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Action against *Vibrio cholerae* O1 Tox$^+$ of Chemical Products Used in the Lemon Production

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ABSTRACT. Tucuman is the first lemon exporting province in Argentina and the fourth lemon exporter in the world. The present work was set up to study the survival of *Vibrio cholerae* O1 Tox$^+$ after application of different chemical products used in the lemon production (from its cultivation until its packing). The following products were studied: copper oxychloride, benomil (a carbamate), active chlorine, sodium-o-phenylphenoate, guazatine (a polyamine mixture), imazalil (an imidazole) and fresh and dehydrated lemon peel. Using different dilutions of the products above mentioned antimicrobial tests were carried out with different exposure times against *V. cholerae* Serogroup O1, Biotype El Tor, Serotype Inaba. The microorganism was used at concentrations of 10^2, 10^4, 10^6 and 10^8 CFU ml^{-1}, the latter one being considered as an infectious dose. The following results were obtained: 1) Active chlorine (chlorinated water) showed bactericidal activity at concentrations of 0.5 x 10^{-1}, 10^{-1} y 2 x 10^{-1}g l^{-1} after 10 min of exposure time. 2) Copper oxychloride, sodium-o-phenylphenoate, guazatine and imazalil showed bactericidal activity against *V. cholerae* at concentrations of 10^2 and 10^4 CFU ml^{-1}. 3) Due to the fact that the fruit is successively sprayed with several chemical products during its cultivation, it could be proposed that the result of the successive treatments is superior to the result of a treatment with each of the individual products. This consideration should be taken into account when evaluating the eventual protection of the lemon.

RESUMEN. Tucumán es la primera provincia exportadora de limón en la Argentina y cuarta en el mundo. El presente trabajo se realizó para estudiar la sobrevida de *Vibrio cholerae* O1 Tox$^+$ frente a la aplicación de diferentes productos químicos usados en la producción del mismo (desde su cultivo hasta su embalaje). Se estudiaron los siguientes productos: oxi-cloruro de cobre, benomil (carbamato), cloro activo, sodio-o-fenilfenoato, guazatine (mezcla de poliaminas), imazalil (imidazol) y la cáscara de limón fresca y deshidratada. Usando diferentes diluciones de los productos arriba mencionados se realizaron ensayos antimicrobianos con diferentes tiempos de contacto con la bacteria *V. cholerae* Serogrupo O1, Biotipo El Tor, Serotipo Inaba. El microorganismo fue usado en las concentraciones de 10,^{2} 10,^{4} 10^6 y 10^8 UFC ml,$^{-1}$ considerando la última concentración como dosis infectiva. Se obtuvieron los siguientes resultados: 1) el cloro activo (agua lavandina) mostró actividad bactericida en concentración de 0,5 x 10^{-1}, 10^{-1} y 2 x 10^{-1} g l^{-1} después de 10 min de tiempo de exposición. 2) El oxi-cloruro de cobre, sodio-o-fenilfenoato, guazatine e imazalil mostraron actividad bactericida contra *V. cholerae* en concentraciones de 10^2 y 10^4 UFC ml^{-1}. 3) Debido a que se pulveriza sucesivamente el limón con varios productos químicos durante el cultivo, se podría proponer que el resultado de los tratamientos sucesivos es superior al tratamiento con cada uno de los productos individuales. Esta consideración se debería tomar en cuenta cuando se evalúe la eventual protección del limón.

INTRODUCTION

Cholera is one of the oldest diseases of humanity. At the end of 1991 an epidemic burst out in Peru, and then expanded rapidly to many other Latin American countries.[3,4,5,8,26,27]

It is well known that food is an important vector in the transmission of this disease, which comes immediately after direct contamination with human faeces, contact with sick people, or contaminated drinking water, in order of importance.[20]

Food product such as fruits are probable vectors in the transmission of cholera, which facilitate the development of *V. cholerae* Serogroup O1. Although when the bacterium can be found in small number, the microorganism can grow very rapidly until it concentrations superior to the risk thresholds.[10]

Because Tucumán province is the most important lemon exporter in Argentina and the fourth in the world, we began this research with the aim to understand the survival of *V. cholerae* with respect to the different chemical products applied in the lemon industry; from its cultivation until its pack-

ing. The study was carried out in collaboration with the Tucumán Citrus Fruit Association.

The aim of this study was to verify if the chemicals used in the lemon production process possess bactericidal or bacteriostatic activity against the etiologic agent of cholera.

The processing used in the lemon industry is as follows: The lemon is sprayed in the fields with copper oxychloride, sodium-o-phenylphenoate and benomil to diminish the microbial population and the presence of insects. After the manual harvest, the lemons are treated with active chlorine, guazatine and imazalil, and subsequently waxed and packed in cardboard boxes.

MATERIAL AND METHODS

Microorganisms. A strain of *V. cholerae* Serogroup O1, Biotype El Tor, Serotype Inaba, toxin positive, was used in concentrations of 10^2, 10^4, 10^6 and 10^8 CFU ml^{-1}. This latter one was considered to be an infectious dose. In order to select the concentrations a *V. cholerae* growth curve was carried out. OD_{560} was measured every 30 min and plates were seeded to determine CFU. The number of CFU was selected according to OD.

The strain was kindly provided by the Reference Laboratory of Prof. Dr. Harry Smith, School of Medicine, Jefferson University, Jefferson, Philadelphia, USA.

Culture media. Alkaline Meat Extract Agar (MEA) and TSB were purchased from Difco laboratories Inc., Detroit, MI.

Chemical and lemon-derived products studied. 1. Copper oxychloride (1 ppm; 10^{-3} g l^{-1}), 2. Active chlorine (100 ppm; 10^{-1} g l^{-1}), 3. Methyl 1-(butylcarbamoyl)-benzimidazol-2-ylcarbamate, diluted at 50% of a 1 ppm (0.5×10^{-3} g l^{-1}) solution (hereafter referred to as benomil, the general name), 4. Sodium-o-phenylphenoate (2%; 2×10^{-2} g l^{-1}), 5. A mixture of reaction products of polyamines, comprising mainly of octamethylene diamine, iminodi(octamethylene)diamine, octamethylenebis(imino-octamethylene)diamine and carbamonitrile, diluted at 40% of a 1 ppm (0.4×10^{-3} g l^{-1}) solution (hereafter referred to as guazatine), 6. (±)-1-(2-allyloxy-2,4-dichlorophenylethyl)imidazole, diluted at 80% of a 1 ppm (0.8×10^{-3} g l^{-1}) solution (hereafter referred to as imazalil). All products were purchased from Tucumán Citrus Fruit Association, 7. Fresh lemon peel and 8. dehydrated lemon peel.

Dilutions and exposure time. Dilutions of the above mentioned chemical products were determined following the recommendations of the Citrus Fruit Cultivator Association, which refer to the treatment of citrus fruit in the fields and in the industrial processing. Two more dilutions (concentrations) were added to the chosen ones: One superior and one inferior to the standardized concentration.

For copper oxychloride, benomil, guazatine and imazalil exposure time of *V. cholerae* was 1, 3, 10 and 24 h. With active chlorine exposure time was 0.5, 1, 10 and 30 min. For sodium-o-phenylphenoate exposure time was 10, 30 and 60 min.

Fresh lemon peel was used in pieces of approximately 1 cm^2 and dehydrated lemon peel was used at dilutions of 10^2 g l^{-1} and 10 g l^{-1}; exposure time was 1, 3, 10 and 24 h.

Determination of the survival of the microorganism. Aliquots of the different concentrations of the products and the microorganism were mixed together. For all samples the initial concentrations of *V. cholerae* were determined, and after the exposure time to each of the chemical products the number of colonies was counted using the most probable number technique.[16]

The results were used to make graphics (CFU ml^{-1} against the exposure time; for each concentration a separate graph) in order to compare effectiveness.

RESULTS

Copper oxychloride: (Fig. 1) With a dilution of 10^{-2} g l^{-1} of the product and concentrations of the microorganism of 10^2, 10^4 and 10^6 CFU ml^{-1}, inhibition of the latter could be observed after 6 h of exposure. Dilutions of 10^{-3}g l^{-1} and 10^{-4}g l^{-1} did not show inhibition of the microorganism after 24 h of contact, using concentrations of the latter of 10^6 and 10^8 CFU $ml.^{-1}$

Benomil: (Fig. 2) Dilutions of 0.5×10^{-2}, 0.5×10^{-3} and 0.5×10^{-4} g l^{-1} of the chemical all inhibited *V. cholerae*, present at concentrations of 10^2 and 10^4 CFU ml^{-1}, within the first hour of exposure. The same chemical concentrations did not inhibit the microorganism even after 24 h of exposure, when the latter was present at concentrations of 10^6 and 10^8 CFU ml^{-1}.

Active chlorine: (Fig. 3) All the dilutions assayed showed complete inhibition of the microorganism, present at concentrations of 10^2 and 10^4 CFU ml^{-1}, during the observed period. With an active chlorine dilution of 0.5×10^{-1} g l^{-1} complete inhibition was first observed after 10 min of exposure, using concentrations of 10^6 and 10^8 CFU ml^{-1} of *V. cholerae*.

Sodium-o-phenylphenoate: (Fig. 4) Dilutions of 2×10^{-1}, 10^{-2} and 10^{-3} g l^{-1} inhibited the microorganism, present at concentrations of 10^2 and 10^4 CFU ml^{-1}, completely during all observed exposure times. A dilution of 2×10^{-1} g l^{-1} exhibited bactericidal activity against a *V. cholerae* concentration of 10^8 CFU ml^{-1} after 10 min of exposure.

Guazatine: (Fig. 5) All three dilutions assayed of the chemical demonstrated inhibition against a concentration of 10^2 CFU ml^{-1} of the microorganism. A dilution of 0.4×10^{-2} g l^{-1} of the product showed complete inhibition against a concentration of 10^4 CFU ml^{-1} of *V. cholerae* after 10 min of exposure. No bactericidal activity was observed after 24 h with any of the three dilutions of the chemical against *V. cholerae* concentrations of 10^6 and 10^8 CFU $ml.^{-1}$

Imazalil: (Fig. 6) A dilution of the chemical compound of 0.8×10^{-2} and 0.8×10^{-3} g l^{-1} presented total inhibition against concentrations of 10^2 and 10^4 CFU ml^{-1} of the microorganism. For higher concentrations of the microorganism, only partial inhibition of *V. cholerae* was observed.

Fresh lemon peel: (Fig. 7) Inhibition was observed at concentrations of 10^2 and 10^4 of *V. cholerae* after an exposure time of 1 h. *V. cholerae* at a concentration of 10^8 CFU ml^{-1} was not inhibited even after 24 h.

Dehydrated lemon peel: (Fig. 8) Suspensions of 10 and 10^2 g l^{-1} inhibited microorganism concentrations of 10^2 and 10^4 CFU ml^{-1}. However, *V. cholerae* concentrations of 10^6 and 10^8 CFU ml^{-1} were not inhibited.

DISCUSSION AND CONCLUSION

The need of economic integration between different countries of the whole world and an increase in international interchanges of food products make an increase in the epidemic vigilance and improvement of the control and prevention of diseases, of which food is the vector, necessary.

Although the substances used in this work are commonly used in the protection against insects, fungi and contaminating bacteria, their action against *V. cholerae* is unknown. Our studies demonstrate a partial or total elimination of the microorganism after 24 h of exposure to the products assayed. This contributes to the improvement of sanitary measures in order to avoid transmission of this agent.

There is more knowlegde about infectious microorganisms such as *Salmonella*, *Listeria* and *Vibrio* whose pathology is found to be involved in diseases transmitted by food.[2,6,13,23,24,25] Other authors have also observed that the types of food, storage conditions and previous treatment contribute to establish significant differences in the survival time of *V. cholerae*.[7,21] Felsenfeld[9] confirmed that in Thailand drinking water, ice and also fruit and vegetables, washed with polluted water, act as important vectors in the transmission of cholera. The fruit that grows in trees can be contaminated during irrigation.

Singleton et al.[22] proposed the study of the epidemiological survival of the bacterium in the environment, and they concluded that *V. cholerae* is able to grow in a natural medium under certain regimes of nutrients, temperature and salinity.

Gerichter et al.[10], the Pan-American Organization of Health (Organización Panamericana de la Salud[17]), Pavia et al.[18] and Pesigan et al.[19] established the survival times of the microorganism in fruit and raw vegetables, determining that the survival of the bacterium to ambient temperature does not exceed a week, which can be extended if the surface of these fruits and vegetables is rough.

Halstead[11], McIntyre et al.[14] and Yan et al.[28] showed that *V. cholerae* is able to survive for long periods under extreme conditions such as low temperatures and reduced nutrients concentrations. When the conditions turn back to normal it starts growing again. Its fast spreading is due to poor sanitary and environmental conditions.

Mossel et al.[15] suggest that a technological level of food safety should be implemented, which would assure good economic benefits to exporting countries. This can be obtained with rigorous application of preventive sanitary measures that permit a guarantee of the microbiological quality of all the exported products.

Adams et al.[1], Koek et al.[12] and Pesigan et al.[19] recommend washing and disinfection of vegetables and fruit in order to get them free of contaminants.

In this work we have showed the bactericidal activity of active chlorine against *V. cholerae*. This result coincides with observations made by Felsenfeld[9] who studied the effect of chlorinated solutions and a permanganate solution on food products and concluded that the chlorinated solutions are more effective under well controlled conditions of time and concentration.

We suppose that the increase in the number of microorganisms during the first hours of exposure to the products is because of the presence of precursors that have been formed previously. Once these have been used up the inhibitory effect of the products against *V. cholerae* is allowed.

With regard to our results and due to fact that the fruit is successively sprayed with several chemical products during the industrial process, including cultivation, it can be postulated that the sum of the effects of each of the individual products leads to a final treatment which is even more secure.

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