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ORIGINAL ARTICLE

Growth response of *Vibrio cholerae* O1 and *V. cholerae* non O1/non O139 strains to algae extracts from stream water in far north Cameroon

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ABSTRACT

Background and Objectives: *Vibrio cholerae* O1 or non O1/non O139 is found in water ecosystems where it colonizes phytoplankton and has different lifestyle. This study aimed to investigate the impact of some algae extracts on the survival/growth of both *V. cholerae* strains.

Materials and Methods: Algae extracts consisting of three fractions, F1 containing chlorophyll-a, F2 containing chlorophyll-b, and F3 containing carotenoids, and raw extract (RAE) were obtained from the algal bloom collected in the Kaliao stream (Maroua, Cameroon). The survival and growth of *V. cholerae* O1 and *V. cholerae* non O1/non O139, in microcosms consisting of sterile saline with these extracts and peptone (PEP) respectively added at concentrations of 0.01, 0.05 and 0.1 mg/L, and 50/50 mixtures F1+F2, F2+F3, and F2+PEP at a concentration 0.05 mg/L, were compared during a 24h experiment.

Results: The microcosms F2 and RAE did not support the growth of O1 strain; *V. cholerae* non O1/non O139 count in all algae extract microcosms ranging from 3.97 log (CFU/mL) to 5.2 log (CFU/mL). In all PEP microcosms, the counts of both strains reached an uncountable value. Microcosms F1+F2 and F2+F3 supported the growth of *V. cholerae* O1 and *V. cholerae* non-O1/nonO139 strains.

Conclusion: The algae compounds showed strain-specific effect on the growth of *V. cholerae.*

Keywords: *Vibrio cholerae*; Algae; Survival; Water; Chlorophyll; Carotenoids

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INTRODUCTION

Vibrio cholerae is a comma-shaped Gram-negative bacillus, facultative anaerobe, halotolerant, motile with a polar flagellum. Classification of *V. cholerae* based on the O antigen shows over 200 serogroups, among which only serogroups O1 and O139 are responsible for cholera (1).

Cholera is a diarrheal disease characterized by the emission of severe watery diarrhea with a "rice water" appearance, sometimes accompanied by vomiting (2). Cholera causes more than three million victims and 95,000 deaths annually worldwide (3). Africa is the continent most affected by epidemics of this disease, with nearly 50% of cases recorded each year. Lake Chad Basin, including north Cameroon, southeast Niger, northeast Nigeria, and south Chad, is one of the major endemic areas on the continent. Indeed, it was suggested that *V. cholerae* O1 is endemic to the Lake Chad Basin and is clonal and different from other African *V. cholerae* O1 (4).

V. cholerae strains can survive in the aquatic ecosystem, in several forms including: planktonic, attached to a substrate or to other organisms in the environment, or in a Viable but Non-Culturable (VNC) state (5, 6). The occurrence of cholera epidemics usually coincides with an increase in the prevalence of toxigenic *V. cholerae* O1 in aquatic environments where physico-chemical (pH above 8, temperature above 15°C, and salinity up to 6% NaCl), biological (reduced predation by protozoa, the lifting of dormancy due to osmotic stress, and an algal bloom) parameters, and human activity (anthropogenic water pollution) are likely to positively influence its survival and abundance (7-12). A previous study demonstrated a link between the frequency of cholera cases, temperature, and seasonal water abundance of phytoplankton and zooplankton in the Bay of Bengal (13).

According to Islam et al. (7), non-O1 environmental strains were found both attached to particles or hosts as well as free living in water and readily culturable, while pandemic-related *V. cholerae* O1 were found mostly as particle/host-associated and in a VNC state. This suggests that pandemic *V. cholerae* O1 differs from other strains of the species because of its unique environmental lifestyle. There is an evidence that certain species of algae can act as reservoirs of *V. cholerae* O1. *V. cholerae* O1, and was found to remain viable for a longer period in association with *Ulva lactiva* and *Rhizoclonium fontanum* (14). A functional explanation for this association was provided by several studies (14-16). Algae produce a number of extracellular products in a mucilaginous sheath, providing a protective nutrient-rich microenvironment for *Vibrio* species (14). Relationship between algae and *V. cholerae* can also span antagonistic interaction. Indeed, algae can produce a number of compounds that have antibacterial effect by disrupting respiration and cell division (17, 18). Synergistic effect of these substances could arise (18). Anas et al. (19) showed that the probability of the presence or absence of *V. cholerae* O1 in Kerala Lake (India) is a function of the chlorophyll concentration in this environment. However, to date, no study has assessed the role of algal compounds in the persistence of *V. cholerae* O1 in freshwater environments in cholera-endemic areas of Cameroon. The aim of this study was to comparatively determine the impact of algal extracts on the survival and growth of O1 and non O1/non O139 *V. cholerae* in aquatic microcosms.

MATERIALS AND METHODS

Study site. The Far North Cameroon region is located between 9°58' and 13°03' North latitude and 13°31' and 15°47' East longitude. This region belongs to the Sudano-Sahelian zone, characterized by the alternation of the dry season (October to May) and the rainy season (June to September). The annual rainfall is low, around 811.6 mm, with peaks in July and August (between 200 and 260 mm/month). The relief varies and consists of plains and mountains. Most rivers have non-permanent flows, which are more related to the length of the dry season than to low annual rainfall. The lack of respect for hygiene measures and insufficient environmental sanitation encourage the development of waterborne diseases, such as cholera, malaria, salmonellosis, bilharzia, and dysentery (20).

Algae collection. Twenty (20) liters of water containing green algae colonies from Kaliao stream (Maroua, Far North Cameroon) were collected thrice and filtered through a 1mm mesh sieve. Algae retained by the sieve were collected in a dark bottle (SIMAX, Czech Republic) and transported to the laboratory. The algae were dewatered and air-dried in shade for 72 h at room temperature (38 ± 2°C) (21). Few physicochemical parameters of the collected water samples

were measured, including temperature, pH, electrical conductivity, total dissolved solids and salinity using a multiparameter meter (Extech EC 500, USA).

Extraction and isolation. Air-dried powdered algae (106 g) were ground in a mortar, sieved and macerated in 400 ml methanol (Fisher Scientific, China) at room temperature for 3x72 h (18). The mixture was filtered and concentrated using a rotary evaporator (Buchi R-300, China) (64.7°C) to yield 0.5 g of raw extract (RAE). The crude algal extract was subjected to silica gel column chromatography (Merck, Germany) (SiO2, 0.063-0.200) in order to isolate compounds, following the method outlined by Yanda et al. (22). Hexane and ethyl acetate (Fisher Scientific, China) were used as eluents because of their affinity to these compounds (21-23). Elution was carried out using a gradient of increasing polarity of solvents (hexane, hexane-ethyl acetate) in a similar way to Yanda et al. (22). The afforded fractions (F1 containing chlorophyll-a, F2 containing chlorophyll-b and F3 containing carotenoids) were concentrated by dry evaporation and separated by thin layer chromatography.

Preparation of bacterial strains. The bacterial cells used in this study belong to the non-O1/non-O139 and O1 serogroups of *V. cholerae*. *V. cholerae* O1 has been responsible for cholera epidemics in Cameroon. The *V. cholerae* O1 strain was obtained from the *Centre Pasteur du Cameroun*. Non-O1/non-O139 *V. cholerae* cells were isolated from the Kaliao stream (Maroua, Far North Cameroon) on Thiosulfate Citrate Bile Sucrose (TCBS) agar (Liofilchem s.r.i. Bacteriology Products Italy). The yellow and shiny colonies on TCBS were further confirmed by biochemical tests. The confirmed *V. cholerae* isolates were submitted to the agglutination test on clean glass slides using polyvalent O1 and O139 antisera (Denka Seiken, Japan) (24). A bacterial stock was prepared for each strain after culturing on brain heart infusion agar (Liofilchem s.r.i. Bacteriology Products Italy) (25).

Experimental design. The experiment consisted of introducing under aseptic conditions a bacterial inoculum of density 3.97 log (CFU/mL) in 13 test tubes each containing 5 mL of sterile saline to which F1, F2, F3, RAE and PEP were added at concentrations of 0.01, 0.05 and 0.1mg/L (21, 25, 26). Furthermore, three additional microcosms consisting of the respective 50/50 mixtures F1+F2, F2+F3 and F2+PEP at the concentration 0.05mg/L were added in a second experiment. The control microcosm consisted of saline inoculated with each bacterial strain (21, 25). Incubation of the different microcosms was performed at 37°C and checked at the time intervals: T0=0 h, T1=8 h, T2=16 h and T3=24 h. At the each time point for each strain of *V. cholerae*, colony forming units (CFU) were counted by plating 0.1 mL of each microcosm content on alkaline nutrient agar (Liofilchem s.r.i. Bacteriology Products Italy). The bacterial concentration was expressed in colony forming unit (CFU) per volume of water. All experiments were performed in triplicate (21, 25).

Quality control. The quality control concerned the culture media used for the study to ensure that they provide the best conditions to support the growth of bacterial strains. This control included accurate weighing and measurement of raw materials, pH control and adjustment, sterilization validation, and media performance testing (27). Control used for sterility of microcosms prior to each experiment was also considered as a quality control measure (21, 27).

Data analysis. Curves showing the variation in bacterial counts in the different microcosms were obtained. The data were log(x+1)-transformed to approach normal distribution. Analysis of variance (ANOVA) and Tukey post-hoc tests were performed to compare the bacterial counts in the microcosms. The R software (Rcore Team) was used to analyze the data.

RESULTS

Physicochemical properties of water samples. The temperature of the sampled water from Kaliao stream was 37.2 ± 0.6°C. The pH of water was 6.87 ± 0.8. The mean electrical conductivity of sampled water was 530 ± 23 µS/cm. The mean level of total dissolved solids was 318 ± 43 mg/L. The mean water salinity was estimated at 254 ± 41 ppm.

Variation in counts of *Vibrio cholerae* in the presence of individual algae extracts in microcosms. *V. cholerae* non O1/non O139 showed a higher resistance to the antibacterial activity of algae extracts,

and a slower increase of its bacterial counts in most microcosms (Fig. 1). However, no significant difference in bacterial counts was observed between all microcosms and the control group (P>0.05).

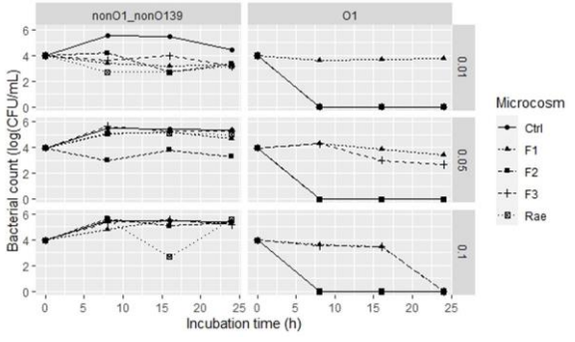

Fig. 1. Abundance dynamics of *V. cholerae* O1 and *V. cholerae* non O1/nonO139 as a function of incubation time in different microcosms containing the isolated pigments at concentrations ranging from 0.01 to 0.1 mg.L^{-1}.

The inoculation of bacteria in most microcosms inhibited the growth of *V. cholerae* O1 in the first series of experiments (Fig. 1). *V. cholerae* O1 was less influenced by the presence of F1 and its colonies could be detected at the end of the experiment for concentrations of F1 lower than 0.1 mg.L^{-1}. In all other microcosms, colonies of this isolate were not detected after 16 h except F3 (0.05 mg.L^{-1}) which showed colonies 24 h after bacterial inoculation. At 0.05mg/l concentration, the count of *V. cholerae* O1 in F1 increased from 3.97 log (CFU/mL) to 4.3 log (CFU/mL) after 8 h, then decreased to 3.84 log (CFU/mL) (16 h) and slightly to 3.39 log (CFU/mL) (24 h). In the F3 microcosms, *V. cholerae* O1 counts ranged from 3.97 log (CFU/mL) to 5.28 log (CFU/mL) (8 h) and decreased to 3.00 log (CFU/mL) (16h) and 2.69 log (CFU/mL) (24 h). For the 0.1mg/l concentration, bacterial count of *V. cholerae* O1 in F1 ranged from 3.97 log (CFU/mL) to 3.6 log (CFU/mL) (8 h). The bacterial count decreased to 3.47 log (CFU/mL) (16 h) and was cancelled after 24 h. We noted that the variation in the growth of *V. cholerae* O1 in F1 was similar to that observed in the microcosms containing F3 (0.1mg/l). In the microcosms containing crude algal extracts and F2 at concentrations of 0.01, 0.05 and 0.1 mg/L we observed no growth of *V. cholerae* O1 (Fig. 1). In all microcosms containing peptone, an uncountable number of *V. cholerae* O1 and nonO1/nonO139 colonies appeared. Tukey's test showed significant differences between the mean counts of *V. cholerae* O1 in F1 and F2, and Control and RAE microcosms (P<0.05). There was a significant difference between the mean counts of *V. cholerae* O1 and *V. cholerae* non O1/non O139 in F2, Control and RAE (P<0.05).

Bacterial counts in the presence of combined extracts. The growth of *V. cholerae* O1 and non-O1/non-O139 varied with time in the F2+F1, F2+F3 and F2+PEP microcosms (Fig. 2). For the *V. cholerae* O1 strain, a variation in the bacterial count between 3.97 log (CFU/mL) and 5.52 log (CFU/mL) (8 h) was noted in F2+F3. After 16 hours of incubation, this bacterial count decreased slightly to 5.48 log (CFU/mL). This count decreased to 4.95 log (CFU/mL) (24 h) in microcosms containing F2+F1. In the microcosm containing F2+PEP the growth of *V. cholerae* O1 varied from 3.97 log (CFU/mL) to 5.34 log (CFU/mL) (24 h).

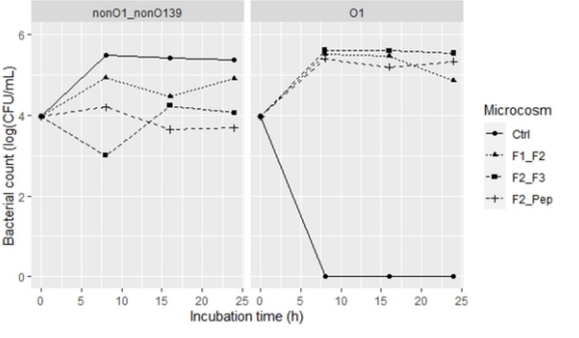

Fig. 2. Abundance dynamics of *V. cholerae* O1 and *V. cholerae* non O1/nonO139 as a function of incubation time in different microcosms containing 50/50 mixed pigments at concentration 0.05 mg.L^{-1}.

For the non-O1/non-O139 *V. cholerae*, bacterial counts ranging from 3.97 log (CFU/mL) to 4.93 log (CFU/mL) (8 h) were observed. This value decreased to 3.00 log (CFU/mL) (16 h) and was reached again after 24 h in the F2 +F1 microcosm. In the microcosm containing F2+F3, bacterial growth of *V. cholerae* non O1/ non O139 was observed from 3.97 log (CFU/mL) to 4.06 log (CFU/mL) (24 h). In microcosms F2+PEP, counts of *V. cholerae* non O1/ non O139 varied from 3.97 log (CFU/mL) to 4.20 log (CFU/mL) (8 h) and decreased to 3.69 log (CFU/mL) (24 h).

Bacterial counts stabilized above the initial bacterial concentrations for both strains after 24 h of incubation for all combined algal extracts, demonstrating that the addition of F2 to other algal extracts increased bacte-

rial growth. The concentration of *V. cholerae* non O1/non O139 was significantly lower than the concentration of *V. cholerae* O1 in F2+PEP ($P<0.05$). *V. cholerae* O1 was the most resistant to the F2 antibacterial effect in the second series of experiments.

DISCUSSION

Temperature affects physical and chemical water properties. In particular, it affects the density, viscosity, and solubility of gases in water as well as the rate of chemical and biochemical reactions in microbial cells (28). The recorded temperature (37.2°C) is optimal for heterotrophic bacteria and algae growth. The pH of sampled water was slightly acidic (6.87 ± 0.8) to basic. The pH of water depends on the dissolved CO_2 due to the equilibrium between H_2CO_3, HCO_3^-, and CO_3^{2-} chemical species (28).

According to Cai et al. (29), the Kaliao stream waters (mean electrical conductivity=530 µS/cm) have high mineralization level. The household waste and the nature of the soil could explain the recorded values of electrical conductivity.

The high levels of TDS could indicate significant water pollution, depending on their composition (30). Salinity was linked to TDS and water electrical conductivity.

Exposure of *V. cholerae* O1 to the different algal extracts in the microcosms showed varied growth depending on the compounds and exposure time (Fig. 1). The growth of *V. cholerae* O1 in F1 is thought to be due to the presence of protoporphyrin molecules, in which iron is the main cofactor in the enzymatic system that converts protoporphyrin to chlorophyll (31, 32). This accessibility of iron in protoporphyrins is thought to be responsible for maintaining the growth of *V. cholerae* O1 in microcosms (33). It was suggested that chlorophyll-a is the main algal pigment responsible for maintaining *V. cholerae* in an endemic area (34). F2 was not favorable for the development of *V. cholerae* O1 under experimental conditions. Chlorophylls a and b belong to a group of tetrapyrrolic pigments with common functions and structural elements, including five-membered isocycles. However, chlorophyll-b differs from chlorophyll-a by the presence of an aldehyde (formyl) group instead of a methyl group at the C (7) position. Indeed, aldehydes have electrophilic centres that attract free electrons and can interact with amines to form compounds that are toxic to bacterial cells (35). Some of these compounds can cause DNA damage, which may explain their antibacterial activity against *V. cholerae* O1.

Organic matter in aquatic environments is considered as one of the main sources of essential carbon for most heterotrophic bacteria. It plays an indispensable role in persistence and survival of *V. cholerae*. The high abundance of *V. cholerae* (O1 and non O1/non O139) in the presence of organic matter (PEP) is explained by the fact that the peptone used is rich in synthetic nutrient molecules, inducing the resurrection of *V. cholerae* that has become dormant in the aquatic environment; hence, it is used in the enrichment of dormant bacterial cells (36).

The absence of growth of *V. cholerae* O1 in the crude algal extract (RAE) could be due to the presence of numerous bioactive molecules with cytotoxic effects, such as phenolic compounds, terpenes, and aldehydes, responsible for the antibacterial activity. These results are similar to those of Gomes et al. (31), who suggested the development of new antibiotics from polar molecules, such as certain pigments and phenolic compounds, in algae. Several algae showed significant antibacterial and antioxidant effects against a range of pathogenic strains (37-39). In a recent study, algae extracts inhibited the growth of *Pseudomonas aeruginosa* and biofilm formation (21). The counts of *V. cholerae* non O1/non O139 in microcosms F1, F2, F3, RAE, and PEP are explained by the fact that this bacterium was isolated from the same aquatic environment as the algae used for the extraction of the test compounds. *V. cholerae* is an indigenous bacterium of the aquatic environment that lives in association with aquatic organisms such as algae. The co-evolution of this association would have enabled *V. cholerae* non O1/non O139 to detect and respond rapidly and adequately to changes in the aquatic environment and even develop natural adaptation mechanisms against certain bioactive compounds in algae (6).

F2 showed an important growth-inhibitory effect on clinical strain (O1) (Fig. 2). Therefore, the combination of this fraction with the other fractions and PEP was tested on *V. cholerae* O1 and non O1/non O139 strains to assess the antibacterial effect of F2 combined with other algal constituents on both *V. cholerae* strains. The growth of *V. cholerae* non O1/non O139 was expected in different microcosms as this bacterium lives in the same environment as algae

producing the tested compounds. The survival and growth of *V. cholerae* O1 in the microcosm containing the combination of F2 and F1 could be explained by the existence of molecular interactions between these two compounds, which would be the origin of the inhibition of the observed antibacterial effect of F2. The growth of *V. cholerae* O1 in the microcosm containing the combination of F2 + F3 would be due to the fact that carotenoids are organic compounds in plants known for their antioxidant properties (40). Their combination with compounds with oxidative activity would allow them to protect *V. cholerae* cells the antibacterial effects, thus promoting the survival and growth of *V. cholerae*. The growth of *V. cholerae* in F2 + PEP is explained by the fact that the organic matter used (peptone) is rich in nutrients, such as growth factors, proteins, and enzymes; hence, it is used in enrichment broths for the resurrection of viable but non-culturable bacteria. These growth-inducing substances present in peptone are believed to be responsible for the neutralization of the antibacterial effect of F2.

F1 and F3 were responsible for the growth and survival of *V. cholerae* O1, whereas F2 showed antibacterial activity against the clinical strain. The environmental strain survived and grew in the presence of all isolated and combined algal components, without distinction. It was found that the specificity of the attachment between wild type *V. cholerae* strains and cyanobacteria, was partially mediated by mucin-dependent chemotaxis. *V. cholerae* strains without the mucinase gene could not be associated with these algae (14).

Limitation. This study used algae extracts from an algal bloom and contributes in understanding the interactions of *V. cholerae* with algae in freshwater. However, further studies are still required to shed the light on the impact of single-species algae extracts on the *V. cholerae* strains.

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