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Stability of Human Enteroviruses in Estuarine and Marine Waters

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Studies of the effects of temperature and salinity on the survival of three enteric viruses (poliomyelitis type 1, echovirus-6, and coxsackievirus B-5) under controlled laboratory conditions and in situ indicate that temperature rather than salinity is the critical factor affecting their stability, in that the higher the temperature the more rapid was the loss of viral infectivity. In the laboratory studies, all three viruses were quite stable at 4°C, with infectious virus still detectable after 46 weeks of incubation. In situ studies on virus survival in free-flowing estuarine or marine waters showed that, although the viruses were more labile in natural waters than in the laboratory studies, they persisted for several months, in some cases during the winter months. At all temperatures and salinities, coxsackievirus B-5 was the most stable, echovirus-6 was intermediate, and poliovirus 1 was the least stable of the viruses tested.

There is growing concern over the discharge of human enteric viruses into not only fresh waters but also into estuarine and marine environments where they could present health hazards in areas that are used either for recreational purposes or from which shellfish are harvested for human consumption.

The enteroviruses are introduced into the sea by means of inflowing polluted rivers and by offshore sludge-dumping practices, and, in fact, human enteroviruses have been isolated from both marine (4) and estuarine (9) waters and from shellfish (6-8, 11).

Although several studies on the stability of enteroviruses in seawater have been reported in recent years (results reviewed in 1, 2), the results presented are sometimes contradictory and probably reflect the complex nature of virus inactivation processes in natural waters. This study is concerned with determining the effect of salinity and temperature on the survival of selected enteroviruses under controlled laboratory conditions and in situ, i.e., when exposed to free-flowing marine and estuarine waters.

MATERIALS AND METHODS

Viruses. Viruses from each of the three major enterovirus subgroups were used: poliovirus type 1 (Mahoney), coxsackievirus B-5 (Faulkner), and echovirus-6 (D'Amori). All virus strains were obtained from the Reference Reagents Branch of the National Institute of Allergy and Infectious Diseases, Bethesda, Md.

Cell cultures. A continuous line of African green monkey kidney cells (BGM line), reported to be susceptible to a wide range of enteroviruses (3), was used for both the preparation of virus stocks and for the virus assays. The cells were kindly supplied by D. R. Dahling of the Environmental Protection Agency, Cincinnati, Ohio, and were propagated with minimum essential medium prepared with Earle salts (EMEM) and supplemented with 10% fetal calf serum.

Virus assays. Confluent BGM cultures were grown in Falcon tissue culture dishes (60 by 15 mm). Serial 10-fold dilutions of virus (0.1 ml of inoculum per dish, two dishes per dilution) were adsorbed to the monolayers for 1 h at 37°C, after which 3 ml of an overlay medium consisting of equal volumes of 2% agarose and a 2× concentration of EMEM was added. After solidification, the overlay was covered with 3 ml of EMEM containing 2% fetal calf serum, and the cultures were incubated at 37°C in an atmosphere of 5% CO_2 and 95% air. After 48 (poliovirus 1) or 72 h (coxsackievirus B-5 and echovirus-6) of incubation, 2 ml of full strength (40%) formalin was added to each dish and allowed to stand for 15 min. The agarose and formalin were then decanted from the flasks, and the remaining cell sheet was stained for 2 min with 1% methylene blue, after which the plaques were counted. Results are expressed as the $\log_{10}$ of the number of plaque-forming units (PFUs) per ml.

Virus survival studies. For the controlled laboratory studies, synthetic seawater was prepared by the addition of Marine Mix (Utility Chemical Co., Paterson, N. J.; composition described in their technical bulletin no. 156) to glass-distilled water. The water was adjusted to the desired salinity by means of a salinity meter (model 33, Yellow Springs Instrument Co., Yellow Springs, Ohio); the salinities used were 10, 20, and 34 parts per thousand (ppt). The solutions were sterilized by autoclaving, and the pH was adjusted to 7.5 to 8.0 if necessary.

Replicate flasks, containing 100 ml of each of the various salinity waters, were seeded with 10^5 to 10^6 PFUs of the respective viruses per ml. Virus stocks were centrifuged at 5,000 × *g* for 10 min to remove cellular debris before their inoculation into the test flasks or dialysis bags. Four incubation temperatures were used (4, 15, 25, and 37°C) with the flasks immersed in shaker water baths operated at 150 cycles per minute. Samples were taken immediately after adding the virus (time 0) and at 7-day intervals thereafter until viable virus was no longer detectable in the undiluted samples.

The in situ survival studies were conducted at the University of Delaware Marine Laboratory, Lewes, Del., and at the State of Maryland Natural Resource Institute Laboratory at Solomons, Md. Dialysis bags, fitted with neoprene stoppers with a sampling port, were filled with autoclaved water samples (approximately 250 ml each) taken at the two locations. The samples were then seeded with 10^5 to 10^6 PFUs of the respective viruses per ml and immersed in plastic tanks which were continuously charged (6 to 7 liters per min) with free-flowing marine (salinity of 26 to 28 ppt at Lewes) or estuarine (salinity of 8 to 10 ppt at Solomons) waters. The sampling regimen was the same as that used for the controlled laboratory studies described above.

RESULTS

Laboratory studies. Although not a natural temperature for estuarine or marine waters, we included an incubation temperature of 37°C in our studies as a control to determine the lability of these viruses in water at the normal incubation temperature for most biological assays. No results are given in the ensuing tables because no viable virus was detectable with any of the viruses at any salinity after 7 days of incubation at this temperature.

Poliomyelitis virus. The inactivation rates for poliovirus type 1 incubated at 4°C are shown in Table 1. At a salinity of 10 ppt, only a one-log reduction in viral infectivity occurred within 8 weeks, and it took 18 weeks for a 99% loss in viability. Similar findings were made with the virus suspended in salinities of 20 and 34 ppt in that a 2-log drop in titer occurred gradually during the first 12 weeks of incubation. Infectious virus was still detectable after 40 weeks of incubation at all salinities.

At 15°C, poliovirus 1 was inactivated more rapidly than at 4°C, and the salinity used did seem to have an effect in that the virus seemed to lose infectivity at a faster rate at salinities of 20 and 34 ppt (no infectious virus was detectable after 20 weeks of incubation) when compared with a salinity of 10 ppt, where viable virus was noted after 46 weeks. At 25°C, total loss of infectivity occurred within 9 weeks at all salinities tested.

Echovirus-6. The survival characteristics of echovirus-6 (Table 2) were similar to those of poliovirus 1 in that, again, the temperature of incubation proved to be more important than the salinity of the suspending medium as a factor influencing virus survival. Generally,

TABLE 1. *Effects of salinity and incubation temperatures on the survival of poliomyelitis type 1 (Mahoney) virus*

Weeks	Log_{10} of no. of PFUs/ml at:								
	4°C[a]			15°C			25°C		
	10[b]	20	34	10	20	34	10	20	34
0	5.4	5.3	5.2	4.7	4.5	4.8	5.0	5.5	4.8
2	5.2	4.5	4.1	4.7	3.3	4.2	3.2	2.6	3.7
4	5.0	3.9	3.4	4.4	3.0	3.6	2.1	0.5	2.5
6	4.8	3.7	3.3	4.0	2.9	2.8	1.0	0.0[c]	0.8
8	4.3	3.6	3.3	3.5	2.8	2.5	0.0		0.0
10	4.0	3.4	3.1	3.9	2.5	2.3			
12	3.8	3.3	3.3	4.3	2.7	2.3			
14	3.9	2.6	3.2	4.1	2.3	1.9			
16	3.7	3.0	3.5	4.3	2.5	2.3			
18	3.1	2.6	3.4	3.8	1.7	1.8			
20	2.8	2.2	2.7	3.9	0.6	1.3			
22	2.4	1.8	2.2	3.4	0.0	0.0			
24	2.8	2.3	2.4	2.8					
32	2.6	1.9	1.5	3.1					
40	1.4	1.7	1.9	2.2					
46	0.0	0.0	0.7	1.1					
53			0.0	0.0					

[a] Incubation temperature.
[b] Salinity in parts of NaCl per thousand.
[c] No plaques detected with the undiluted sample.

this virus exhibited a slower inactivation rate than poliovirus 1 at all temperatures and salinities tested.

Coxsackievirus B-5. This agent proved to be the most stable of the three viruses tested (Table 3). Although its infectivity decreased steadily with prolonged incubation at 25°C, it survived 2 to 4 weeks longer than the other two viruses at this temperature. Similarly, at 15°C, the agent proved to be more stable in that low levels of infectious virus were detected after 40 weeks at all salinities and after 46 weeks at 10 and 20 ppt. Although all three viruses were fairly stable at 4°C, coxsackievirus B-5 was

TABLE 2. *Effects of salinity and incubation temperatures on the survival of echovirus-6 (D'Amori)*

Weeks	Log_{10} of no. of PFUs/ml at:								
	4°C[a]			15°C			25°C		
	10[b]	20	34	10	20	34	10	20	34
0	4.5	5.0	4.7	4.7	4.8	5.0	4.5	4.8	4.7
2	4.4	4.5	4.8	4.4	4.8	4.3	4.1	3.7	3.1
4	4.7	4.7	4.2	4.0	4.5	4.0	3.3	2.7	2.3
6	4.5	4.5	4.0	3.5	3.9	3.5	1.8	0.7	0.0[c]
8	4.3	4.4	4.1	3.6	3.8	3.6	1.0	0.0	
10	4.3	4.2	3.8	3.3	3.0	3.3	0.0		
12	4.1	4.0	3.6	2.3	2.3	2.0			
14	3.8	4.0	3.3	2.3	2.7	2.3			
16	3.4	4.1	3.4	2.4	2.7	2.4			
18	2.5	3.3	2.4	0.9	1.9	0.9			
20	2.9	2.6	2.9	0.8	0.7	0.3			
22	2.8	3.0	2.8	0.3	1.0	0.8			
24	2.7	3.4	2.7	0.0[c]	1.0	0.3			
32	2.8	2.8	2.7		0.0	0.0			
40	2.6	3.3	2.5						
46	0.0	0.3	0.7						
53		0.0	0.0						

[a] Incubation temperature.
[b] Salinity in parts of NaCl per thousand.
[c] No plaques detected in the undiluted samples.

TABLE 3. *Effects of salinity and incubation temperatures on the survival of coxsackie B-5 (Faulkner) virus*

Weeks	Log_{10} of no. of PFUs/ml at:								
	4°C[a]			15°C			25°C		
	10[b]	20	34	10	20	34	10	20	34
0	5.6	5.4	5.5	4.8	4.9	4.7	5.2	6.0	4.7
2	5.0	4.9	5.0	4.5	4.4	4.4	4.1	4.4	3.6
4	5.4	4.9	4.7	4.7	4.2	4.6	3.7	2.6	3.1
6	4.9	5.1	4.8	4.7	4.6	4.6	2.6	2.6	2.3
8	5.1	4.6	4.9	4.5	4.1	4.0	2.2	1.3	1.0
10	4.9	4.7	4.8	4.5	4.1	4.3	1.1	0.0[c]	0.0
12	5.3	4.9	4.8	4.6	4.2	4.3	0.0		
14	4.9	5.2	5.1	4.3	4.0	4.1			
16	5.3	5.8	5.4	4.8	4.3	4.4			
18	5.2	5.0	5.1	4.6	4.3	4.1			
20	4.8	4.2	4.5	4.0	2.9	3.3			
22	3.9	3.8	3.9	3.5	2.8	2.9			
24	4.5	4.4	4.5	3.5	3.2	3.1			
32	3.3	4.5	4.8	3.3	3.1	2.6			
40	2.1	4.0	4.6	3.0	2.3	1.3			
46	1.4	3.4	3.8	1.6	0.3	0.0			
53	1.6	2.6	3.1	1.2	0.0				

[a] Incubation temperature.
[b] Salinity in parts of NaCl per thousand.
[c] No plaques detected in the undiluted sample.

again the most stable in that infectious virus was demonstrable at all salinities after 53 weeks of incubation, which was the last sampling time.

In situ studies. Figures 1 through 3 show the survival characteristics of the enteroviruses in free-flowing ocean water. The summer experiments were conducted between 1 July and 19 August 1975. During the test period the water salinity varied from 26 to 30 ppt, the temperature was in the range of 21 to 26°C, and the pH was in the range of 7.8 to 8.0. The winter experiments were conducted from 11 November 1975 to 30 January 1976. During this time the water characteristics were the same as above except the water temperature, which varied from 4 to 16°C.

As in the laboratory studies, water temperature proved to be the important factor since the viruses were more labile during the summer months than during the winter months. Poliovirus 1 again proved to be the most labile virus tested in that all infectivity was lost by 27 days during the summer and by 65 days during the winter (Fig. 1). Echovirus-6 survived slightly longer than poliovirus 1, i.e., 31 days in the summer and 70 days in the winter (Fig. 2), although again the differences noted may not be significant. As in the laboratory studies, coxsackievirus B-5 proved to be the most stable one tested in that it took 7 weeks for its total inactivation in the summer, and only a 2-log drop in infectivity occurred over an 80-day period during the winter months (Fig. 3).

Figure 4 shows the survival characteristics of the three viruses in estuarine waters during the winter months (24 October 1975 through 17 February 1976). During this time period the water's salinity ranged from 8 to 10 ppt, the temperature range was 2 to 19°C, and the pH of the water was 7.6 to 7.8. The results obtained are similar to those from previous studies in that poliovirus 1 was detectable at 46 but not after 53 days of incubation (6.8-log drop), infectious echovirus-6 was noted at 88 days but not after 116 days (6-log drop), and coxsackievirus B-5 retained considerable infectivity (2.7-log

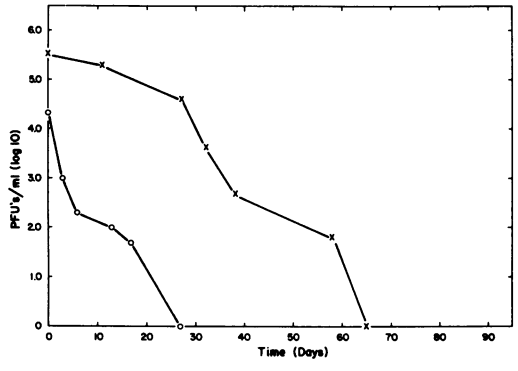

FIG. 1. *Survival of poliovirus type 1 in ocean water during summer (×) and winter (○) months.*

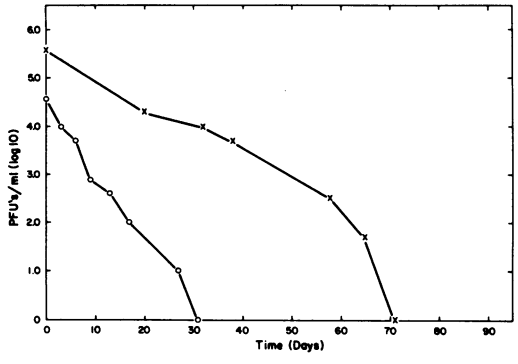

FIG. 2. *Survival of echovirus-6 in ocean water during summer (×) and winter (○) months.*

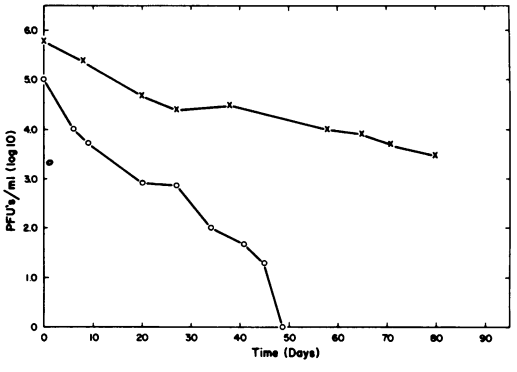

FIG. 3. *Survival of coxsackievirus B-5 in ocean water during summer (×) and winter (○) months.*

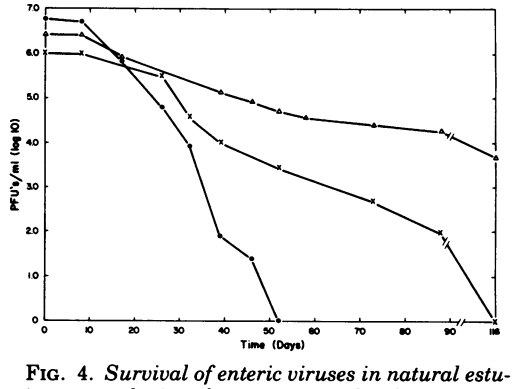

FIG. 4. *Survival of enteric viruses in natural estuarine water during the winter months. Symbols: ●, poliovirus 1; ×, echovirus-6; △, coxsackievirus B-5.*

drop) after 116 days of incubation, which was the last sampling time.

DISCUSSION

It is apparent from the controlled laboratory studies that enteric viruses survive for relatively long periods of time in estuarine and marine salinities when the water temperature is 15°C or lower. Of the three enteroviruses tested, coxsackievirus B-5 was the most stable, with echovirus-6 as intermediate and poliovirus 1 as the least stable. These results are probably representative of the diversity in instability one would encounter if all the 70+ human enteroviruses were studied. Clearly, the most important factor affecting virus viability was the temperature of the water, since in all cases the higher the incubation temperature, the more rapid was the virus inactivation. With the exception of poliovirus 1 incubated at 15°C in a salinity of 10 ppt, the salinity of the water seemed to have only minor effects on virus survival. At 25°C the viruses appeared to be slightly more stable in the low-salinity water, whereas at 4°C poliovirus 1 and coxsackievirus B-5 appeared to be more stable in the high-salinity water; however, the differences noted probably are not significant.

Results of the in situ studies indicated that the enteroviruses tested are more labile in natural waters than in the artificially prepared estuarine and marine waters. Natural processes of virus inactivation have been described by various investigators (2, 9, 10, 12), and some attribute the antiviral activity to the presence of specific marine bacteria (12). Attempts to isolate and characterize the virucidal substance(s) present in the Chesapeake Bay and ocean water are underway.

As in the controlled studies, the in situ studies also indicated that water temperature is the critical factor affecting virus survival in that viral infectivity titers dropped more rapidly during the summer months than during the winter months. Similarly, the relative stabilities of the agents studied were coxsackievirus B-5, echovirus-6, and poliovirus 1.

The only consistent finding from the numerous studies on enterovirus survival in waters of all types is that there is a direct relationship between virus stability and water temperature, namely, the higher the temperature, the greater the rate of virus inactivation. The results of this study support that finding.

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