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Role of Chemotaxis in the Attachment of *Vibrio cholerae* 0139 with Different Aquatic Flora and Fauna

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Abstract: Bacterial chemotaxis is one of the important mechanisms of association of bacteria and other living biota. To investigate the role of chemotaxis that might function for association of *Vibrio cholerae* -0139 with different aquatic flora and fauna, the homogenates of four aquatic plants and animals namely water hyacinth (*Eichhornia crassipes*), water cress (*Pistia stratiotes*), oyster (*Lamellidens marginalis*) and snail (*Pila globosa*) were examined in chemotaxis capillary assay. Chemotaxis assay revealed that *V. cholerae* -0139 swims towards the homogenates of aquatic plants and animals with a higher chemotactic response being observed for 4% solution of *P. globosa* homogenate. The influence of temperature and salinity on the chemotaxis of *V. cholerae* -0139 revealed that at 25°C and 1.7% salinity favoured the chemotactic motility towards the homogenates of aquatic flora and fauna. *Vibrio cholerae* -01 also showed the chemotactic response to the homogenates of snail muscles.

Key words: Chemotaxis, *Vibrio cholerae*, flora, fauna

Introduction

Cholera, a dreadful water-borne diarrhoeal disease, is one of the three diseases subjected to International Health Regulations caused by *Vibrio cholerae* specially serotype 01 and 0139. Certain parts of Bangladesh are endemic zones for cholera (Glass *et al.*, 1982). Currently the Indian subcontinent is experiencing an epidemic of cholera caused by *V. cholerae* 0139 (synonym 'Bengal') and has emerged as the second aetiological agent of this disease (Albert, 1994). So far, 7th pandemic of cholera having been recorded: the causative agent of the last three pandemics was *V. cholerae* -01. The newly emerged *V. cholerae* -0139 is now suspected to be the causative agent of the 8th pandemic of cholera (Albert, 1994). Various reports have been published on the isolation of *V. cholerae* -0139 from various aquatic and clinical sources from different parts of the world including Bangladesh (Islam *et al.*, 1994a, Lee *et al.*, 1996, Sengupta *et al.*, 1994, Pokhrel *et al.*, 1996, Tay *et al.*, 1994). Huq *et al.* (1995) demonstrated the coexistence of *V. cholerae* -0139 and 01 in plankton. We have reported earlier the occurrence of culturable *V. cholerae* -0139 with *ctx* gene in various components of the aquatic environment including snails (*P. globosa*) in Bangladesh (Islam *et al.*, 1996). The isolation of *V. cholerae* -0139 during their first appearance until their transient disappearance in 1994 and reemergence in 1995 and 1996 (Faruque *et al.*, 1997) pose a question to the investigators about their possible reservoirs during interepidemic period. The reservoirs and sites of survival and multiplication of *V. cholerae* during interepidemic period are not well understood. One of the hypothesis suggested that during interepidemic period the organism may hide in specially selected micro habitats and possibly that the blue-green algae may provide such micro environment (Islam *et al.*, 1994b). Islam and Aziz (1981) reported the association of *V. cholerae* non-01 with some aquatic macrophytes from Bangladesh, especially water hyacinth (*E. crassipess*). Spira *et al.* (1981) observed the same for virulent *V. cholerae* biotype El Tor. Islam *et al.* (1990) observed the association of toxigenic *V. cholerae* -01 with a duckweed, *Lemna minor*. Recently a report has been published on the isolation of *V. cholerae* non 01 and *V. mimicus* from various aquatic components especially the oysters, snails and water hyacinth (Anowara and Khan, 2001). Molecular analysis of *V. cholerae* -01, 0139, non –01 and non-0139 revealed that both clinical and environmental strains retained the core of the *ctx* genetic elements and also TCP and OMP both of which are recognized as important components in pathogenicity, even in the aquatic environment (Singh *et al.*, 2001). The same study reported that in the absence of cholera toxin, NAG specific heat stable toxin and TCP and OMP, *V. cholerae* -01, 0139 and non-01 and non-0139 strains that have the ability to cause diarrhea by a mechanism entirely different from that of the toxigenic *V. cholerae* -01 and 0139 strains. This study indicated that both the clinical and environmental isolates of *V. cholerae* have the same extent of pathogenic potentials. Though various reports have indicated about the reservoirs of *V. cholerae*, their mode of attachment and subsequent colonization to various aquatic flora and fauna remains an enigma to the investigators. Pearl (1985) hypothesized that chemotaxis might play a role in the establishment and maintenance of cyanobacterial and bacterial association. However, no report has been published so far regarding the chemotaxis of a potent human pathogen *V. cholerae* -0139. The primary objective of this study was to measure the chemotactic response of *V. cholerae* -0139 to the homogenates of various aquatic flora and fauna and influence of environmental factors on chemotactic movement.

Materials and Methods

Bacterial strains and chemicals: An environmental isolate of *V. cholerae* -0139 (DWP-341) was obtained from the Environmental Laboratory, ICDDR, B. The purity of the strain was reconfirmed by cultural, biochemical and serological tests and was maintained in T1N1 soft agar (Trypticase 1%, (BBL) NaCl 1% and agar 0.8%) in vials filled with paraffin oil at room temperature. Both the strains of *V. cholerae* -01 and 0139 were grown at 37 °C in Trypticase soy broth (TSB) (BBL). The vibrio-selective medium used was TCBS agar (Difco Laboratories, USA). The soft agar used for assaying the motility of vibrio strains was TSB plus 0.3% agar. The chemotaxis and washed medium used in this study was phosphate-buffered saline (PBS)(pH 7.4). All compounds tested in the chemotaxis assay were purchased from Sigma

Chemical Co. (USA). In all experiments glass-distilled deionized water was used.

Collection of aquatic samples: Several ponds and rivers were selected in Matlab area in Bangladesh, which is hyper-endemic zone of cholera located about 45 km south-east of the capital city, Dhaka. From each selected area aquatic flora and fauna namely water hyacinth (*Eichhornia crassipes*), water-cress (*Pistia stratiotes*), snails (*Pila globosa*) and oysters (*Lamellidens marginalis*) have been collected during the year 1997 and 1998, according to the standard methods described before (Bordner and Winter, 1978).

Processing of samples for chemotaxis assay: The roots and submerged portion of aquatic plants and hard shells of snails and oysters were washed vigorously several times with previously autoclaved PBS. The shells of the snails were broken with a hammer and shell of the oyster was opened, using a sterile spatula. The muscles from both snails and oyster were washed many folds again with PBS. Ten g of sample from each species after mixing with 90 ml of PBS was homogenized with a commercial blender (USA) and finally with an ultrasonicator to make a fine homogenate suitable for chemotaxis study. Different concentrations of homogenates used for chemotaxis assay were 0.5, 1.0, 2.0 and 4.0%.

Chemotaxis towards carbohydrates: The chemotactic response of *V. cholerae* -0139 towards glucose and lactose, two important carbohydrates for characterization of *Vibrio* spp. was measured in a capillary chemotaxis assay using the concentrations of 10^{-1} M, 10^{-2} M, 10^{-3} M, 10^{-4} M and 10^{-5} M.

Chemotaxis assay: A modification of the quantitative capillary assay (Adler, 1973) was used to measure the chemotaxis of *V. choleare* -0139. Strain of *V. choleare* -0139 was grown overnight in TSB. The overnight culture was diluted 10 folds in TSB and incubated for up to 4 h to maximize the number of motile cells before proceeding to assay. The bacterial cells were harvested at 8000x g for 5 min and resuspended in an equal volume of PBS. This washing step was repeated three times, and the final resuspension was made in chemotaxis medium (PBS) to give an estimated cell density of 10^{10} bacterial cells per ml. Serial dilutions of the bacterial suspension was made in PBS and viable cell plating was performed. A suspension of bacteria in PBS at an estimated concentration of 10^7 viable bacteria/ml was dispensed in 200-μl aliquots into 1 cc syringe {(B-D)R Brand, Becton Dickinsion medical products Ptc. Ltd. Singapore}. A 1-μl capillary tube (Drummond Scientific Co.), heat sealed at one end and containing the homogenate (in PBS) to be tested in half the length of the tube, was inserted horizontally into the syringe to approximately 1.0 cm below the surface of the bacterial solution. After incubation for 15, 30, 45, 60, 75 and 90 min at room temperature, the capillaries were removed and exterior rinsed with a thin stream of PBS, and their contents were expelled into a specific amount of chemotaxis medium. A ten fold dilutions were made in the same medium and plated on TCBS plate and counts of viable cells were performed on the capillary contents. In each experiment, homogenates of each species were simultaneously tested in triplicate, and control capillaries containing PBS were included. The chemotactic activity of a particular species was expressed in terms of percent accumulation, i.e., the ratio of accumulation of bacteria in homogenate-containing capillaries to the number of bacteria in 200 μl contained in syringe multiplied by 100.

Influence of temperature and salinity on the chemotaxis of *V. choleare* 0139: The effect of different values of temperature and salinity on the chemotaxis of *V. choleare* -0139 to the homogenates of various aquatic plants and animals was evaluated. Bacterial culture was prepared as described above, to obtain a suspension with approximately 10^7 bacterial cells per ml. The bacterial suspension was incubated for 8h in artificially prepared saline water at different conditions of temperature and salinity. The homogenates of different species were prepared in 1, 1.7 and 3.5% salt solution and chemotaxis assay was performed as described above at 15, 20 and 25 ^{0}C.

Chemotaxis of *V. cholerae* 01: The chemotactic response of *V. cholerae* -01 was observed against various concentrations of homogenized snail muscles in a capillary chemotaxis assay according to the method described above.

Statistical analysis: Statistical analysis of the data was performed using the analysis of variance. The differences between two groups were analyzed by Student's t-test.

Results

Chemotactic response of *V. choleare* -0139 towards carbohydrates: Fig. 1 shows the percentages of accumulation of *V. cholerae* 0139 in capillaries with different concentrations of glucose. The capillaries without attractant (control, Fig.1) showed a relatively small percent bacterial accumulation at different time intervals and are insignificant at 5% level of significance (P>0.05). However, maximum accumulation was observed at a concentration of 10^{-3} M at 90 min, where as the lowest accumulation was achieved at the highest concentration (10^{-1}M) of glucose at the same time (Fig. 1). On the other hand the chemotactic response with lactose demonstrated a much lower accumulation of the strain into the capillaries (data not shown) indicating that lactose rarely acts as chemoattractant for *V. cholerae* -0139.

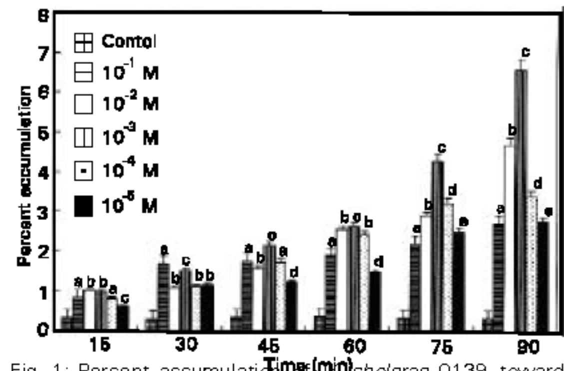

Fig. 1: Percent accumulation of *V. cholerae* 0139 towards various concentrations of glucose. Error bars stand for standard deviation. Bars with different letters (Within each time interval) are significantly different (P<0.05).

Chemotactic response of *V. choleare* -0139 to the homogenates of aquatic plants: The accumulation of *V. cholerae* -0139 in capillaries with different concentrations of homogenates of water hyacinth at specified time of incubation is shown in Fig. 2A. When the capillaries contained no attractant, designated as control (Fig. 2A, control), a small number of bacteria were found to move inside the capillaries.

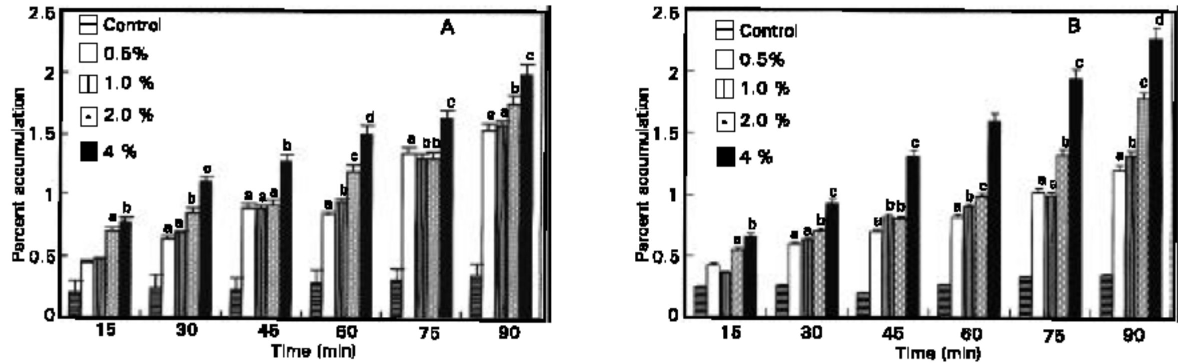

Fig. 2: Chemotaxis of *V. cholerae* -0139 towards the homogenates of (A) water hyacinth (*E. crassipes*) and (B), water cress (*P. stratiotes*). Error bars indicate standard deviation. Bars with different letters (within each time interval) are significantly different (P<0.05).

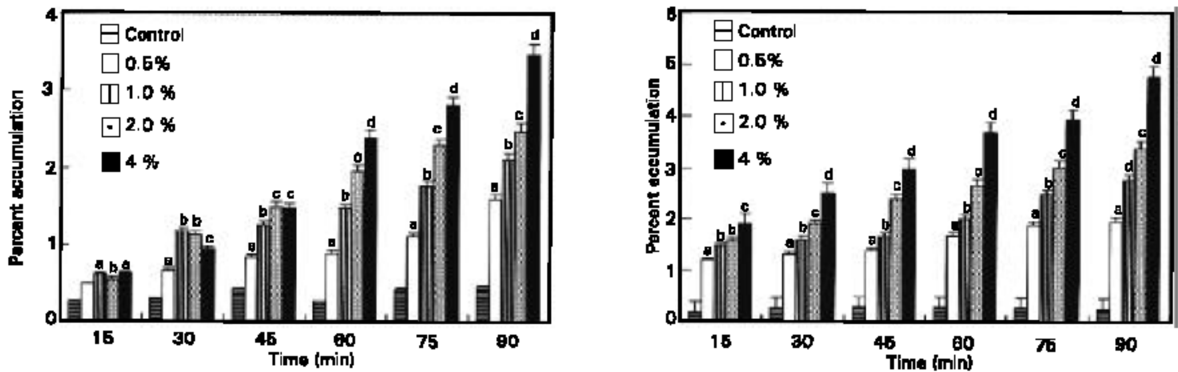

Fig. 3: Chemotaxis of *V. cholerae* -0139 towards the homogenates of (A) oyster (*L. marginalis*) and (B), snail (*P. globsa*). Error bars indicate standard deviation. Bars with different letters (within each time interval) are significantly different (P<0.05).

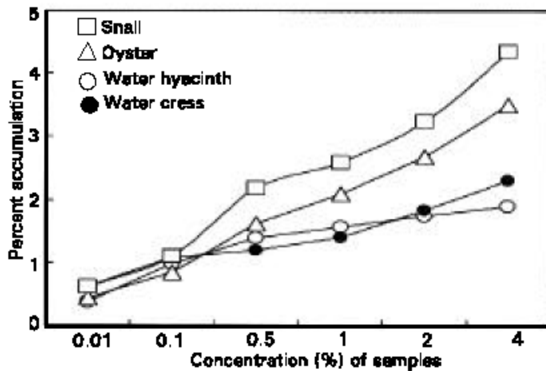

Fig. 4: Response of *V. cholerae* -0139 towards different concentrations of homogenates of aquatic flora and fauna.

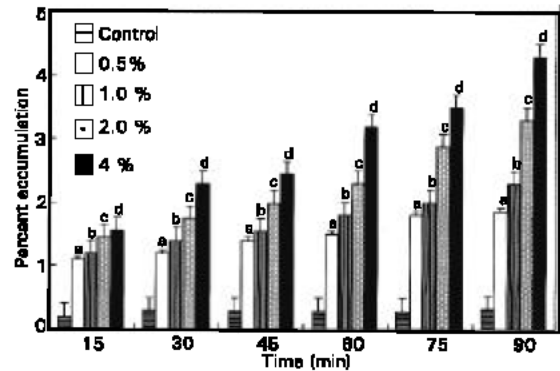

Fig. 5: Chemotaxis of *V. cholerae* -01 towards the homogenates of snail muscle. Error bars indicate standard deviation. Bars with different letters (within each time interval) are significantly different (P<0.05).

The capillaries containing homogenates of the plant showed that a significant portion of bacterial strains were attracted into the capillaries and the maximum number was recorded at 90 min with 4.0% homogenates. Almost similar pattern of accumulation was observed with homogenates of water-cress (Fig. 2B).

Chemotactic response of *V. cholerae* -0139 to the homogenates of muscles of aquatic animals: A large accumulation of *V. cholerae* -0139 cells in capillaries containing the homogenates of oyster and snail muscles was observed with respect to the control capillaries containing

Table 1: Influence of temperature and salinity on the chemotaxis of *V. cholerae* 0139 towards the homogenates of various aquatic flora and fauna.

Aquatic components	Percent accumulation at indicated temperature and salinity								
	15°C			20°C			25°C		
	1.0%	1.7%	3.5%	1.0%	1.7%	3.5%	1.0%	1.7%	3.5%
Water hyacinth	0.8	1.1	0.7	1.0	1.6	0.8	1.7	1.8	0.8
Water cress	0.8	1.1	0.6	1.0	1.4	0.8	1.8	2.0	0.8
Oyster	1.0	2.1	1.0	1.2	2.9	0.9	2.8	3.3	1.1
Snail	1.2	2.2	0.9	1.5	1.0	1.0	3.2	4.0	1.2

PBS (Fig. 3). Among the concentrations tested, the highest percentages of accumulation of the strain was recorded at 4.0% suspension of both oyster and snail muscles and the lowest percent was recorded at 0.5% suspension. As shown in Fig. 3 A and B, the response was higher towards the homogenates of snail muscle (Fig. 3B) than those, observed with oyster muscle (Fig. 3A)

Concentration-response curve: Various concentrations (ranging from 0.01 to 4%) of homogenates of aquatic plants and animals described above were subjected to chemotaxis capillary assay in order to determine their threshold and peak concentration for chemotaxis of *V. cholerae* -0139. The 'threshold concentration' the lowest concentration of attractant that gives an accumulation in the capillary greater than that obtained in the absence of attractant, was about 10^{-7} M for glucose (data not shown) and 0.01% for all the reported aquatic flora and fauna (Fig. 4). The threshold (10^{-7} M) for the non-metabolizable attractant, lactose was not affected by diluting the bacteria. The 'peak concentration' where the maximum response to attractant occurs, was 10^{-3} M for glucose and 4% for all the aquatic flora and fauna (Fig. 4).

Effects of temperature and salinity on the chemotaxis *V. cholerae* 0139: Various values of temperature and salinity were selected to evaluate their effects on the chemotaxis of the strain 0139 towards the homogenates of aquatic flora and fauna according to the method described above. The numbers of bacteria attracted to the homogenates of various species in different assay conditions are given in Table 1. The minimal values of chemotactic response towards the homogenates of samples tested were observed at the lowest values of temperature (15 ^{0}C) and at extreme values of salinity (3.5%). The highest numbers of cells were obtained at salinity of 1.7% and at temperature 25 ^{0}C to the homogenate of snail muscles. The percent accumulation obtained in these experiments in which the cells of the strain were preincubated for 8 h in saline water, a nutrient deficient medium, are lower than those when the strains assayed were grown in TSB.

Chemotactic response of *V. cholerae* -01 to the homogenates of snail muscle: The chemotactic response of *V. cholerae* -01 towards the homogenates of snail was assayed in the same manner as described in Materials and Methods. The homogenate at a concentration of 4% induced maximum number of accumulation of the strain at 90 min in the capillaries (Fig. 5). The response was a bit lower than that, obtained with *V. cholerae* -0139 using the same concentration of snail muscle homogenates as shown in Fig. 3B.

Discussion

Bangladesh is a country where cholera epidemics occur repeatedly every year causing death of hundreds of people. Contaminated water, poor environmental sanitation, low standard of personal hygiene, lack of proper understanding on the handling of aquatic components are considered as the major contributory factors for diarrheal deaths. The isolation of strains of *V. cholerae* from various components of water indicated that the deadly pathogen can survive in the aquatic environment in association with those components where vibrios can persist long time even in the inter-epidemic period. Therefore to find the specific factors that provide their attachment to the aquatic habitats and long term persistent are important for epidemiological purposes.

In this study chemotactic response of *V. cholerae* -0139 towards two characteristic carbohydrates for vibrios and the extracts of four aquatic flora and fauna were tested in a chemotaxis capillary assay. In the chemotaxis study of the strain with glucose, the strain was invariably attracted towards various concentrations of glucose as compared to control capillaries where an insignificant number of bacteria was accumulated (Fig. 1) The accumulation of bacteria in the control capillaries can be explained as background accumulation that had occurred presumably by random swimming. The largest accumulation was observed at concentration of 10^{-3} M glucose. The decline in the concentration-response curve at higher concentrations (10^{-1} M and 10^{-2} M) of glucose might be due to the longer lag periods for high concentrations or might be the high ionic strength present for high concentration inhibited motility. This observation coincides with those of chemotaxis of *E. coli* towards various chemicals (Adler, 1973). At 5% level of significance, the accumulation was observed with chemotaxis study with lactose are insignificant ($P>0.05$). This might be due to that the strain could not sense the lactose as an attractor because this strain is a non-lactose fermenting organism (Islam *et al.*, 1994a) or the lag period might be greater than 90 min that also corroborate with the findings of Adler (1973).

Water hyacinth (*E. crassipes*) and water cress (*P. stratiotes*), are ubiquitous and free floating surface plants in the rural areas of Bangladesh. For both the plants the highest chemotaxis were observed at 4.0% homogenate at 90 min (Fig. 2) suggesting that the higher concentrations of homogenates contained an optimum quantities of chemoattractants for vibrios. Results indicated that vibrios present in water as an autochthonous member, contaminated by stool from cholera victims or from any other sources might be concentrated on the surfaces of these plants. Finally establish a mutual association between them, thereby appear to increase the length of time they remain viable in the aquatic environment and transmitted cholera in a suitable environmental conditions. The similar type of phenomenon was observed with the association of *V. cholerae* -01 and *Anabaena* spp. (Islam *et al.*, 1994b)

The homogenates of oysters and snails muscle were also chemotactic to *V. cholerae* -0139 (Fig. 3 A and B) in a greater percentages than that obtained with aquatic plants tested. It was observed that various amino acids such as glutamic acid,

alanine, serine, threonine, glycine, tyrosine, valine, leucine, phenylalanine and aspartic acid, carbohydrates such as arabinose, xylose glucose, galactose were present in the extracellular products of *Anabaena* spp. (Fogg, 1952; Walsby, 1974). Though it is not clarified yet, it might be due to that the homogenates of snail and oyster muscle contain such types of organic components in a greater amount that are responsible for attracting higher number of *V. cholerae* -0139. The effect of environmental factors such as temperature and salinity indicated that the highest chemotaxis regardless of the aquatic fauna and flora was observed at temperature and salinity of 25 ^{0}C and 1.7% respectively. It could be assumed that at this optimal temperature the synthesis of chemotactic machinery is favoured. It was reported that the synthesis of lateral flagella was maximal at temperature and salinity ranging between 15 and 37 ^{0}C and 1 to 2% (Belas and Colwell, 1982) that is in a good agreement with our findings. Because *V. cholerae* -01 is one of the important pathogens causing death to the mankind, we have tried to find their chemotactic behaviour towards one of the aquatic fauna. Since the highest accumulation of *V. cholerae* -0139 was observed against the homogenized muscle of snail, we have selected this animal in order to measure the chemotaxis of *V. cholerae* 01. This strain was also highly chemotactic to the component tested (Fig. 5).

In conclusion we have illustrated that chemotactic motility might have been influenced by metabolic products of aquatic components that mediate the movement of the *V. cholerae* 0139 and 01 towards those aquatic flora and fauna. This investigation also suggests that during interepidemic periods, the epidemic strains of *V. cholerae* -01 and 0139 might use these water components as a sanctuary to survive against adverse environmental conditions that finally favor these pathogens to spread cholera disease in the epidemic periods. Our future work will focus on the identification of attractants and repellents from the homogenates of aquatic components and to know the extent of chemotactic response of both *V. cholerae* -01 and 0139.

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