Viability of El Tor Vibrios in Common Foodstuffs Found in an Endemic Cholera Area

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The survival of cholera vibrios in foodstuffs has been studied by a great many workers and most of the work concerning the classical strains of *Vibrio cholerae* has been well summarized by Pollitzer.[a] Studies on El Tor vibrios have been carried out in Thailand,[b] Iraq,[c] and the Philippines [d] and India.[e] Further confirmation of these studies was necessary in order to demonstrate the folly of excessively stringent quarantine controls on goods from cholera-infected or endemic areas.

This study was carried out with 3 main aims: firstly, to comply with one of the aims of the Conference on International Co-operation in the Prevention of Cholera, Ankara,[f] which requested research on El Tor vibrio viability in food products from various localities, particularly produce entering international trade; secondly, to confirm previous studies; and thirdly, to examine foodstuffs from an endemic area for the presence of naturally occuring *V. cholerae*.

Materials and methods

Examination of foodstuffs for the presence of V. cholerae. Fruits and vegetables with their outer surfaces intact were purchased at the Beliaghata Market, Calcutta. This market is located in the centre of a highly endemic cholera area. Duplicate samples of each of the following foodstuffs were taken on 2 occasions: lime, lemon, orange, grape, banana, guava, papaya, dates, fig, raisins, tomato, onion, eggplant, pea, celery, green bean, bean sprout, okra, pumpkin, potato and lima bean.

Pieces of the skin or outside of the produce and pieces of the inside portion each weighing 1 g were placed aseptically in 9 ml of peptone water. Each specimen was prepared in duplicate. All the tubes were then incubated for 6 h at 37°C, and the contents were then plated on bile salt agar (BSA) plates and TCBS (Eiken) plates. The plates were incubated for 18 h at 37°C, and then examined for the presence of vibrios. Suspected colonies were checked serologically with Cholera O Group I serum.

Artificial contamination of foodstuffs with V. cholerae. The techniques used were based on those of El-Shawi & Thewaini.[c] The foodstuffs were purchased at the New Market, a large market servicing central Calcutta. The organism, *V. cholerae*, biotype El Tor, Ogawa serotype, 15865H/68, had been recently isolated at the Cholera Research Centre, Calcutta, from a clinical case of cholera in the Infectious Diseases Hospital, Calcutta. A suspension of fresh stool [g] in sterile saline was contaminated with this strain at the rate of 10^9 organisms per ml. A 0.1 ml aliquot of the suspension was placed on to squares of solid food or pipetted into fluids at a ratio of 1 part suspension to 10 parts of fluid. In the case of powders, such as cinnamon and pepper, small mounds were placed in the Petri dish and the vibrio suspension was absorbed by the powder. The contaminated food was placed in a sterile Petri dish and allowed to incubate at room temperature (20°C–25°C). Duplicate pieces, 1¼ in square (2.5 cm square), were cut from the contaminated sample and were placed in peptone water 1 h after contamination and at intervals of 24 h thereafter. These samples were incubated for 18 h at 37°C and were then plated on to 3 BSA plates and 1 TCBS (Eiken) plate. The plates were incubated for 24 h at 37°C and then examined for the presence of

[a] Pollitzer, R. (1959) *Cholera*, Geneva (*World Health Organization: Monograph Series* No. 43).

[b] Felsenfeld, O. (1965) *Bull. Wld Hlth Org.*, 33, 725.

[c] El-Shawi, N. N. & Thewaini, A. J. *(1967) El Tor vibrios isolated in Iraq and their survival on some foods and beverages* (unpublished document WHO/Cholera Information/67.9, pp. 8-10). A limited number of copies of this document is available to persons officially or professionally interested on request to distribution and Sales, World Health Organization, 1211 Geneva, Switzerland.

[d] Pesigan, T. P. Plantilla, J. & Rolda, M. (1967) *Bull. Wld Hlth Org.*, 37, 799-786.

[e] Neogy, K. N. (1965) *Bull. Calcutta Sch. trop. Med.*, 13, 10.

[f] *WHO Chron.*, 1967, 21, 436.

[g] Stool from a healthy person.

VIABILITY OF EL TOR VIBRIOS IN ARTIFICIALLY CONTAMINATED FOODSTUFFS

Specimen	Period of survival at 20°C–25°C
Fresh fruits	
Lime, lemon	1 hour
Orange, grape	1 day
Banana	2 days
Guava	3 days
Papaya	5 days
Dried fruits	
Dates	1 hour
Figs, raisins	1 day
Fresh vegetables	
Tomato	1 day
Onion, eggplant, pea	3 days
Celery, green bean, bean sprout	5 days
Okra, lima bean	6 days
Pumpkin	7 days
Potato	8 days
Grain	
Rice, wheat	3 days
Nuts	
Peanut, walnut, pecan, hazelnut	3 days
Spices	
Red chili, turmeric, cardamon, cinnamon carroway seeds	1 day
Peppercorn, ground pepper	2 days
Bay leaf	3 days
Ginger root	5 days
Drinks	
Coca-cola	1 day
Rose water	2 days
Cooked food	
Sandesh (milk sweet)	1 day
Rossegolla (milk sweet), *nimki* (pastry)	2 days
Paiji (onions and chili in batter)	3 days
Miscellaneous	
Coffee (ground)	1 hour
Tea leaves, sugar	1 day

vibrios. Characteristic colonies were picked and agglutinated with cholera O group I serum. The sample plates were left at room temperature and tested each day until negative.

In a second procedure for testing the survival of vibrios in fluids, 10-ml aliquots of infected soft drinks were filtered through a Millipore filter (0.22μ), and the filter-pad was placed in 100 ml of peptone water. After incubation for 18 h at 37°C, this was subcultured on to 3 BSA plates and 1 TCBS plate. The plates were incubated for 24 h at 37°C and then examined.

Results

All of the fresh fruits, dried fruits and vegetables listed in the accompanying table were tested for the presence of *V. cholerae* 6 times, twice in the winter and 4 times during the cholera season. Although the surfaces of the unwashed produce showed a great deal of bacterial and fungal contamination neither the outer nor the inner portions of any of the material tested gave any evidence of the presence of *V. cholerae*.

The results of the artificial contamination studies are listed in the accompanying table. Vibrios survived for only a short time in acidic foodstuffs such as citrus fruits or tomatoes. Their survival time was also short in foods with a very high percentage of sugar, in processed fruits like dates, figs, and raisins, and in dry powdered spices. Vibrios remained viable longer in starchy vegetables like potatoes and in more neutral vegetables like pumpkin or okra, particularly when the fruit or vegetables remained moist for some time.

The variability in vibrio survival time in different samples of the same material was slight, particularly in the acidic foods. In the foods in which vibrios survived longer than 3 days, viability varied by about 1 day in the duplicate samples and in these cases the longer time is quoted in the table.

Discussion

Our failure to find naturally occuring vibrios in fruits and vegetables with their outer surfaces intact should help to dispel many of the false ideas circulating among both scientists and lay people; the most important of these is that *V. cholerae* can enter the plant through its root system and thereby infect the interior of the produce.

The studies on vibrio viability confirmed many of the results obtained earlier in the Philippines.[a] Although there was some variation this could be due

to differences in pH, humidity, temperature and other physico-chemical factors. The short survival time in acidic fruits and vegetables was expected. Japanese workers[h] have shown that an acidic environment (pH $\leqslant$ 5.0) was harmful to *V. cholerae*. These same workers also pointed out that high osmolarity decreased vibrio viability. This would account for the low survival time in foods with a high percentage of sugar. Bengali sweets are famed for their extreme sweetness, and *Rossegolla* is not only made with a great deal of sugar but it is also soaked in sugar-cane syrup.

The results from these studies, in conjunction with those from other countries, point to the conclusion that excessive importation restrictions on goods coming from countries in which cholera is present or endemic are unwarranted.

[h] Miyaki, K., Iwahara, S., Sato, K., Fujimoto, S., & Aibara, K. (1967) *Bull. Wld Hlth Org.*, **37**, 773-778.

Isolation of Dengue Viruses in *Aedes albopictus* Cell Cultures

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The *Aedes albopictus* cell line[b] has been shown to be more sensitive than infant mice and Vero cells to infection with dengue viruses and the infection can easily be detected on the basis of the characteristic cytopathic effect produced by these viruses in the inoculated cultures.[c, d] In view of these findings, a study was undertaken to assess the utility of this cell line for the primary isolation of dengue viruses from sera and mosquitos.

Altogether, 25 human sera collected from cases of dengue-like illness from various places in India during the last 5 years were used in this study. Dengue viruses of types 1, 2, 3 or 4 had been isolated from most of these sera during earlier studies in this laboratory by intracerebral inoculation in infant mice or in vertebrate cell cultures or in both. As it was found that the undiluted sera had anticellular effects leading to rapid cell lysis in *A. albopictus* cultures, the sera were diluted 1 : 10 in 0.75% bovine albumin in phosphate saline (BAPS) buffered to pH 7.2 prior to their inoculation in these cultures.

Each diluted serum was inoculated in batches of 4 culture tubes; 0.1 ml/tube. Two hours after incubation at 30°C, the cultures were washed twice, the growth medium was replaced, and the cultures were reincubated at 30°C in a stationary position. They were observed daily for 10 days for the appearance of cytopathic effects. The cultures showing cytopathic effects were frozen and thawed 3 times and the tissue culture fluid from 4 tubes of the same batch was pooled and centrifuged at 300 *g* for 10 minutes. The supernatant fluid was used either for further passages or as an antigen for identification in complement-fixation (CF) tests.[e] Most specimens required only 1, or sometimes 2, passages before they could be identified.

As the human sera used in this study had been stored at a temperature of −50°C for long periods (between 1 and 5 years), and had been thawed more than once during this period, they were inoculated in 10^{-1} dilutions intracerebrally into infant mice and some of them also in Vero cell cultures simultaneously with the inoculation in *A. albopictus* cell cultures to cross-check the presence of virus in the samples. The inoculated mice were observed for 21 days for signs of sickness and the survivors were challenged intracerebrally with the TR-1751 strain of dengue type 2 virus to test for resistance to the challenge virus.[f] Inoculated Vero cell cultures were observed only for cytopathic agents and the interference technique for detecting the viruses was not used.

[a] The Virus Research Centre is maintained by the Indian Council of Medical Research and also receives a grant (3 x 4307) of the PL-480 Funds from the National Institutes of Health, US Public Health Service, through the Indian Council of Medical Research. The Centre is also a WHO Collaborating Laboratory for Arboviruses.

[b] Singh, K. R. P. (1967) *Curr. Sci.*, **36**, 506-508.

[c] Singh, K. R. P. & Paul, S. D. (1968) *Curr. Sci.*, **37**, 65-67

[d] Paul, S. D. & Singh, K. R. P. (1969) *Curr. Sci.*, **38**, 241-242.

[e] Pavri, K. M. & Ghosh, S. N. (1969) *Bull. Wld Hlth Org.*, **40**, 984–986.

[f] Paul, S. D. et al. (1965) *Ind. J. med. Res.*, **53**, 777-789.