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Relative abundance of enterovirus serotypes in sewage differs from that in patients: clinical and epidemiological implications

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SUMMARY

One thousand one hundred and sixty-one non-polio enterovirus strains, isolated during regular screening of Finnish sewage specimens, were analysed for serotype distribution seasonally through 20 years, and the findings were compared with similar data based on 1681 clinical isolates. Coxsackievirus B4 (CBV-4), CBV-5, echovirus 11 (EV-11), EV-6, CBV-2 and CBV-3 were the most common serotypes in sewage, whilst CBV-5, EV-11, coxsackievirus A9, EV-22, CBV-3 and EV-30 were the most common clinical isolates. Reasons for the differences are not known but several explanations are possible. Seasonal variation of enterovirus occurrence in both sources showed an expected peak in the autumn with a trough in the spring. The occurrence of enteroviruses was closely correlated with monthly recordings of mean relative humidity. A further observation concerning the clinical specimens in Finland was the relative excess of some serotypes, such as echovirus 22 and coxsackievirus A9, and paucity of others, for instance, echoviruses 4 and 9, when compared to published data from other countries. This is consistent with the idea of geographically restricted circulation of enteroviruses.

INTRODUCTION

Enteroviruses comprise altogether 67 serotypes of human pathogenic viruses including polioviruses (PV), coxsackieviruses of subgroups A and B (CAV, CBV), echoviruses (EV) and the newer enteroviruses of serotypes 68–71. Effective vaccines against poliomyelitis have been used worldwide for decades and the World Health Organization (WHO) is aiming at global eradication of the disease by the year 2000. In addition to poliomyelitis, enteroviruses cause a large variety of different clinical symptoms ranging from non-specific acute infections to serious diseases involving the central nervous system (CNS), heart and other tissues [1, 2].

Information on the relative abundance of different enterovirus serotypes circulating in human populations is mainly derived from virus isolation data based on specimens collected from hospitalized patients. A large study comprising 24000 enterovirus isolations made in different parts of USA in 1969–81 revealed that the 15 most common serotypes comprised about 75% of all isolates [3]. The most prevalent serotypes varied greatly from year to year. However, because most enterovirus infections are subclinical [2], it is not clear, how accurately these data really reflect the relative abundance of circulating enterovirus serotypes. Prospective population-based surveys of virus excretion [4] would produce more reliable data but are tedious and expensive.

Analysis of sewage for presence of viruses [5] provides an alternative approach that would complement the clinical data because individuals infected shed virus into faeces, and hence into sewage, usually for several weeks [2]. Enteroviruses survive well in sewage and can be readily isolated from concentrated specimens [6].

This institute has been screening sewage samples in Helsinki since the early 1970s mainly to detect the circulation of wild poliovirus in the population. This

is possible because only inactivated poliovirus vaccine is used in Finland in regular immunizations. During this survey data have been collected on the circulation of other enteric viruses in the population. Part of this data mainly from the 1970s has been published earlier [7, 8]. Here we report the patterns of occurrence of different non-polio enterovirus serotypes in the sewage from 1971–92 and correlate these observations with enterovirus isolation from clinical samples in Finland and elsewhere.

METHODS

Cell cultures

Two continuous cell lines of monkey kidney origin, GMK and Vero, as well as primary human amnion cells (HA) and low passage fibroblast cultures derived from human embryonic tissues (HES) were used throughout the study period. Occasional specimens were not inoculated into the HES cells as these cells were not always available. Cells were propagated in Minimal Essential Medium (MEM) supplemented with 10% newborn of foetal calf serum (FCS), and regularly screened for mycoplasma contamination using the fluorescent DNA chelating dye (Hoechst) technique.

Sewage specimens

The specimens comprised raw sewage collected during 1971–92 at the inlets of two major sewage cleaning plants in Helsinki, at 1 or 2 week intervals throughout the year. Only a small number of specimens were obtained in 1975 and 1984 and the data for these years are excluded from the analysis.

Usually 1 l of sewage was sampled, kept cool during transport and arrived in the laboratory within 24 h. The specimens were concentrated about 100-fold, initially using the alginate filter method [7] and after 1976, the simple two-phase separation-based method described in detail previously [9]. The concentrates were extracted with chloroform before inoculation into cell cultures.

Virus isolation and serotyping

Monolayer cultures of different cell types in 50 ml plastic flasks were inoculated with 0·5 ml of the sewage concentrate as described in detail previously [9]. After 1 h adsorption at 36 °C the inoculum was removed and replaced by 5 ml of maintenance medium (MEM + 2% FCS). Cultures with cytopathic effect were subcultured to homologous cells and virus isolates were identified with pools of commercial monotypic rabbit antisera. For neutralization typing, aliquots of the test isolate containing 10–100 $CCID_{50}$ (cell culture ID_{50}) of the cytopathic agent were incubated for 30 min at 36 °C with pretested pools of monotypic antisera, and inoculated in fresh cultures of homologous cells. The pools were designed in such a way that the results usually revealed the serotype unequivocally. Ambiguous results were confirmed with individual monotypic antisera. Because a specimen may contain several different viruses, isolates were subcultured in heterologous cells and, in principle, all strains showing individual replication pattern were identified. On average, four isolates per specimen were serotyped. Even so, it is possible that we detected only the most abundant serotypes. One blind subculture was made of all initially inoculated cell types before scoring a specimen negative and involved a minimum total incubation time of 3 weeks.

Source of data on clinical isolates

Since 1970, this institute has collected monthly reports of all virological laboratories that carry out virus isolations in Finland for diagnostic purposes. The findings were registered in the files according to the date of identification, rather than on the basis of specimen collection. Exact number of specimens examined for presence of viruses could not be obtained for the entire period but, judged from the information obtained from the laboratory chiefs, about 1500 faecal specimens were cultured annually in Finland for virus detection during the study period. Individual records for associated clinical symptoms were not available. Filed summaries of laboratory records were examined for monthly occurrence of individual serotypes from 1971–92, excluding 1975 and 1984.

RESULTS

Relative rates of occurrence of different serotypes

Altogether 1036 sewage specimens were analysed for cytopathogenic viruses from 1971–92, excluding 1975 and 1984. Seven hundred and eight specimens yielded at least one non-polio enterovirus serotype, 172 contained adenoviruses and 48 reoviruses. Apart from the previously reported wild poliovirus and vaccine-

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Table 1. *Proportions of most common enterovirus serotypes isolated from sewage or patients in Finland in 1971–92**

Enterovirus serotype	Occurrence (%) Sewage (n = 1161)	Patients (n = 1681)	P/S†
Coxsackievirus B4	18·7	6·2	0·33
Coxsackievirus B5	17·3	14·9	0·86
Echovirus 11	16·6	12·1	0·73
Echovirus 6	15·2	4·5	0·30
Coxsackievirus B2	11·1	4·6	0·41
Coxsackievirus B3	7·8	7·3	0·94
Coxsackievirus B1	3·5	2·7	0·77
Echovirus 22	2·7	8·0	2·96
Echovirus 3	2·6	1·3	0·50
Echovirus 30	1·3	6·7	5·15
Echovirus 25	0·9	1·3	1·44
Echovirus 7	0·8	1·3	1·62
Coxsackievirus A9	0·6	8·6	14·33
Echovirus 9	0·4	3·9	9·75

* 1975 and 1984 are excluded.
† P/S, 'clinical significance index' (ratio of relative frequencies in patient and sewage specimens).

derived strains isolated in 1984–5 [9, 10], 1161 non-polio enterovirus strains were serotyped. Unidentified cytopathogenic agents comprised < 5% of all isolates in any of the study years. Altogether, 24 different enterovirus serotypes were represented. The six most common serotypes, CBV-4, CBV-5, EV-11, EV-6, CBV-2 and CBV-3, comprised 87% of all typed isolates (Table 1). At the same time, 1681 enterovirus strains belonging to as many as 43 different serotypes were isolated from clinical specimens in Finland. The most striking difference between the occurrence of different serotypes was the relatively much greater abundance of CAV-9 among the clinical isolates (Table 1). Echovirus serotypes 9, 22, and 30 were also more frequently found among the clinical isolates. The calculated ratio of occurrence rates in the two sources, a 'clinical significance index', showed a wide variation with the extremes being coxsackievirus A9 (14·3) and echovirus 6 (0·29) (Table 1).

Annual variation of relative frequencies

Annual variation of the occurrence of individual serotypes was assessed on the basis of the proportion of a given serotype of all typed isolates per calendar year. In all but one of the 20 years studied, one or more serotypes occurred in an epidemic pattern, i.e. comprised at least 20% of all isolates (3) made from the sewage specimens. A similar epidemic pattern of occurrence was also seen among the clinical isolates, but the most frequently found serotype was identical with that in the sewage specimens in only 4 out of 20 years studied. Occurrence of the most common serotypes during two selected periods is shown in Table 2. Echovirus 22 appears to be found in both types of specimen relatively more frequently after 1985 than before. However, in early 1970s, EV-22 was a rather common virus and ranged up to 10% of all clinical isolates in some years (not shown).

Seasonal variation of enterovirus infections

Enteroviruses were isolated from both sewage and clinical specimens during all seasons but were more frequently encountered in the autumn, as reported before for clinical specimens in other countries with temperate climate. The proportion of enterovirus-positive sewage specimens peaked in September followed by a high level tailing until January with the nadir being observed in May (Fig. 1). The nadir of enterovirus isolations from the clinical material was also observed in May and a relatively sharp peak was seen in October. The increase of the proportion of positive clinical specimens after the nadir occurred with an about 1 month delay as compared to that of sewage specimens and coincided well with the increase of the mean relative humidity in Southern Finland (Fig. 1). On the other hand, the relatively longer persistence of enteroviruses in sewage was parallel to the elevated level of relative humidity in the winter months.

Monthly occurrence of individual serotypes in the sewage, when assessed for the whole 20 years period, generally followed the overall pattern shown in Figure 1, but the degree of variation among serotypes showed some differences. Coxsackievirus B5 appeared to have two peak seasons, one in October and the other in February. In general, peaks in the monthly distribution of individual serotypes isolated from the clinical specimens followed those derived from the sewage specimens but, for instance, the second peak of coxsackievirus B5 was not seen in the clinical specimens. On the other hand, the pattern of monthly occurrence of, for instance, echovirus 6 in the clinical specimens followed fairly well that from the sewage, with an approximately one month delay. Epidemic

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Table 2. *Annual occurrence of some common enterovirus serotypes in sewage and clinical specimens in selected years in Finland**

Serotype†		1977	1978	1979	1980	1981	1985	1986	1987	1988	1989	1990	1991	1992
Coxsackie B2	S	**10**	4·0	7·0	**22**	**31**	10	3·0	**14**	4·0	8·0	**11**	12	**14**
	C	13	11	**7·0**	3·0	1·8	3·1	1·9	1·1	1·9	6·8	6·7	2·6	0
Coxsackie B3	S	5·0	**27**	**18**	10	0	5·0	1·4	7·0	4·0	0	3·0	12	10
	C	4·2	3·8	**5·3**	4·8	**8·9**	4·1	11	11	1·3	0	2·2	**25**	6·1
Coxsackie B4	S	**39**	6·0	11	5·0	5·0	**24**	**44**	**17**	**20**	**18**	**23**	**16**	5·0
	C	**13**	0	3·5	4·8	1·8	3·1	**15**	4·0	3·8	2·7	9·0	2·6	1·5
Coxsackie B5	S	**34**	**15**	7·0	**13**	10	**21**	1·4	13	**8·0**	**17**	6·0	20	19
	C	**21**	**13**	1·7	3·8	**11**	**21**	2·9	**13**	**7·7**	***16***	**10**	2·6	**11**
Echovirus 6	S	2·5	**31**	**16**	2·0	**37**	10	8·0	3·0	**59**	**50**	**11**	9·0	2·0
	C	0	1·9	0	2·8	3·6	6·2	5·8	3·4	***19***	12	2·2	1·3	6·1
Echovirus 11	S	7·0	4·0	**30**	**44**	**16**	**12**	**22**	**15**	3·0	6·0	**30**	**19**	0
	C	4·2	0	***68***	**25**	8·9	***30***	**15**	***22***	2·6	4·1	***25***	2·6	4·6
Echovirus 22	S	0	2·0	0	0	0	2·0	0	13	1·3	0	3·0	1·0	**28**
	C	2·1	1·9	1·8	0	3·6	**6·2**	7·8	**17**	**12**	**9·6**	**11**	**14**	**15**

* The numbers are percentages of all identified enterovirus strains per each year; three most common serotypes of the year, if among the indicated ones, are in bold face; the most common serotype of the clinical specimens in a given year is in italics, if among the indicated ones.

† S, isolates from sewage; C, clinical isolates.

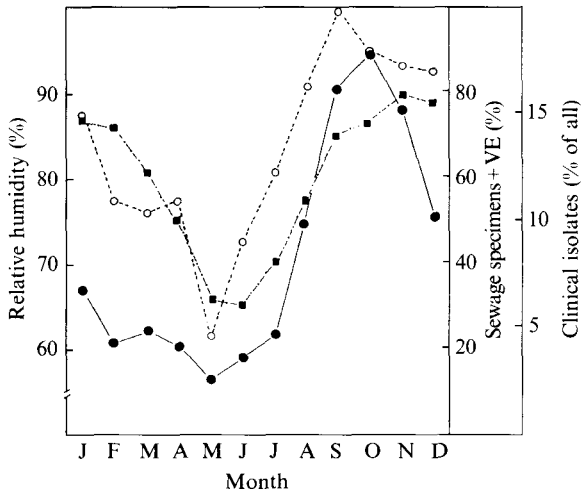

Fig. 1. Seasonal covariation of enterovirus circulation and relative humidity. Months are indicated by their first letters. Relative humidity values (■) are from published statistics for Helsinki (11). Enterovirus isolations from clinical specimens (●) represent monthly proportions of all isolations. Presence of enteroviruses in sewage (●) is indicated by proportion of studied specimens yielding one or more enterovirus isolate. The data on enteroviruses are presented cumulatively for the 20 study years.

activity of individual serotypes within shorter periods did not always strictly follow the seasonal pattern. An epidemic period of a given serotype occasionally continued over more than a single season as indicated for EV-6 in Table 3.

DISCUSSION

Enterovirus infections are very common all over the world and appear endemic or epidemic in most populations studied. Regular personal and food hygiene does not block virus circulation efficiently, most likely because the transmission pattern of infection appears to be through both faeco-oral and respiratory routes. Enterovirus infections are known to occur in communities enriched in susceptible individuals such as newborn wards and children's day care centres [1, 2]. Unlike influenza viruses, enteroviruses only exceptionally cause pandemics [2] but tend to be geographically restricted in transmission [12]. Since most enterovirus infections are subclinical or cause only minor non-specific symptoms, data based on isolates from hospitalized patients does not necessarily reflect overall circulation of different enterovirus serotypes in human populations. The latter is becoming more interesting along with accumulating evidence for a role of coxsackie B and other enteroviruses infections in the pathogenesis of insulin-dependent diabetes [13].

Interesting differences were seen in this study between the clinical isolates and those from sewage in the relative frequency of different serotypes in the 20 years that were studied. Data on sewage isolates were based on results of our laboratory only while those on the clinical specimens were also derived from

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Table 3. *Virus serotypes recovered from sewage specimens in Helsinki in 1988–90*

	Presence (+) or absence (−) by year and month*																																	
	1988												1989												1990											
Serotype	J	F	M	A	M	J	J	A	S	O	N	D	J	F	M	A	M	J	J	A	S	O	N	D	J	F	M	A	M	J	J	A	S	O	N	D
Coxsackie																																				
B1	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	−	+	−	−	−
B2	−	−	−	−	−	−	−	+	−	−	+	+	−	−	−	−	−	−	+	+	−	−	+	−	+	+	−	−	+	−	−	+	+	+	−	+
B3	+	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	+	−
B4	−	+	−	−	−	−	+	+	+	+	+	−	+	+	−	−	+	−	−	+	+	+	+	+	+	−	−	+	−	+	+	+	+	+	−	+
B5	+	+	−	−	−	−	+	−	+	+	−	−	−	+	−	−	−	−	+	−	+	+	+	−	−	−	+	−	−	−	−	−	−	−	−	+
Echovirus																																				
3	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−
6	−	−	−	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−	+	+	+	−
9	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−
11	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	−	−	−	−	−	−	+	+	+	+	+	+	+	+
12	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−
21	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
22	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	+	+	−	−	−	−
25	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
30	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Adenovirus																																				
1	−	−	+	+	−	−	−	−	−	−	−	+	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	+	−
2	−	−	+	+	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	+	+	−	−	−	−	−	−	−	−	−	−	−	−	+
3	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
5	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	+	−	−	−	−	−	−	+	−	−	−	−	+	−	−	−	−	−	−	+
Reovirus																																				
2	−	+	−	−	−	+	−	−	−	−	−	−	−	−	+	+	−	+	−	−	−	+	+	+	+	+	+	−	−	−	−	+	−	−	−	−
3	+	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	+	−	−	+	−	−	−	−	−	−	−

* At least four sewage specimens were analysed per month. The month is scored positive if at least one of them contained the indicated virus.

Table 4. *Proportions of most common serotypes among enterovirus strains isolated from patients in Finland, in USA and in WHO surveys*

Serotype	Occurrence (%)*		
	Finland (n = 1678)	USA (3) (n = 23813)	WHO (14) (n = 41540)
Coxsackievirus B5	14·9	8·7	9·4
Echovirus 11	12·1	12·2	6·6
Coxsackievirus A9	8·6	4·5	5·6
Echovirus 22	8·0	< 1·6	1·4
Coxsackievirus B3	7·3	4·5	7·8
Echovirus 30	6·7	6·8	5·6
Coxsackievirus B4	6·2	4·6	5·4
Coxsackievirus B2	4·6	4·8	6·5
Echovirus 6	4·5	5·5	8·7
Echovirus 9	3·9	11·3	10·9
Echovirus 18	3·2	< 1·6	1·9
Coxsackievirus B1	2·7	1·6	2·8
Coxsackievirus A16	2·0	< 1·6	3·0
Echovirus 7	1·4	3·0	3·5
Echovirus 3	1·3	3·2	1·7
Echovirus 4	0·8	6·3	3·6

* Source of data shown by reference number in parentheses.

several other laboratories using partially different sets of cell cultures for enterovirus isolation. In addition, sewage was analysed mainly in the Helsinki region which represents only one fifth of the population of Finland while the clinical specimens were isolated from the whole country. However, this cannot explain all the differences between the two types of specimen since, for instance, CAV-9 was readily isolated from clinical specimens in our laboratory, but was rarely found in the sewage.

Large variation was seen between serotypes in the arbitrary serotype-specific 'clinical significance index', calculated from the relative proportion of a given serotype among the clinical isolates over that in sewage. Several explanations for this variation can be envisaged: it may be (i) due to inherent differences in the general virulence of different serotypes, (ii) based on differences in the mean amount of virus excreted by an infected person, or (iii) a consequence of different rates of inactivation in the environment. There is prior evidence for the first alternative, for instance, concerning CAV-16 thought to result in symptomatic infection almost regularly [1]. The last alternative was not supported by our pilot experiments where stock solutions of CAV-9, CBV-4 and EV-6 were diluted in a faecal suspension and subsequently incubated in raw sewage for 2 weeks at 4 °C. Incubation in sewage did not affect viral infectivity (T. Hovi, M. Stenvik, unpublished).

Annual variation of the relative abundances of different serotypes in the clinical specimens corroborated previously published results [3, 14]. Serotype distribution of isolates derived from the sewage followed poorly that of the clinical specimens. This suggests that screening of sewage for enteroviruses could only rarely unequivocally disclose the serotype(s) concurrently circulating in the corresponding human population, and possibly causing epidemic disease. Sewage screening has been shown to be useful in assessing the extent of poliovirus outbreaks and consequences of vaccination campaigns in the absence of regular immunization with live poliovirus vaccine [9, 10]. Application of recombinant cell lines with high specificity for poliovirus replication [15] might make screening an even more beneficial tool in poliovirus surveillance.

Seasonal variation of enterovirus isolations was as expected and followed that of the relative humidity. This association may have occurred by chance but it is in agreement with laboratory data on virus survival at different humidities [16, 17]. It is conceivable that relative humidity could influence the inter-host transmission of enteroviruses rather than the quantity of virus shedding. The relative humidity and clinical

isolation curves diverged from each other in November, which could result from the fact that physical contacts between people that facilitate virus transmission are likely to be less frequent at colder temperatures during the winter months. Peak season of the bulk of clinical isolations took place somewhat later than that of sewage isolations, as could be expected from the long incubation period of clinical diseases [1, 2] on one hand, and from the slightly misleading mode of time scores in the files, on the other. Reasons for the longer persistence of elevated isolation rate in the sewage material as compared with the clinical one are not clear. Shedding of enteroviruses may continue for several weeks or perhaps months after the onset of the disease [2]. It is possible that the observed relative tailing of isolations from the sewage specimens reflects this phenomenon.

It is noteworthy that the distribution of the different serotypes in the Finnish clinical specimens differs from that in large long-term surveys from other countries [3, 14]. Serotypes relatively more common in Finland included echovirus 22 and coxsackievirus A9 while, for instance, echovirus types 4 and 9 were relatively rare in Finland (Table 4). Although effects of different laboratory techniques cannot be fully excluded, this observation may mean that a region-typical collection of enterovirus serotypes, and perhaps genotypes within each serotype, is circulating in Finland. Variations from the general pattern have also been reported by others [18].

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