Behavior of *Vibrio cholerae* in Hot Foods

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Four food types held hot at 45 to 60°C were deliberately contaminated with O1 and non-O1 *Vibrio cholerae* strains. These organisms were assayed for survival and recovery from the foods within 1 h of the time the food was kept hot. The results showed no growth of *V. cholerae* non-O1 on thiosulfate-citrate bile-suerosce agar plates after 24 h of incubation at 37°C for food held hot at 50 to 60°C. Growth was low for *V. cholerae* O1 and was not achieved in some instances in which foods were held at either 55 or 60°C after 40 or 60 min of from the time the food was kept hot. Both organisms, however, were recovered equally from all food types held at all temperatures after 48 h of incubation. When incubated for an additional 24 h, the organisms grew to unusually small-sized colonies, measuring 0.1 to 0.3 mm in diameter, on the same agar plates that were negative for growth after an initial 24 h of incubation. It was concluded that *V. cholerae* survived the period of time held at high temperatures. Although the organisms were not recovered from some foods when held at some of the temperatures and times after 24 h of incubation, they remained viable. An incubation period of 48 h at 37°C was found to be appropriate for the recovery of *V. cholerae* from hot foods.

There have been several outbreaks of cholera and cholera-like diarrhea due to non-O1 *Vibrio cholerae* in which evidence has implicated contamination of food, although *V. cholerae* was seldom been recovered from it. Foods and food items that have been epidemiologically incriminated include seafoods, potatoes, vegetables, rice, cucumbers, and melons (1, 3, 4, 8, 12-14, 22, 24, 26, 32, 34, 37, 40, 42; W. P. Dakin, D. J. Howell, and R. G. Sutton, Letter. Med. J. Aust. 2:468, 1974). Other outbreaks have been associated epidemiologically with food served in restaurants, airplanes, and passenger ships. Outbreaks involving food served in homes and at parties have also been reported (17, 18, 21).

Although the literature of foodborne cholera (including non-O1 *V. cholerae*) seems to suggest that consumption of raw shellfish or other foods is responsible for the illness (4, 6, 8-14, 29, 39), foods that are otherwise held and served hot have also been responsible for causing cholera. In Bulgaria, 13 cases of cholera resulted after the consumption of boiled sausages (45); in Portugal, 48 deaths occurred after the victims ate poorly cooked cockles (6); and the consumption of steamed prawns was incriminated for causing 7 clinical and 6 asymptomatic cases of cholera (19). In Bahrain, food and beverages eaten in restaurants or bought from vendors were associated with cholera (19). A classic outbreak of 16 cases of cholera in which rice contaminated with *V. cholerae* was held hot was reported by Martin et al. (27).

In a study by Roberts and Gilbert (34), survival and growth of noncholera vibrios were reported in various foods. Their growth rates increased with increasing temperature. The bacteria were reported to show no resistance to heat, 10°C organisms per g of food were reduced to 100 organisms per g of food in 2 or 3 min at 55°C. A marked decrease in numbers was reported after 7 min of heating at 52.5°C. The exposure of *V. cholerae* for 15 min at 55°C, 10 min at 60°C, or 5 min at 65°C was reported to kill the organisms (15, 32, 41). These temperature-time relationships were found not to hold when different foods were contaminated with these organisms (C. A. Makukutu, Masters of Public Health thesis, University of Texas, Houston, 1983).

Food can become contaminated with *V. cholerae* from food handlers who are infected or from convalescent or asymptomatic carriers (5, 7, 16, 28, 32, 35, 44; C. O. Tacket, Letter, J. Am. Med. Assoc. 248:2972, 1982). The organisms have been found in food (15, 32, 33). Food prepared in homes by household members with cholera or by other asymptomatic food handlers was found to be highly infectious (21). Household members were found to be more likely to contract cholera if they ate food prepared by food handlers convalescing from cholera. These organisms were found to multiply rapidly in a range of foods (25, 39).

Because hot foods have been implicated in or have caused foodborne cholera (both by non-O1 and O1 *V. cholerae* strains), because previous studies on the response of *V. cholerae* to heat have been inconclusive, and because food can easily become contaminated by *V. cholerae*, we decided to study the survival and recovery of the organisms from food at hot holding temperatures. Although hot food should be held hot at or above 60°C (20, 23, 43), it is generally held below 60°C for aesthetic (30) and other reasons (C. A. Makukutu, Dr. of Public Health thesis, University of Texas, Houston, 1985). It was therefore hypothesized that *V. cholerae* survive food holding temperatures of 45 to 60°C.

**MATERIALS AND METHODS**

*V. cholerae* O1 (569B Inaba serovar) was obtained from the University of Texas Medical Branch at Galveston. The bacterium was maintained in culture on fresh alkaline peptone slants containing 1% NaCl (2, 32). It was transferred to 35 ml of alkaline peptone broth; it was held there as a stock culture. It was agglutinated in commercial Inaba antiseraum (Difco Laboratories, Detroit, Mich.); and large, smooth, yellow, and slightly flattened colonies with opaque centers and translucent peripheries grew on thiosulfate-citrate bile-suerosce (TCBS) agar (BBL Microbiology Systems, Cockeysville, Md.) after 24 h of incubation at 37°C (2, 22, 31).

The native vibrio, non-O1 *V. cholerae*, was isolated from condemned Louisiana oysters supplied by the City of Houston Health Department during an outbreak in October 1984. Isolates were streaked onto TCBS agar plates and held in 35 ml of alkaline peptone broth. The bacterium produced large,

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smooth, and yellow colonies on TCBS agar but did not agglutinate in Inaba or Hikojima (Difco) antisera.

Bacterial stock cultures were made weekly. Fresh stock cultures were incubated at 37°C for 4 h before use. Thereafter, the stock cultures were held at room temperature.

Cooked rice, peas, macaroni and cheese, and meatballs were obtained from the University of Texas M. D. Anderson Hospital and Tumor Institute Food Service Department. The food was not specifically prepared for this experiment but was part of the bulk food prepared to be served in the hospital cafeteria.

Four water baths were set at constant temperatures of 50, 55, 60, and 64°C to achieve food holding temperatures of 45, 50, 55, and 60°C, respectively. A thermometer was put in each water bath to ensure temperature fluctuations to be maintained within a range of ±2°C of the desired water bath temperature.

Between 200 and 300 g of food was weighed and put into a Reynolds Redi-Pan (an aluminum loaf pan of dimensions 7.875 by 4.875 by 1.125 in.; about 20.003 by 12.383 by 2.858 cm). The pan, in turn, was put in a constant-temperature water bath. This provided a means of heating the food and maintaining it at the chosen constant temperature. The temperatures of the food and the heated water bath were allowed to equilibrate by heating the food in the pan for 30 min or 1 h, depending on whether the food was used soon after collection from the cafeteria or was refrigerated before use. The temperature changes, which were maintained to within ±2°C, were monitored with a thermometer in the food.

After the food reached the selected temperature, 0.10 ml of V. cholerae stock culture was added to the food drop by drop. This process achieved a food contamination level of about 10⁴ organisms per g of food. One V. cholerae serovar was used at a time for all food types at each holding temperature. The bacteria and the food were thoroughly mixed, and a 25-g food sample was withdrawn immediately. This sample was considered to be withdrawn at time zero. Additional 25-g food samples were withdrawn after the contaminating food was held for 10, 20, 40, and 60 min.

Each food sample was blended with 100 ml of 0.80% physiological saline. Serial dilutions of up to 10⁴ were prepared with physiological saline and were plated on TCBS agar plates. Direct plating was also done with 0.01 and 0.02 ml of the food blended with 100 ml of physiological saline. Plates were incubated at 37°C and were read after 24 and 48 h. CFUs were counted with the aid of a dark-field colony counter (Quebec). V. cholerae were estimated as CFU/g of food.

RESULTS

Logarithmic transformations of CFU/g of food obtained for each food type at each holding temperature were performed (38, 38). Mean log CFUs/g of food obtained after 24 and 48 h of incubation for each V. cholerae serovar, in each food type, and at each holding temperature were plotted against holding time (Fig. 1 through 4).

Recovery from rice. In Fig. 1 are shown the survival and recovery of both O1 and non-O1 V. cholerae strains from rice held at 45 to 60°C. At 45°C (Fig. 1A), both serovars were recovered at all time intervals. Recovery was higher after 48 h of incubation. At 50°C (Fig. 1B), only V. cholerae-O1 was recovered at all time intervals after 24 h of incubation, while non-O1 V. cholerae was only recovered during the first 20 min of the 60-min holding time. At 55°C (Fig. 1C), V. cholerae O1 was recovered only during the first 20 min.

Recovery at 60°C was during the first 20 and 10 min for V. cholerae O1 and non-O1, respectively.

Recovery of the organisms incubated for 48 h was possible at all time intervals and at all temperatures. In general, the bacterial population tended to increase with holding time at 45°C, while it decreased with time at 50 to 60°C.

Recovery from peas. At 45°C both serovars were recovered from peas throughout the holding times (Fig. 2). Recovery was higher after 48 h than after 24 h of incubation. Recovery of V. cholerae O1 was higher than that of V. cholerae non-O1. V. cholerae non-O1 was not recovered after 24 h of incubation at holding temperatures of 50, 55, and 60°C. V. cholerae was recovered at all holding times and temperatures, except at 60°C, at which the organisms were not recovered after 40 min of holding at the hot temperature. Both serovars were recovered equally, however, after 48 h of incubation (Fig. 2B to D).

Recovery from macaroni and cheese. Both V. cholerae serovars were recovered from macaroni and cheese held at 45°C (Fig. 4A). Their recovery tended to increase with holding time after both 24 and 48 h of incubation. The O1 serovar was recovered more than the non-O1 serovar. At 50°C (Fig. 4B) V. cholerae non-O1 was not recovered after 40 min of holding after 24 h of incubation. Recovery was higher for O1 than for non-O1 V. cholerae and declined with increasing hot holding after 24- and 48-h incubation periods.

At both 55 and 60°C, V. cholerae non-O1 was not recovered after 24 h of incubation but was recovered after 48 h of incubation. Recovery of V. cholerae non-O1 was higher than that of V. cholerae O1 after 48 h of incubation.

DISCUSSION

During preliminary studies we observed that V. cholerae formed unusually small colonies on agar plates that were negative for the organism when such plates were left standing in the laboratory for an additional 24 h. The plates were those that contained samples of peas that were held hot at 50°C and incubated at 37°C for 24 h. We then decided to record colony counts after 24 and 48 h of incubation of the same plates containing all food types held at all holding temperatures. The resulting colonies were very tiny and were estimated to measure 0.1 to 0.3 mm in diameter. These unusual colonies were creamy yellow in color at the beginning and gradually intensified their color to yellow but did not enlarge in size. V. cholerae typically form smooth, yellow colonies measuring 2 to 3 mm in diameter (22) after they are sequentially transferred to TCBS plates and incubated twice for 48 h at 37°C.

The fact that V. cholerae was recovered on TCBS agar plates which were negative for the organism after the initial 24 h of incubation at 37°C suggests that the bacteria are irrecoverable after the initial incubation period but are viable. The bacteria, possibly injured during the blending of the food and subsequent heat, remained viable and may
FIG. 1. Recovery of *V. cholerae* from rice held at 45°C (A), 50°C (B), 55°C (C), and 60°C (D) after 24 and 48 h of incubation. Symbols: ○, *V. cholerae* O1 incubated for 24 h; ●, *V. cholerae* O1 incubated for 48 h; □, *V. cholerae* non-O1 incubated for 24 h; ■, *V. cholerae* non-O1 incubated for 48 h.
FIG. 2. Recovery of *V. cholerae* from peas held at 45°C (C), 50°C (B), 55°C (C), and 60°C (D) after 24 and 48 h of incubation. Symbols: ○, *V. cholerae* O1 incubated for 24 h; ●, *V. cholerae* O1 incubated for 48 h; □, *V. cholerae* non-O1 incubated for 24 h (not recovered); ■, *V. cholerae* non-O1 incubated for 48 h.

have needed time to repair and to be able to grow. Basing the results on the standard 24 h of incubation therefore gives false-negative results and an underestimate of the true viable bacterial load of the food.

The 24-h incubation period for *V. cholerae* serovars studied here was therefore rejected as a reasonable time to fully recover viable *V. cholera* organisms from hot foods. Subsequent to this no contribution analysis for food type,
FIG. 3. Recovery of V. cholerae from macaroni and cheese held at 45°C (A), 50°C (B), 55°C (C), and 60°C (D) after 24 and 48 h of incubation. Symbols: ○, V. cholerae O1 incubated for 24 h; ●, V. cholerae O1 incubated for 48 h; □, V. cholerae non-O1 incubated for 24 h (not recovered); ▲, V. cholerae non-O1 incubated for 48 h.
FIG. 4. Recovery of V. cholerae from meatballs held at 45°C (A), 50°C (B), 55°C (C), and 60°C (D) after 24 and 48 h of incubation. Symbols: ○, V. cholerae O1 incubated for 24 h; ●, V. cholerae O1 incubated for 48 h; □, V. cholerae non-O1 incubated for 24 h; ■, V. cholerae non-O1 incubated for 48 h.
temperature, or their interaction on the recovery or viability of the organisms in hot foods was carried out. However, the data based on the 24-h incubation period seemed to suggest recovery differences by food type, holding time, holding temperature, and bacterial serovar type. These differences were not apparent for both serovars after 48 h of incubation.

Although the recovery of these organisms decreased with increasing time at each holding temperature from 50 to 60°C, more organisms were recovered after 48 h than after 24 h of incubation. The increased numbers suggest the total recovery of viable organisms after 48 h of incubation. In all instances recovery of the organisms was about the same (about 10,000 organisms per g of food). This finding suggests that these organisms are far more heat resistant than is currently believed. It has been reported that workers at the U.S. Food and Drug Administration have suggested an in-the-can pasteurizing process for canned oysters. Their preliminary results indicate an effective temperature and time of 15 to 30 min at 57.2°C (7). Wilson and Ashley (41) reported that the bacteria are killed by heat at 55°C in 15 min. It was found that these organisms could survive higher temperatures for longer periods (Makukutu, Masters of Public Health thesis; R. K. Guthrie, C. A. Makukutu, and R. Gibson, Dairy Food San., in press). In this study, both organisms survived in hot foods for 1 h even at 60°C.

V. cholerae recovery based on the 24-h incubation period implies that V. cholerae non-O1 is more susceptible to heat than V. cholerae O1 because it was recovered least often in all food types at all holding temperatures. There is no evidence for this assumption, however, when recovery was based on 48 h of incubation because both organisms were recovered equally at each holding temperature from each food type.

The persistence of these organisms at food hot holding temperatures causes concern, for there is a threat of foodborne cholera or cholera-like diarrhea due to V. cholerae non-O1 if hot food becomes contaminated. The failure to recover some of these organisms or the recovery of low levels of them after 24 h of incubation gives a false sense of safety from contamination that may result in food being deemed fit for human consumption. In general, the mere presence of these organisms in food suggests fecal pollution which may result from the mishandling of the food or through cross-contamination.

It could therefore be inferred that hot foods may have been responsible for some cases of cholera, but such food is not suspect during epidemiological investigations because it is generally believed that heat kills these organisms (7, 41). Failure to recover the organisms based on the traditional standard incubation period of 24 h may have contributed to this situation, enabling humans to ingest viable V. cholerae cells.

Therefore, we conclude that food hot holding temperatures of 45 to 60°C for 1 h are ineffective in killing V. cholerae, that both V. cholerae O1 and non-O1 survive equally well and remain viable in food at these temperatures, that the standard 24-h incubation period is not long enough to allow the growth and recovery of the organisms from hot foods, and that use of such temperatures for food contaminated with these organisms yield an underestimate of V. cholerae O1 contamination and false-negative results for V. cholerae non-O1 contamination.

Based on the findings of this study, it is recommended that a lethal temperature for V. cholerae be established and that the 48-h incubation period be adopted for investigatory work on these organisms, especially in cases in which heat may have been applied or if the method used may have incurred bacterial injury. Further research is needed to characterize the nature of the tiny colony sizes resulting from V. cholerae subjected to heat and to determine whether such cells, if ingested with food, would subsequently result in the characteristic profuse diarrhea caused by these organisms.

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LITERATURE CITED


