Rev. Biol. Trop., 42(3): 487-492, 1994

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Extinction of *Vibrio cholerae* in acidic substrata: Contaminated cabbage and lettuce treated with lime juice

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

Abstract: Lime juice killed millions of *Vibrio cholerae* O1, El Tor, Inaba, present on cabbage and lettuce contaminated in the laboratory. The lethal effect was evident within 5 min of exposure to lime juice. No vibrios could be recovered at dilution 1:10 using alkaline peptone water (APW) and thiosulfate-citrate-bile salts-saccharose agar (TCBS). More than 99.9 % of the initial inoculum was effectively destroyed. The number of vibrios killed by lime juice was 2 to 6 logarithms greater than the maximum infecting dose, and 4 to 8 logs greater than the minimum infecting dose for cholera El Tor. The time interval needed for killing was smaller than the usual waiting time for serving food in homes and restaurants. The addition of lime juice to non-acidic foods, beverages and water, is strongly recommended to prevent infection with cholera vibrios and other acid-sensitive microorganisms. This measure is particularly important for rural and slum populations in the tropics and subtropics.

Key words: *Vibrio cholerae* O1 El Tor, contaminated salad, lime juice, prevention of cholera

Vibrio cholerae O1 is transmitted primarily by water contaminated with fecal waste, and secondarily by foods and beverages contaminated with human feces, especially in the case of the biotype El Tor. Prompted by the emergence of the large cholera epidemic in Latin America in 1991, experiments were begun in our laboratory, aimed at determining the effect of lime juice and other vibriocides on El Tor organisms growing on laboratory-contaminated vegetables. The purpose was to explore simple preventive measures that could be implemented in the field to curtail cholera and probably other diarrheal diseases. Several experiments were conducted to determine the effect of bacteriocidal substances or products on large masses of vibrios that were experimentally contaminating cabbage and lettuce used to prepare salads. Key experiments were performed during July-December 1991, and several of them were repeated and expanded during 1992 and 1993.

MATERIAL AND METHODS

***Vibrio cholerae O1* El Tor Inaba strain 1900-82**, was obtained from the Centers for Disease Control (CDC), Atlanta, Georgia. Cultures were prepared and handled as described elsewhere (Mata & Vives 1992, Mata *et al.* 1994). Inocula were prepared by diluting 18 h cultures of *V. cholerae* in alkaline peptone water (APW), with sterile deionized water at pH 6. Culture media were Difco; group and type-specific anti-*V. cholerae* sera were obtained from CDC.

Juice of limes was squeezed from fruits of three cultivars: "limón agrio" (lime, *Citrus aurantifolia*), "mandarino" (mandarin lime, *Citrus* x *limonia*), and "mesino" or "real" (lemon, *Citrus limon*). The three types of limes have comparable acidity (average pH = 3.0, unaltered after freezing for prolonged periods), and experiments with each one of them gave similar results. Whole lime juice was expressed by hand or with a home juicer, and was used unprocessed (Mata & Vives 1992, Mata *et al.* 1994).

Other vibriocidal substances tested were: chlorine, prepared from a 3.5 % solution of sodium hypochlorite sold in local supermarkets; ozone, instantly released in water by an Ozone-O-Matic device (Colombia); and kilol L DF-100 (Provequi, Costa Rica), extracted from grape fruits (*Citrus* x *paradisi*), used to preserve vegetables and fish during transport.

Deionized sterile water, pH 6, was prepared in B-Pure Barnstead columns device and was autoclaved.

Vegetables were cabbage (*Brassica oleracea*) and lettuce (*Lactuca sativa*), purchased in agricultural open markets. Vegetables were washed in running potable water, gently dried, stored under refrigeration, and used within two days of purchase.

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

Dilutions of lime juice were made in sterile deionized water (Mata *et al.* 1994).

Experiments. Vegetables were exposed to known numbers of *V. cholerae* for varying periods, after which quantization was performed in log-10 dilutions of the fluid phase (soup) and of the macerate of the tissues of the salad. The macerate was prepared in a mortar with the aid of a pestle. The number of bacilli was estimated in cultures of log-10 dilutions of broth and macerate in APW and thiosulfate-citrate-bile salts-saccharose agar (TCBS), and the results were expressed as log-10 bacilli per ml or g. Details of methodology for different experiments will be given under Results.

RESULTS

Death of *V. cholerae* present in cabbage. Two portions of cabbage of 25 g each, sliced as if it were for a salad, were placed in different glass beakers. One hundred million vibrios contained in 1 ml of APW were added to each portion, mixed thoroughly, and left at room temperature for 30 min. Thereafter, the juice of three limes was added to one salad (experimental), leaving the other untreated (control). At 5, 15 and 30 min after addition of lime juice, 1 ml of soup and 1 g of tissue were removed from the experimental and control salads. Log-10 dilutions were prepared from the soup using sterile deionized water, and they were inoculated in APW and TCBS. The tissue was macerated and diluted using deionized water, and dilutions were inoculated in APW and TCBS.

Five min after addition of lime juice, no viable vibrios could be detected in the 1:100 dilution of the soup or macerate. The control salad had viable vibrios in numbers comparable to those in the inoculum (Table 1). Thus, lime juice eliminated more than 99.99 % of the bacilli originally seeded onto the vegetable.

TABLE 1

Death of 10 million Vibrio cholerae *O1 in contaminated* cabbage of, by action of lime juice*

Exposure time minutes	Treatment: Lime juice	None
5	0 **	7 ***
10	0	7
15	0	7
30	0	7

* Inoculum: 100 million vibrios
** 0 = No vibrios detected in APW
*** 7 = at least 10 million vibrios found in APW

Death of *V. cholerae* present in lettuce. Several different experiments were made. In one, four portions of lettuce of 25 g each were sliced as if to make salads. Each portion was contaminated with 100 million vibrios per g of lettuce, and left at room temperature for 4 hr. Thereafter, the four portions were immersed, each for 5 min, in separate 5 liter plastic pans, each containing 3 liters of deionized water, to which either of the following treatments were

provided: a) chloride prepared from commercial chlorine, added seconds before immersing the lettuce, to obtain a final concentration of 20 ppm. This was preceded by treatment of the lettuce with KW surfactant (du Pont de Nemours) in a dose of 0.5 ml/l); b) ozone prepared by machine, generated in the water seconds before immersion of the lettuce; c) undiluted lime juice (pH 3.0); and d) lime juice diluted 1:100 (pH 3.4). The lime juice and its dilution were prepared 1 h before immersion of lettuce because its lethal capacity is very stable and is not affected by organic matter as with chlorine and ozone. Log-10 dilutions were made from the soup and tissue of each salad portion, and inoculated in APW and TCBS.

At least 1 million vibrios per g remained viable in untreated salad (Table 2), but all treatments virtually eliminated the vibrios from the soup, evidenced by the absence of bacterial growth in the 1:10 dilution. This implies that more than 99.99 % of the inoculum was destroyed by the vibriocides. Unexpectedly, chlorine and ozone did not eliminate all vibrios from the lettuce tissue, because at least 1 000 000 vibrios per g of macerate remained viable (Table 2). Contrasting, undiluted lime juice reduced the numbers of vibrios to 100 or less per g of lettuce, or to 10 000 per g when a 10-fold dilution of lime juice was used.

Death of *V. cholerae* in lettuce: a likely scenario. The large mass of contaminating vibrios in the previous experiment is unlikely to occur in nature, a reason to use 1 000 000 vibrios per g of lettuce as inoculum, to avoid treating the lettuce with WK surfactant. The experiment was conducted as before, testing chlorine, lime juice and kilol to a final concentration of 500 ppm. All lettuce salads were left at room temperature for 2 to 4 h, at which point chlorine, kilol or undiluted lime juice were added as previously indicated. One portion of untreated lettuce was left as control. Exposure to the vibriocide for 5 min was by immersion as in the previous experiment. The number of vibrios in untreated lettuce decreased ten fold (Table 3). In contrast, the number of vibrios in the portions exposed to vibriocides was reduced by 99.99 % in soup and tissue, by all vibriocides.

TABLE 2

Death of Vibrio cholerae *O1 from laboratory contaminated lettuce*, by action of lime juice and other vibriocides*

Vibriocide	Broth	Macerate
None (control	7*	6
Ozone	0**	6
Chlorine	0	6
Lime juice:		
undiluted	0	2
diluted 1:10	0	4

* Inoculum: 10 million vibrios
** 10 million vibrios at dilution 1:10
*** No growth in APW or on TCBS at dilution 1:10

DISCUSSION

TABLE 3

Death of one million V. cholerae *O1 per g of lettuce, by treatment with vibriocides*

Treatment	Cultured material	Log 10 vibrios	Percent vibrios unrecovered
Chlorine	fluid	<2*	99.99**
	lettuce	<2	99.99
Kilol	fluid	<2	99.99
	lettuce	<2	99.99
Lime juice	fluid	<2	99.99
	lettuce	<2	99.99
None	fluid	5	0
	lettuce	5	0

* Less than 100 bacilli per g or ml.

The interest in the safety of vegetables and other foodstuffs stems from the demonstration that they can be contaminated with *V. cholerae* (Felsenfeld 1965, Prescott and Bhattacharjee 1969, Christian *et al.* 1976, Faechem *et al.* 1981, Kolvin and Roberts 1982, Faechem 1984). However, there has not been clear evidence of occurrence of cholera linked to consumption of raw salads, independent from the risk of drinking untreated water or other foods contaminated with feces. Nevertheless, 41 cases of cholera El Tor occurred in metropolitan Santiago, Chile, in April 1991, three months after the beginning of cholera in neighboring Peru. Most cases were associated with consumption of raw vegetables (67 %), water (10 %) and seafood (5 %). No risk could be established for 10 % of the cases. According to

one unpublished report (Shuval 1991), the outbreak was linked to vegetable gardens irrigated with sewage in the neighborhood of Santiago. The epidemic was controlled by destruction of orchards irrigated with sewerage and other drastic measures such as banning raw salads in restaurants, markets and streets (Shuval 1991). However, it should be remembered that having Chile and Costa Rica similar health profiles, cholera incidence was considerably less in Costa Rica. Raw salads continued to be eaten in Costa Rica throughout the cholera threat, despite a government campaign to encourage consumption of cooked salads. It should be remarked, however, that in Costa Rica it is traditional to add lime juice, vinegar, a 1:1 mixture of both, or other acidic dressings to lettuce, cabbage and salads in general.

We showed that millions of *V. cholerae* O1 El Tor, experimentally contaminating fish for ceviche, die rapidly during marination in lime juice (Mata & Vives 1992, Mata *et al.* 1994). We also showed that lime juice has a marked killing capacity against vibrios experimentally added to cabbage and lettuce, to common foods like rice and to drinking water (Mata *et al.* 1991a, 1991b, Mata & Vives 1992, Mata *et al.* 1993a, 1993b, Saborío *et al.* 1993, Mata 1994). The experiments presented here clearly show that lime juice eliminates millions of *V. cholerae* O1 from vegetables, within minutes. Large masses of vibrios added experimentally to vegetables, survived with some bacterial replication. Multiplication of vibrios on the surface of vegetables lead to rapid decay of the product (spoilage) making it unattractive for the preparation of salads. Despite this evidence, it is difficult to find vibrios on the surface of a wide variety of vegetables and fruits during the course of cholera epidemics (Felsenfeld, 1949), suggesting that contamination of vegetables play a limited role in transmission in certain ecosystems.

The vibriocidal effect of lime juice was clear when added to vegetables harboring 100 million vibrios per g of tissue. Such killing capacity was equivalent or greater (at high vibrio doses) than those of recognized vibriocides such as chlorine, ozone and kilol. Lime juice has the added advantage of having a considerable lesser cost than regular vibriocides, while its addition to food does not require particular training. Lime juice has prophylactic value when sprayed over pealed fruits that are prepared with the bare hands, or when added to traditional meals like rice, mashed potatoes, beans and meats (Mata & Vives 1992).

The value of acidic foods for the prevention of cholera became evident both in well controlled field studies (Tauxe *et al.* 1988, St. Louis *et al.* 1990), and in our laboratory experiments (Mata & Vives 1992, Mata *et al.* 1994). The studies described here showed that immersing vegetables in water chlorinated at home does not eliminate large masses of vibrios as effectively as lime juice, probably because part of the chlorine is taken up by the tissues of the vegetables.

Thus, the traditional custom of adding lime juice to salads has a scientific base. Such tradition should be preserved, supported and promoted without prejudice or inhibition. It is a simple measure that favors consumption of the products, since lime juice has appealing taste and odor and low cost, while it is an important element in the traditional diet, alone or mixed with vinegar, another potent vibriocide. The measure has special value for rural and slum populations where orthodox measures to prevent cholera may not be available.

The mass of vibrios found in experimentally contaminated vegetables are reduced to undetectable levels. Even if some bacilli remain in the vegetables (not evident in most of our experiments), such masses would be so small as not to have epidemiological significance, except for persons with enhanced cholera risks like achlorhydria, hypochlorhydria, stomach surgery, or ingesting medicines or drugs to diminish gastric acidity (Gitelson 1971, Schiraldi *et al.* 1974). Since ozone and chlorine rapidly loose their bactericidal capacity upon contact with organic matter (tissues of vegetables, dirt on surface), the use of lime juice seems more indicated to decontaminate lettuce and cabbage, particularly in the course of cholera outbreaks. These findings are relevant to the epidemiology of cholera because the consumption of raw vegetables is strongly rooted in the culture. Lime juice is also useful because of its widespread availability and relatively low cost. Finally, in areas where water, soap and other basic resources are scarce, the use of lime juice is highly convenient.

RESUMEN

El jugo de limón agrio elimina millones de *Vibrio cholerae* O1, El Tor, presentes en repollo y lechuga contaminados en el laboratorio. El efecto letal fue evidente al cabo de 5 min de exposición al jugo de limón, lográndose una reducción de más del 99.9 % del inóculo inicial. No se recuperaron vibrios a la dilución 1:10, cultivando en agua peptonada alcalina (APA) y en agar tiosulfato-citrato-sales biliares-sacarosa (TCBS). El número de vibrios destruido 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 del cólera El Tor. El tiempo requerido para matar los vibriones fue menor que el intervalo usual de espera en hogares o restaurantes. El vinagre tiene un efecto letal sobre el *V. cholerae*, similar al del jugo de limón. Se recomienda fuertemente la adición de jugo de limón agrio a los alimentos no ácidos, bebidas y agua de beber, para prevenir la infección con vibrios del cólera y otros microorganismos sensibles al ácido. Esta medida es particularmente importante para poblaciones rurales y urbanas marginadas de los trópicos y subtrópicos.

ACKNOWLEDGEMENTS

The authors wish to thank Carla Solórzano, María Elena Villegas, Sylvia Pacheco, Irlanda Jiménez and Freddy Fernández. 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.

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