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Protection from cholera by adding lime juice to food – results from community and laboratory studies in Guinea-Bissau, West Africa

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Summary Epidemiological studies have shown that food plays an important role in the transmission of *Vibrio cholerae*, and different foods have been incriminated in many epidemic outbreaks of cholera. Storing contaminated meals at ambient temperatures allows growth of *V. cholerae*. Some ingredients such as lime juice may inhibit the survival of *V. cholerae* in foods. During an epidemic caused by *V. cholerae O1* in Guinea-Bissau in 1996, a case control study was conducted in the capital Bissau, the main affected region with an attack rate of 7.4%. Cases were hospitalized patients and controls were matched for area, gender and age. Lime juice in the sauce eaten with rice gave a strong protective effect (odds ratio [OR] = 0.31, 95% confidence interval [CI] = 0.17–0.56), and tomato sauce was also protective (OR = 0.36, 95% CI = 0.24–0.54). On the other hand, use of a bucket for storage of water in the house was associated with increased risk (OR = 4.4, CI = 2.21–8.74). Laboratory experiments to elucidate the inhibitory effect of different concentrations of lime juice on survival of *V. cholerae* in meals showed that *V. cholerae* thrives in rice with peanut sauce, but lime juice inhibited its growth. Since lime juice is a common ingredient of sauces, its use should be further encouraged to prevent foodborne transmission in the household during cholera outbreaks.

keywords *V. cholerae*, food preparation, lime juice, inhibition, Guinea-Bissau

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Introduction

After seven years without *V. cholerae* infection, Guinea-Bissau was affected by a cholera epidemic in October 1994 which caused 15 719 cases and 290 deaths. In October 1996 a new epidemic caused by *V. cholerae O1* struck the country and by January 1997, 26 967 cases and 961 deaths had been reported, with a national attack rate of 2.4%. The epidemic began in the capital Bissau, which had 18 513 cases and 227 deaths, equivalent to an attack rate of 7.4%. Studies in Africa (Tauxe *et al*. 1988; St. Louis *et al*. 1990) have shown that contaminated cooked foods often constitute a risk of cholera infection in the household. In Guinea-Bissau, using hands to eat from the same bowl favours the risk of cholera transmission (Rodrigues *et al*. 1997). Rice is served in a bowl with the sauce, leftovers are often stored at ambient temperature. Poor hygiene in handling food allows contamination with *V. cholerae*, which may replicate and reach an infectious dose. Leftovers are often consumed without reheating. Factors such as low temperatures, high organic content, near-neutral pH, high moisture, and absence of competing flora enhance survival and growth of *V. cholerae O1* (Kolvin & Roberts 1982; PAO 1991). Acidity is known to prevent the survival of *V. cholerae* in food (Kolvin & Roberts 1982; Mata *et al*. 1994a,b). In a case-control study during the 1994 epidemic in Guinea-Bissau, lime juice in sauce eaten with rice was protective (Rodrigues *et al*. 1997) and a subsequent laboratory study corroborated this effect of lime in water (Dalsgaard *et al*. 1997). Thus lime juice should be advocated for water disinfection and food preparation. It is commonly used by many ethnic groups in the preparation of sauces eaten with rice and readily acceptable. Since a more specific

message was needed on the quantity of lime to be used, we undertook the studies presented here.

Materials and methods

The first suspected cholera case was admitted on October 6, 1996, to the National Hospital Simão Mendes in Bissau. *Vibrio cholerae O1*, biotype El Tor, serotype Ogawa, was isolated from faecal samples at the National Public Health Laboratory. Samples transported in Stuart medium were inoculated onto thiosulphate-citrate-bile salts-sucrose (TCBS) agar directly and after overnight (O/N) incubation in alkaline peptone water at 37 °C. Suspected colonies were tested by agglutination with *V. cholerae* O group 1 polyvalent antiserum (Difco Laboratories, Detroit, MI).

During a matched incident case control study from October 12th to November 1st, 339 patients with acute watery diarrhoea clinically diagnosed as cholera were admitted to the National Hospital Simão Mendes, where all cases in Bissau were treated. A case was defined as a hospitalized cholera patient resident in Bissau $>$ 5 years of age, since watery diarrhoea is common in children under 5 and not specifically indicative of cholera in this age group. Concurrent sampling was used to select controls. Cases were consecutively interviewed during hospitalization by trained interviewers. A structured questionnaire elicited information on type of drinking water, food consumption, hygiene and social activities in the week before hospitalization. Patients living outside Bissau, children and those who left the hospital or died before an interview could be conducted were excluded.

Each case was matched with a control of the same district, gender, and similar age. Controls for cases living in the Bandim and Belém districts (25%) were randomly selected using a computerized register from the demographic surveillance system of the Bandim Health Project. For the remaining areas in Bissau, a starting point was defined on a map using random numbers for angle and distance. At this point the interviewers spun a bottle to get a direction and used random numbers for steps. In the nearest household the person fulfilling the matching criteria was chosen (age $\pm$ 5 years). Interviews with controls took place in the afternoon and weekends using the same questionnaire about activities in the last week before the interview.

The National Commission against Cholera and the Research Committee of the Ministry of Health approved the study. Respondents were asked for informed consent before the interview.

Matched odds ratios were calculated and a conditional logistic regression was performed using variables likely to be correlated with the infection and with *P*-values $<$ 0.15 in univariate analysis.

For the laboratory experiments, a strain of *V. cholerae O1* isolate from the 1996 epidemic was inoculated into alkaline peptone water and incubated overnight at 37 °C. The final concentration of *V. cholerae* in alkaline peptone water was 1.5×10^8 cells/ml as determined by count under microscope. Peanut sauce was prepared and divided into three portions for 8–10 persons each. One portion was prepared without lime juice, one with the juice of two limes, and one with five limes. Portions of rice with the three peanut sauces and one with curdled milk were homogenized to a paste with a mechanical homogenizer; 15 g of each portion was weighed, transferred to polypropylene tubes, and inoculated with 1.5×10^5 cells/gram food from the overnight culture. The inoculated food was incubated in a humid environment at 29 $\pm$ 1 °C.

After 0, 1/2, 3, 6, 12, and 24 h of incubation, two parallel aliquots of approximately 0.1 g were weighed and diluted with 900 μL 0.9% NaCl solution. TCBS plates were inoculated with 5, 50 and 100 μl of these suspensions and incubated overnight at 37 °C. Sample concentrations were back-calculated from the colony count of each of the plates, and from each time interval and for each sample the highest value was registered.

Results

Case-control study

Of 339 hospitalized patients, 221 were included in the study, 98 males and 123 females. One hundred and eighteen patients were excluded for the following reasons: 76 were children $<$ 5 years of age, 19 could not be matched with controls, seven lived outside Bissau, seven were severe cases and could not respond, eight questionnaires were faulty and 1 case had chronic diarrhoea and thus did not fit the case definition of cholera. The mean age of cases was 30.5 years (range 6–90) and of controls, 30.9 years (range 4–87). Ethnic distribution was almost the same in both groups.

Of 442 people included in the study, 25 cases and 41 controls had attended funeral and memorial ceremonies. Participation in a ceremony *per se* was not associated with a risk (OR = 0.6, 95% C.I. = 0.34–0.97). However, those attending a funeral, a ceremony with a corpse, may have had a slightly higher risk, the OR being 1.46 (95% C.I. = 0.44–4.86). Consumption of drinks (OR = 4.04, 95% C.I. = 1.33–12.22) and food (OR = 6.21, 95% C.I. = 2.06–18.7) at ceremonies was more frequent among cases. The risk associated with eating at ceremonies involving a corpse was higher (OR = 7.66, 95% C.I. = 2.13–27.48) than at memorial funeral feasts (*toca choro*) (OR = 4.80, 95% C.I. = 0.39–58.01). Five of those who attended ceremonies manipulated the corpse (three cases and two controls); the three cases had dealt with corpses of persons who had died with diarrhoea.

Table 1 Risk factors for cholera using matched univariate and multivariate analysis

Factor	Cases	Controls	Odds ratio of univariate analysis (95% CI)	Odds ratio of multivariate analysis (95% CI)
Shellfish	63/221	68/221	0.90 (0.60–1.34)	1.00 (0.59–1.70)
Curdled milk	18/217	38/221	0.48 (0.27–0.86)	0.51 (0.23–1.10)
Peanut sauce	39/218	61/220	0.62 (0.40–0.97)	0.75 (0.41–1.36)
Tomato	56/215	119/219	0.36 (0.24–0.54)	0.43 (0.26–0.70)
Lime juice	145/221	197/221	0.31 (0.17–0.56)	0.30 (0.14–0.62)
Vinegar lime	65/119	41/221	2.21 (1.35–3.63)	1.41 (0.63–3.15)
City pump	14/220	28/219	0.38 (0.17–0.86)	0.33 (0.12–1.01)
Bucket/basin	45/218	14/213	4.40 (2.21–8.74)	4.73 (1.95–11.4)

Exclusive use of municipal water for drinking purposes was protective (OR = 0.38; 95% C.I. = 0.17–0.86). Storage of water in a basin or bucket was riskier than storage in a clay pot (OR = 4.4; 95% CI: 2.21–8.74). A few people used bottles for storage of water.

During the period considered, a third of people had eaten shellfish (29% of cases and 31% of controls). The use of lime juice in the sauce eaten with rice was protective against cholera (Table 1). This was also true for curdled milk, peanut sauce and tomato sauce in the rice. The use of lime vinegar was associated with a higher risk. A similar number of cases (42%) and controls (37%) had consumed cold leftovers (OR = 1.17).

Table 1 presents the adjusted OR for the all variables before exclusion. In the final multivariate analysis, three variables remained: lime juice (OR = 0.3; 95% CI = 0.14–0.63) and tomato sauce (OR = 0.4; 95% CI = 0.26–0.7) remained protective and the use of a bucket or basin for water storage (OR = 4.4; 95% CI = 1.9–11.4) was a risk.

Laboratory experiments

During the experiment, the pH of meals was relatively stable. The pH of rice with peanut sauce varied between 6.8 and 7.1. With lime juice added, the pH changed to 5.52–5.69 for two limes and 4.83–4.95 for five limes. Rice with curdled milk had a pH between 3.58 and 3.93. Temperature was 29 ± 1 °C.

V. cholerae thrived in rice with peanut sauce but not when lime juice was added during preparation. After inoculation of *V. cholerae* in a meal prepared with lime juice, the colony decreased instantaneously 2.5-fold for two limes and five-fold for five limes (Table 2). After 30 min the colony count had decreased four-fold for two limes and 21-fold for five limes. After 3 h colonies had disappeared. *V. cholerae* could not survive in curdled milk.

Discussion

The main result of our study is a significant protective effect of lime juice used in the sauce eaten with rice, which was also confirmed in a laboratory experiment demonstrating lime's inhibiting effect on the growth of *V. cholerae* in food. The study was conducted at the beginning of the epidemic when most controls should have been uninfected. Interviews were conducted in the afternoon and weekends, when most people were available at home. Difficult-to-measure confounding factors due to the selection of controls from the community were controlled for, since cases and controls were individually matched for area of residence.

In Guinea-Bissau sauce is usually served with rice and the leftovers are maintained at ambient temperature and often consumed without reheating. Cooked grain enhances the growth of *V. cholerae O1* (PAO 1991) and the consumption of cold leftovers constitutes a risk of getting cholera (St. Louis *et al*. 1990; Rodrigues *et al*. 1997). The inhibitory effect of lime is known from other studies and experiments

Table 2 Growth of *V. cholerae* in rice with peanut sauce with varying amounts of lime juice and in curdled milk. Units in colony forming units (CFU)/gram food

	Time					
Meal (rice with)	0	30 min	3 h	6 h	12 h	24 h
Peanut sauce without lime	15870	2269	34065	> 400 000	> 500 000	> 500 000
Peanut sauce with two limes	6258	3678	0	0	201	0
Peanut sauce with five limes	3142	762	0	0	0	0
Curdled milk	0	0	0	0	0	0

(Kolvin & Roberts 1982; D'Aquino & Teves 1994; Mata *et al*. 1994a,b; Mujica *et al*. 1994) and was corroborated in a previous case-control study performed in Bissau in 1994 (Rodrigues *et al*. 1997).

Lime vinegar was associated with an increased risk (OR = 2.22), although its pH was similar to that of fresh lime juice. When we controlled for simultaneous use of fresh lime juice, the risk was not significant. Lime vinegar is less tasty than lime juice and perhaps people use less of it. However, it is of interest to further study other factors in lime juice that might be responsible for the protection it confers against cholera.

Consumption of tomato sauce with rice was protective against cholera, both in this study and a previous one (Rodrigues *et al*. 1997). Further investigation is needed to elucidate the use of fresh tomato fruit and conserved pasta in the prevention of cholera, since it is only available for part of the year. The protective effect of peanut sauce appearing in univariate analysis became insignificant in multivariate analysis. In the Guinean study (St. Louis *et al*. 1990), the sauce was a risk factor for cholera when eaten cold. The explanation could be that some ethnic groups in Guinea-Bissau prepare peanut sauce with lime juice while this may not be done in Guinea.

The laboratory experiments corroborate the findings of the case-control studies, demonstrating that *V. cholerae* grows very well in peanut sauce, but not when the sauce is prepared with lime juice. The drop in colony count seen in all samples could in part be explained by cell inactivation due to the sudden environmental changes. However, the cell count decreased more and *V. cholerae* could not grow in sauces with lime juice. The taste of this sauce was considered good.

Consumption of curdled milk with rice was also protective. After inoculation in curdled milk, no colony of *V. cholerae* was found because of the low pH (3.5) and the existence of a competitive flora. In Mali, the consumption of millet gruel was associated with increased risk; adding curdled goat's milk provided protection against cholera (Tauxe *et al*. 1988).

Funeral gatherings are known to be responsible for cholera during epidemics (Tauxe *et al*. 1988; St. Louis *et al*. 1990) and in Guinea-Bissau have been incriminated in the transmission and development of outbreaks (Shaffer *et al*. 1988; Gunnlaugsson *et al*. 1998). Participation in funeral ceremonies as such was not associated with increased risk, but participation in ceremonies with corpses and consumption of food or drink at these events were important risk factors for contracting cholera. Gunnlaugsson *et al*. (1998) have shown the importance of disinfecting corpses in funeral ceremonies in a previous study from Guinea-Bissau. But as discussed by Bradley & Kasem (1998), the final route of transmission is contaminated food or water. As the general introduction of hand-washing with soap before preparation of food and eating requires a longer time and more effective measures of sensitization, lime juice should be recommended for the treatment of food and water.

In Guinea-Bissau municipal water is not chlorinated and not always available. Thus it is stored at home in different reservoirs. Most people use their own source of water. It is known that water storage at home fosters contamination, and clear recommendations for disinfecting water with lime juice should take into account local habits regarding manipulation of drinking water.

Since most people already use lime juice in preparing sauces, this habit should be further encouraged. While the amount of lime used varies within ethnic groups, even a small quantity (two limes in a sauce for 8–10 persons) has a strong effect. Thus the use of lime in food preparation could be an acceptable, effective, and inexpensive measure to prevent or reduce foodborne transmission of *V. cholerae* at home and at ceremonial gatherings. The results from our study could be used to develop messages for the prevention of cholera transmission in West Africa and other developing countries.

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