirus type 1 of children seropositive for echovirus 11' did not differ significantly from those who had not been infected with a non-polio enterovirus. The geometric mean titre of poliovirus type 1-specific antibodies was shown to be more than fourfold lower than that of the children without contact with echovirus 11'. One may, therefore, conclude that the interference between the two viruses was asymmetric. Echovirus 11' was inhibited by OPV type 1, but echovirus 11' was unable to prevent the acceptance of Sabin's poliovirus type 1; however, it reduced the virus titre in children infected with both viruses.

The oldest woman, whose baby suffered from a lethal echovirus 11'-caused illness, was 41 years of age. The proportion of the Hungarian population susceptible to echovirus 11' was about 6 million in 1989. The number of symptomless infections among adults might be as high as 1 million before the onset of the OPV campaign.

The epidemiological data and the neutralization indices revealed that the main cause of diseases with lethal outcome was the infection of seronegative pregnants just before delivery (Lake *et al.*, 1976; Morens, 1978; Modlin, 1980; Modlin *et al.*, 1981; Reyes *et al.*, 1983; Johansson *et al.*, 1992; Jantausch *et al.*, 1995; Pruekprasert *et al.*, 1995).

It should be stressed that an RFLP analysis of natural echovirus isolates in comparison to prototype echovirus strains has not been published before.

The RFLP analysis of the 5'-end IRES elements of the epidemic echovirus isolates indicated similarities to echoviruses 7 and 21. Sequence data will be required to prove whether the biological differences have been produced by a recombination event affecting both P1 region and the 5'-end IRES element, or the recombination modifying the neutralisation epitopes and other changes detected by RFLP analysis and influencing the pathogenicity are of different nature.

Neutralisation titres obtained with mixtures of antisera Nos. 1 and 2 (Table 1) with antisera specific for echovirus prototypes 7 and 21 or coxsackievirus B5 were not found to increase neutralisation titres obtained with echovirus 11' isolates (experiments not shown), therefore the combination of neutralisation epitopes seem to be more complex than simple recombination events between these enteroviruses.

One has to mention that the "classical" RFLP pattern of the 5'-non-translated region of the echovirus 11 prototype (Gregory) strain could be found in two Bulgarian natural coxsackievirus isolates (data not shown). This indicates, that the genetic evolution of enteroviruses is a complex process.

It has been found (A. Szendroi, unpublished results) that some regions of epidemic echovirus 11' isolates were closely related to those of coxsackie B5 and swine vesicular disease viruses.

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