APPLIED AND ENVIRONMENTAL MICROBIOLOGY, May 1987, p. 1181–1182
0099-2240/87/051181-02$02.00/0

Vol. 53, No. 5

Elevated Temperature Method for Recovery of *Vibrio cholerae* from Oysters (*Crassostrea gigas*)

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Received 5 December 1986/Accepted 3 February 1987

Of 222 *Vibrio cholerae* isolates from diverse clinical and environmental sources, 219 produced visible growth in alkaline peptone broth when incubated overnight at 42°C. In field trials conducted to compare enrichment at incubation temperatures of 42 and 35°C, significantly higher rates of isolation ($P < 0.05$) and recovery ($P < 0.01$) of *V. cholerae* from oysters were observed at 42°C.

Seafood-associated *Vibrio cholerae* infections in the United States (2, 14, 17) have resulted in increased surveillance of seafoods and estuarine environments for *V. cholerae* (3–5, 10–12, 16). Variations of methods described in the *Bacteriological Analytical Manual* (7, 8) have been used in these surveys.

Originally, thiosulfate-citrate-bile salts-sucrose (TCBS) agar was directly inoculated with fecal specimens obtained from patients with clinical cholera symptoms (13). Such direct plating was effective because high *V. cholerae* concentrations are usually present in stools, and remaining fecal flora is either inhibited or easily differentiated (9). Because foods may contain low numbers of *V. cholerae*, a 6- to 8-h selective enrichment in alkaline peptone broth was included before isolation on TCBS agar (7). The current *Bacteriological Analytical Manual* (8) recommends dual enrichment in alkaline peptone broth and gelatin-phosphate salt broth at 35°C for 6 to 8 h and isolation on TCBS, gelatin-phosphate salt, and gelatin agars. Although environmental *V. cholerae* surveys have used numerous methods, overnight enrichment in alkaline peptone broth with isolation on TCBS has been used most frequently in the United States (3–5, 11, 12).

V. cholerae often occurs in low numbers, and its colonial morphology is similar to that of other estuarine microflora; thus, the sensitivity and specificity of existing *V. cholerae* isolation methods may be limited. Confirmation rates for suspected *V. cholerae* organisms from TCBS are low, and screening procedures do not always eliminate false-positives. Growth at 42°C distinguishes *V. cholerae* from many other estuarine bacterial species; hence, incubation of *V. cholerae* in alkaline peptone broth at 42°C should increase its specificity for *V. cholerae* by inhibiting the interfering microflora.

The objectives of this study were to determine the ability of *V. cholerae* isolates to grow at 42°C and to compare recovery of *V. cholerae* from oysters (*Crassostrea gigas*) after enrichment in alkaline peptone broth at 35 and 42°C.

A total of 199 environmental *V. cholerae* isolates were obtained during previous Food and Drug Administration studies of the Gulf and Atlantic coasts. The 23 clinical isolates obtained from Betty Davis (Centers for Disease Control, Atlanta, Ga.) and Harry Smith (Vibrio Reference Laboratory, Philadelphia, Pa.) included global isolates of O1 and non-O1 serogroups. Isolates were stored on T_1N_1 agar slants containing 1.0% tryptone (Difco Laboratories, Detroit, Mich.), 1.0% NaCl, and 2.0% Bacto-Agar (Difco) and covered with sterile mineral oil. These cultures were tested for their ability to produce visible growth in alkaline peptone broth (1.0% Bacto-Peptone [Difco], 1.0% NaCl [pH 8.5 ± 0.1]) when incubated at 35 ± 1.0°C in a warm-air incubator and at 42 ± 0.2°C in a water bath.

Oysters were collected at various U.S. Pacific Coast sites and examined within 6 h of collection. They were scrubbed, shucked, and blended for 90 s (1). Duplicate 25-ml portions of each oyster homogenate were inoculated into separate flasks containing 225 ml of alkaline peptone broth; one flask was incubated at 35°C, and the other was incubated at 42°C. After overnight incubation, the pellicle growth from each flask was streaked onto TCBS plates and incubated overnight at 35°C; sucrose-positive (yellow) colonies were selected and identified by *Bacteriological Analytical Manual* procedures (8).

Qualitative data for isolation of *V. cholerae* were compared by using McNemar's chi-square statistic (6). Productivity ratios (number of isolates per test portion) were compared by using the paired *t* test (15).

The 222 *V. cholerae* cultures were tested for growth at 35 and 42°C in alkaline peptone broth; all 222 cultures produced visible growth at 35°C, and 219 (98.6%) produced visible growth at 42°C. The three *V. cholerae* strains that failed to grow at 42°C were non-O1 serogroup isolates from the Gulf Coast with atypical biochemical profiles. Growth of *V. cholerae* in pure culture cannot be equated with its recovery from estuarine oysters that contain abundant background microflora. Although more *V. cholerae* strains grew at 35 than at 42°C, thermal suppression of the background microflora at 42°C could enhance *V. cholerae* recovery in estuarine oysters. Because all methods have limitations, growth of 98.6% of the *V. cholerae* isolates tested at 42°C was considered sufficient to continue into the next phase of study.

Several parameters were compared at incubation temperatures of 35 and 42°C by using alkaline peptone enrichment broth for isolation and confirmation of *V. cholerae* from the oyster, *C. gigas* (Table 1). Nineteen oysters were divided into two portions each; one portion was enriched at 35°C, and the other was enriched at 42°C. The 35°C procedure yielded 16 suspect colonies versus 20 for the 42°C enrichment. Of 20 suspect colonies, 16 were biochemically confirmed as *V. cholerae* at 42°C, whereas only 1 of 16 was confirmed at 35°C. The proportions of suspect colonies confirmed as *V. cholerae* (confirmation ratios) were 0.06 and

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TABLE 1. Comparison of 35 and 42°C incubations with alkaline peptone enrichment broth for the isolation and confirmation of *V. cholerae* from *C. gigas*

Incubation temp	No. of test portions	No. of suspected colonies	No. of *V. cholerae* colonies confirmed	Confirmation ratio[a]	Productivity ratio[b]	No. (%) of test portions positive for *V. cholerae*[c]
35°C	19	16	1	0.06	0.05	1 (5.2)
42°C	19	20	16	0.80	0.84	7 (36.8)

[a] Proportion of suspect colonies confirmed as *V. cholerae*.
[b] Mean number of *V. cholerae* colonies per test portion. Means were significantly different by paired *t* test, $P < 0.01$.
[c] Results were significantly different by McNemar's test, $P < 0.05$.

0.80 with enrichment at 35 and 42°C, respectively. The mean number of *V. cholerae* colonies (productivity ratio) was also significantly higher ($P < 0.01$) with the 42°C enrichment procedure.

Most important, *V. cholerae* was observed in 7 of the 19 test portions at 42°C enrichment but in only 1 test portion at 35°C. Isolation of *V. cholerae* was significantly better ($P < 0.05$) at 42°C than at 35°C.

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