

Extinction of *Vibrio cholerae* in acidic substrata: Contaminated fish marinated with lime juice (ceviche)

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(Rec. 28-IX-1994. Acep. 10-XI-1994)

Abstract: Millions of *Vibrio cholerae* O1 El Tor were rapidly eliminated when added to commercial ceviche prepared by marination of mahi-mahi fish in lime juice. Likewise, large masses of viable vibrios present in laboratory contaminated fish, were readily eliminated after immersion in lime juice, during the preparation of ceviche. The killing effect was evident within 5 min of exposure of vibrios to lime juice, with reductions of more than 99.9 % of the initial bacterial mass. After 2 h of marination of fish with lime juice (the minimum recommended), no vibrios were detected in the lowest working dilutions (1:10, 1:100). The *Vibrio* mass eliminated by lime juice was 2 to 6 logarithms greater than the maximum infectious dose, and 4 to 8 logs greater than the minimum infectious dose to induce cholera El Tor. Also, the killing time was shorter than the elapsing time between preparing and serving food in homes or restaurants. The traditional marination of fish with lime juice or its addition to seafood and meals immediately before consumption, should be protected and promoted to prevent infection with cholera vibrios. In the face of an epidemic of cholera, consumption of ceviche prepared with lime juice would be one of the safest ways to avoid infection with *V. cholerae*.

Key words: *Vibrio cholerae* O1 El Tor, marination of fish, lime juice, vibriocide, prevention of cholera.

The 7th cholera pandemic by *Vibrio cholerae* O1 El Tor, started in Sulawesi (The Celebes) in 1961, and for the following 30 years spread to many countries in Asia, Africa and Europe. Cholera El Tor emerged in Peru in 1991 and reached most countries of continental Latin America within two years (Seminario 1991, Mata 1994). Outbreaks were larger when there were deficient sanitation, water supply and schooling. The disease spread primarily through contaminated water and food (Faechem *et al.* 1981, CDC 1991, Ries *et al.* 1992, Seminario 1991). Risk factors involved were: drinking untreated water from streams, municipal sources, wells and open household containers; consumption of foods and beverages sold by street vendors; and consumption

of unheated leftover food, raw seafood and vegetables exposed to untreated water or sewage (Mujica *et al.* 1991, Rodríguez *et al.* 1991, Seminario *et al.* 1991, Vásquez *et al.* 1991). Raw fish marinated for hours in lime juice (ceviche) has not been convincingly incriminated as a source of cholera infection (Mata & Vives 1992). Factors found to protect against infection were hand-washing, treatment of drinking water, health education and drinking of a home beverage made of grapefruit ("toronjada") (Mujica *et al.* 1991). The experiments consisted in the contamination of fish with very large doses of *Vibrio cholerae* O1 El Tor, and later exposure to lime juice for varying times to determine survival of vibrios. Our studies were made from July 1991 to February

1992, and were repeated in 1992 and 1993 (Mata *et al.* 1991, Mata & Vives 1992, Mata *et al.* 1993, Mata 1994).

MATERIAL AND METHODS

***Vibrio cholerae* O1 El Tor Inaba strain 1800-82**, was obtained from the Centers for Disease Control (CDC), Atlanta, Georgia. Cultures were prepared and handled as described elsewhere (Mata & Vives 1992). Vibrios were grown in alkaline peptone water (APW) at pH 9.0-9.2 and thiosulfate-citrate-bile salts-saccharose agar (TCBS) (Difco Laboratories). Cultures were confirmed by slide agglutination with polyvalent and specific antisera and biochemical tests. All cultures were grown at 35 C for 18 h, and occasionally for 24 h. Gloves and mouth mask were used in initial experiments. Decontamination of materials was achieved by immersion in 5% hydrochloric acid before autoclaving. After six months of handling billions of vibrios, none of the eight persons working in these experiments had developed significant titers of vibriocidal or anti-toxin antibodies (Mata 1992). To estimate the number of vibrios, log-10 dilutions of cultures and substrata were made in deionized water (B-pure Barnstead), using serologic pipettes and calibrated platinum-iridium loops (0.01 ml). Inocula were prepared by diluting 18 h cultures in APW at pH 9-9.2 in sterile deionized water at pH 6. For amplification, log-10 dilutions were placed in APW and TCBS agar, checking after 18 h at 35 C incubation for turbidity and colony-forming units, respectively. Numbers of vibrios were expressed as millions per ml or g. For confirmation of presumptive diagnoses, inoculation of Kligler iron agar (KIA) and physiological tests were made using commercial media and reagents (Difco); slide agglutination tests were performed with specific rabbit *V. cholerae* antisera supplied by the CDC.

Juice of lime was squeezed from fruits of three cultivars: "limón agrio" (lime, *Citrus aurantifolia*), "mandarino" (mandarin lime, *Citrus x limonia*), and "mesino/real" (lemon, *Citrus limon*). The three limes have comparable acidity (average pH = 3.0, unaltered after freezing for prolonged periods), and experiments with them gave similar results. Whole lime

juice was extracted by hand or with a home juicer, and was used unprocessed. Lime juice was diluted in sterile deionized water (Mata *et al.* 1994).

Hydrogen-ion concentration was measured in a digital potentiometer (Beckman), calibrated daily.

Fish: *Coryphaena hippurus* ("mahi-mahi", "dorado") was used throughout the experiments. This fish was caught in the Pacific Ocean at 100 to 150 km from shore, and was skinned, eviscerated, cut and stored on ice without preservatives, right at sea.

Contamination of fish was done with known doses of *V. cholerae*. Macerates of fish were prepared in deionized water, by grinding in porcelain mortar with pestle. Log-10 dilutions of macerates were made in deionized water, which were later inoculated in APW and TCBS.

Ceviche: Commercial ceviche was purchased in neighborhood bars, stored in glass jars and used the same day. For laboratory ceviche, pieces of about 3-5 g of raw fish were marinated in lime juice (juice of sour orange, *Citrus aurantium*, is equally acidic and effective), for 2-6 h at room temperature. Frequently, marination is conducted at 4 C in the refrigerator. Onion slices, celery, pepper, coriander and a pinch of salt, are added after marination, but these were not added to laboratory ceviche for most of our experiments. Macerates of ceviche, dilutions and cultures were made as described before.

Experiments: Twelve experiments were performed to test the killing effect of lime juice on *V. cholerae* O1. In some, varying doses of cholera vibrios were added to commercial ceviche; other experiments consisted of the preparation of laboratory ceviche starting with fish experimentally contaminated with cholera vibrios. Survival of vibrios was tested in log-10 dilutions of macerates of ceviche and amplification of the growth of vibrios was made in bottles containing 50 ml of APW. The crucial experiments were performed in the second half of 1991, and were repeated during 1992 and 1993. Details of different experiments will be given under Results.

RESULTS

Killing of *Vibrio cholerae* by lime juice.

One hundred million cholera vibrios were added to log-10 dilutions of lime juice (pH 2.3-3.0). Sampling and culturing of each dilution in APW and TCBS was made after 5, 15 and 30 min of exposure to lime juice. No colony-forming units of *V. cholerae* were visualized on TCBS at any of the time intervals of exposure to 1:100 dilution of lime juice (Table 1). Thus, more than 99.9 % of the vibrios initially present in the inoculum were killed within 5 min of exposure. The vibriocidal effect was noted at dilutions 1:1 000 and 1:10 000 (Table 1), evidenced by the considerable reduction of vibrios. Lime juice killed *V. cholerae* rapidly, an effect persisting at 1:100 and 1:1 000 dilutions, a point where neither the lime odor or taste were recognizable. A water solution containing 6 % citric and 0.3 % malic acid (as in natural lime juice) rapidly killed vibrios as well. Similar results were obtained using table vinegar.

TABLE 1

Effect of lime juice on 100 million Vibrio cholerae O1

Log 10 dilution of juice	Minutes of contact		
	5	15	30
0	0*	0	0
-1	0	0	0
-2	0	0	0
-3	15**	10	11
-4	100	150	100

* There was no growth on TCBS agar

** Colony-forming units (CFU) on TCBS agar

Note: in certain experiments, the vibriocidal effect of lime juice was one logarithm less than when APW was employed for amplification.

Death of *V. cholerae* in commercial ceviche.

Fifty portions of commercial ceviche were obtained from bars, restaurants and food vendors of the Central Market, all showing pH values ranging from 3.5 to 4.2. Five of these portions were selected for inoculation with 100 to 1000 million vibrios. No vibrios could be recovered from the macerates of ceviche inoculated in APW and TCBS, after 15 to 30 min of exposure (Table 2).

TABLE 2

Death of Vibrio cholerae O1 when added to commercial ceviche

Methodology:

1. Add 100 to 1 000 million cholera vibrios (18 h culture at 35 C in APW), to 5 portions of ceviche; gently mix, mechanically; leave at room temperature.
2. After 30 min, log 10 dilutions of ceviche broth were made and inoculated on TCBS and APW.

Results:

1. No growth of *Vibrio cholerae* at 1:100 dilution; more than 99.99% of vibrios in inoculum could not be cultured.

Death of *V. cholerae* contaminating fish for ceviche.

Two similar portions of fresh fish contaminated with 100 million vibrios, were incubated for 90 min at room temperature and then cut into small pieces. One sample was left untreated as control. The other was placed under fresh lime juice, for marination (ceviche). Both fish and soup of each sample were simultaneously cultured in APW or TCBS at 30 and 60 min, and 72 h of marination, for quantitative studies (Table 3). After 30 min of exposure to lime juice, more than 99.99 % of the original inoculum could not be grown from ceviche meat or soup.

TABLE 3

Death of V cholerae O1 in ceviche prepared with fish contaminated in the laboratory

Methodology:

1. To two portions of 100g of fish, 100 million vibrios per g were added and left for 90 min at room temperature.
2. Fish was cut in small pieces; one portion was left as control; the other was covered with lime juice.
3. After 30 and 60 minutes and 72 h, log 10 dilutions were made and inoculated on TCBS and APW.

Results:

1. After 90 min of contamination the control had 100 million bacilli per ml, as in the inoculum.
2. In the ceviche, no vibrios could be grown at a 1:100 dilution in meat or soup, on TCBS or APW, either at 30 or 60 min or 72 h. There was a reduction of 99.99% or more of the initial contaminating mass.

TABLE 4

Death of V. cholerae O1 in ceviche prepared from fish highly contaminated in the laboratory

Methodology:

1. Fish was washed in tap running water for one hour;
2. 1 000 million vibrios were added to two portions of fish, and left for 90 min at room temperature;
3. After 90 min, one portion cut in chunks was left as control; the other portion was immersed in lime juice;
4. Quantitative cultures were made of meat and broth of both, ceviche and untreated fish (control), in APW and TCBS, at 30 min and at 7 and 24 h after contact;
5. Chunks of ceviche were placed in bottles with 50 ml of APW, at 30 min, 7 and 24 h;

Results:

1. At 90 minutes of exposure to vibrios, there were from 100 to 1 000 million vibrios per gram/ml of meat/soup;
2. Immersion of fish in lime juice resulted in elimination of vibrios at 30 min, and 7 and 24 h. In the control there were >100 million vibrios per gram/ml.
3. *V. cholerae* did not grow in APW, at indicated times.

Death of *V. cholerae* in highly contaminated fish for ceviche. Two portions of 100 g of fish were contaminated with one billion per g (1 000 000 000) per g vibrios and incubated at room temperature for 90 min. After incubation, the mass of vibrios remained basically unaltered. Then, one portion was immersed in lime juice and the other was left as control (Table 4). After 30 min, and 7 and 24 hr of exposure to lime juice, no vibrios could be cultured from meat or soup of ceviche, either in APW or TCBS. In contrast, the mass of vibrios was still 100 million per gram in the untreated portion (control). Furthermore, chunks of ceviche meat were removed (with a platinum needle) from the flasks - at pre-determined intervals - and transferred each to one bottle containing 50 ml of APW at pH 9.2. No growth (turbidity) was noted in the bottles after two weeks of incubation at 35 C.

DISCUSSION

Vibrios are very sensitive to heat, acid, light, chlorine, organic matter and bacteria; their high number in waste and drinking water decrease by several logarithms within hours or days,

unless additional vibrios are added to the substrate, for instance, from a persisting contaminating source (Pollitzer 1959). An ingestion of thousands or millions of vibrios is required to establish infection, except when hypochlorhydria or gastric ulcer is present, or after a heavy meal.

Cholera vibrios also die readily at pH less than 4.5, and they do not fare well at pH 5.0 (Pollitzer 1959). The vibriocidal capacity of lime juice results from the acidity conferred by a concentration of 5 to 6 g % of citric acid (Primo 1982). Lime juice has other organic acids (malic, oxalic, phosphoric, lactic, tartaric, malonic, succinic, benzoic, adipic, isocitric, aconitic and ketoglutaric) and other acids like ascorbic, pantothenic and folic. It also contains biotin, niacin, riboflavin, pyridoxin and thiamine (Braverman 1949, Reuther *et al.* 1968, Geigy 1965, Erickson 1968, Trease & Evans 1976, Primo 1982). Organic acids ionize slowly and therefore a high concentration is tolerated by the mucosa of the mouth, oesophagus, stomach and intestine.

A 100 fold dilution of lime juice is equivalent to a dilution of 1:10 000 of citric acid. Lime juice has vibriocidal capacity comparable to that of sulfuric and hydrochloric acids which diluted 15 000 times can kill cholera vibrios (Kolle & Schürmann 1912).

Vibrios multiply easily in neutral and slightly alkaline substrates like rice, potatoes and vegetables (Felsenfeld 1965, Faechem *et al.* 1981, Kolvin & Roberts 1982), but die easily when they are on the surface of vegetables and fruits, especially if exposed to sunlight and dry weather (Felsenfeld 1965, Gerichter *et al.* 1975). On the other hand, acidic foods like yoghurt and cheese, acidic fruits and dressings made of vinegar, "umeboshi" and fermented fruits, were traditionally recognized as being protective against intestinal infection.

The prophylactic effect of acidic fruits, sour foods and acidic dressings against cholera - inferred by Metchnikoff at the end of the XIX Century - had been recognized by the peoples of India, Bangladesh, Israel and other nations a long time ago (Lal & Jacob 1926, Gitelson *et al.* 1973, Rogol *et al.* 1976), and was deduced from recent field studies (Tauxe *et al.* 1988, St. Louis *et al.* 1990).

Raw fish is not acidic and can be readily contaminated with cholera vibrios in natural

and experimental scenarios (Takano *et al.* 1926, McIntyre *et al.* 1979, Kolvin & Roberts 1982). However, cooking renders food safe for consumption, while cholera transmission by fish can occur if consumed raw or after storage in conditions favoring bacterial multiplication (McIntyre *et al.* 1979). Ceviche is strongly acidic and therefore can not be a substrate for vibrio multiplication (Mata & Vives 1992). Such observation is supported by lack of evidence implicating ceviche or cooked fish in cholera transmission after three years of epidemics in the Americas. The same argument applies to acidic beverages and foods, as recently recognized by the World Health Organization in the following statement: "cholera organisms are sensitive to drying and to acidity (pH <4.5), therefore [dried, acid and pickled foods and fruit juices] are unlikely to cause infection" (WHO 1991).

The powerful lethal effect of lime juice over large masses of cholera vibrios (greatly in excess of numbers naturally occurring in nature), was discussed in a scientific meeting (Mata *et al.* 1991) and summarized in a book and in a review article (Mata & Vives 1992, Mata 1994). Experiments with vibrios in ceviche were also performed by Jiménez-Somarrivas (1992) with similar results. The experiments consistently showed that very large numbers of vibrios are rapidly eliminated after contact with lime juice or during marination with lime juice for the preparation of ceviche. The masses of vibrios eliminated by lime juice are greater than the largest doses of vibrios shed during severe attacks of *cholera gravis* (Huber 1965), indicating that lime juice, pure or even diluted to 1:10 and 1:100, is very effective to kill vibrios and should be decidedly added to foods for prophylaxis against cholera infection.

The data presented here does not apply to less acidic ceviche made with ginger-ale, low acidic grafted limes or to sub-optimal exposure to lime juice. Sporadic appearance of small numbers of vibrios from laboratory ceviche probably reflect inadequate handling of large inocula of vibrios (Mata & Vives 1992). The evidence presented here suggests that properly prepared ceviche is one of the safest foods for human consumption in the course of a cholera outbreak.

ACKNOWLEDGMENTS

We thank Irlanda Jiménez, Silvia Pacheco, Carla Solórzano and María Elena Villegas for collaboration in several experiments. We also thank doctors Darner Mora, Patricia Jiménez, Cecilia Vargas, Florencia Antillón, Luis Poveda, Pablo Sánchez, Alfonso Mata, Francisco Ciccio and Juan Solano for special consultations. The studies received financial support from the Swedish Agency for Research and Cooperation with Developing Countries (SAREC), the Rockefeller Foundation, the Association for Health Research (ASINSA) and the Vice-Presidency of Research Affairs of the University of Costa Rica.

RESUMEN

Millones de *Vibrio cholerae* O1 El Tor son rápidamente eliminados después de agregarlos al ceviche comercial, el cual ha sido preparado con pescado "dorado" marinado con jugo de limón agrio. De igual manera, grandes masas de vibrios pululando en pescado contaminado en el laboratorio, son eliminadas rápidamente al sumergir los trocitos de pescado en jugo de limón durante la preparación del ceviche (marinación). El efecto letal fue evidente al cabo de 5 min de exposición de los vibrios al jugo de limón, con reducciones de la masa bacteriana inicial de más del 99 %. Después de 2 h de marinación del pescado (el período mínimo recomendado), no se detectaron vibrios en las diluciones de trabajo más bajas (1:10, 1:100). La masa de vibrios eliminada por el jugo de limón fue de 2 a 6 logaritmos mayor que la dosis infecciosa máxima, y de 4 a 8 logaritmos mayor que la dosis infecciosa mínima para inducir cólera El Tor. El tiempo letal fue más corto que el tiempo de espera para preparar y servir alimentos en el hogar o restaurante. La marinación tradicional del pescado con jugo de limón y la adición de jugo de limón a los mariscos y otros alimentos inmediatamente antes de su consumo, deben ser protegidos y promocionados para prevenir infecciones con *V. cholerae* O1. Durante el curso de epidemias de cólera, el consumo de ceviche preparado con jugo de limón agrio es una de las medidas más seguras para evitar la infección con *V. cholerae* O1.

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