THE EFFECT OF IRON ON THE TOXIGENICITY OF VIBRIO CHOLERAE

MRUDULA PATEL AND MARGARETHA ISAACSON

Department of Clinical Microbiology and Infectious Diseases, South African Institute for Medical Research and The University of the Witwatersrand, Johannesburg, South Africa

Abstract. In vitro and in vivo studies were conducted to assess the response of cholera toxin (CT) production to increasing iron concentrations in an aquatic environment. Production of CT by seven of eight Vibrio cholerae strains tested, including the Bengal strain (O139), was significantly enhanced in the presence of iron concentrations of 1.0 and 10 g/L. The exception (El Tor Ogawa) had a significant CT response only in the presence of 10 g of iron/L. Enhancement of CT production also occurred at iron concentrations less than 1.0 g/L, but not to a statistically significant degree. The high iron concentrations, which in this study were found to stimulate CT production, have been described by others in association with sediments, water plants, and chitinous fauna. Other investigators have shown a predilection by V. cholerae to attach to these sites in the aquatic environment. The importance of excess in vivo iron with respect to the pathogenicity of several gram-negative bacilli is well recognized. However, the possible impact of environmental iron on the in vitro toxigenicity of a microorganism, in this case V. cholerae in its aquatic environment, is to the best of our knowledge a new finding with important epidemiologic implications. These findings, coupled with the fact that iron concentration is considerably enhanced in industrially polluted waters and sediments, may reflect a causal link between the concurrent global upsurge of industrialization and pandemic occurrence of cholera during the latter half of the 20th century. Enhanced toxigenicity may also cause clinical disease following ingestion of lower than usual infective doses of cholera vibrios, thereby increasing the incidence of symptomatic cases and, possibly, of severe cases.

Iron is an essential component of many enzymes and other proteins and is required for growth of virtually all bacteria. The environment as well as animal hosts have limited amounts of free iron available for microbial growth since the metal forms insoluble complexes at neutral or alkaline pH and is sequestered in hosts by iron-binding proteins. One specific iron acquisition system found in some pathogens, including Vibrio cholerae, consists of a microbially produced iron-binding compound, or siderophore, and proteins associated with the transport and use of chelated iron. Environmental strains of V. cholerae produce more siderophore than clinical isolates. The iron transport system is related to increased virulence of some invasive pathogens. Low or unavailable iron may trigger virulence factors such as the shiga toxin of Shigella dysenteriae, diptheria toxin of Corynebacterium diphtheriae, shiga-like toxin I of enterohemorrhagic Escherichia coli, and exotoxin A of Pseudomonas aeruginosa.

A previous study showed that V. cholerae survival time in water significantly increased with increasing iron levels up to optimum values of 0.1–1.0 g/L (pH 7) and 0.01–0.1 g/L (pH 9–11), above which survival decreased. Vibrio cholerae is a noninvasive bacillus that exerts its pathogenic effect only by virtue of its enterotoxin, which is produced in the bowel lumen of the host. If the growth of V. cholerae is stimulated by increased iron in its aquatic environment, the question arises if iron may also enhance the toxigenicity of this organism. The purpose of this study was to assess the response of cholera toxin (CT) to increasing iron concentrations in an aquatic environment.

MATERIALS AND METHODS

Titration of CT. Titration of CT was done by diluting concentrated (1 μg/μL) purified toxin (ICN Biochemicals, Cleveland, OH) to a concentration of 10 ng/ml using phosphate-buffered saline. This was diluted further through five, two-fold (doubling) dilutions. Each dilution was subjected to the GM₈ ELISA. The entire procedure was repeated 10 times and the results (optical densities [ODs]) were analyzed using linear regression to generate a standard curve for measurement of CT production by the test organisms. The ODs and corresponding CT concentrations were plotted. The formula obtained from the results to calculate the concentration of CT in unknown samples was CT in ng/ml = (a × OD) – b where a (the slope of the line) = 4.777 and b (the x-intercept) = 0.071. This formula allowed calculation of toxin levels with an accuracy of two decimal points.

Bacterial cultures. Eight toxin and two non-toxin producing strains of V. cholerae were used (Table 1). The cultures were maintained in semi-solid agar at room temperature.

Preparation of bacterial inoculum. Cultures on blood agar plates were incubated at 37°C for 24 hr. A loopful of culture was then inoculated into 50 ml of CAYEG (3% casamino acids, 0.3% yeast extract, 0.2% glucose). After incubation at 37°C for 16 hr, 5 ml of the above culture was subcultured in another 50 ml of CAYEG and incubated at 37°C for 4 hr. Approximately 15 ml of the culture was centrifuged at 4,000 rpm for 20 min. The pellet was washed twice with one-fourth strength Ringers solution and the final pellet was resuspended in 5 ml of the same solution. The number of organisms in the final suspension was always found to be approximately 10⁹ organisms/ml.

Defferration of CAYEG. To eliminate small, unspecified amounts of iron in CAYEG, which could have affected the experiments in which the consistency of carefully measured iron concentrations was very important, deferration was done using calcium chloride–mediated iron precipitation.

Preparation of materials for the microtiter ganglioside ELISA. Five 25-ml flasks were prepared containing 0, 0.01, 0.1, 1.0, and 10.0 g/L of Fe₂O₃, respectively, and autoclaved. To each flask, 25 ml of sterile distilled water containing deferrated CAYEG was added aseptically to give a final concentration of 5.6 g/L. One such set of five flasks was prepared for each cholera strain studied. Each flask was inoc-
Table 1

<table>
<thead>
<tr>
<th>Origin and Ref. no.</th>
<th>Biotype</th>
<th>Serotype</th>
<th>Toxigenic</th>
</tr>
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<tbody>
<tr>
<td>ATCC 25870&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Classical</td>
<td>O1 Inaba</td>
<td>Yes</td>
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<tr>
<td>ATCC 9459&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Classical</td>
<td>O1 Inaba</td>
<td>Yes</td>
</tr>
<tr>
<td>ATCC 9458&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Classical</td>
<td>O1 Ogawa</td>
<td>Yes</td>
</tr>
<tr>
<td>ATCC 582&lt;sup&gt;o&lt;/sup&gt;</td>
<td>El Tor</td>
<td>O1 Inaba</td>
<td>Yes</td>
</tr>
<tr>
<td>SAIMR 22453&lt;sup&gt;†&lt;/sup&gt; (ex-patient)</td>
<td>El Tor</td>
<td>O1 Ogawa</td>
<td>Yes</td>
</tr>
<tr>
<td>SAIMR 20856&lt;sup&gt;†&lt;/sup&gt; (ex-sewage)</td>
<td>El Tor</td>
<td>O1 Ogawa</td>
<td>Yes</td>
</tr>
<tr>
<td>ATCC 51394&lt;sup&gt;‡&lt;/sup&gt; (MO45)</td>
<td>–</td>
<td>O139</td>
<td>Yes</td>
</tr>
<tr>
<td>V315&lt;sup&gt;‡&lt;/sup&gt;</td>
<td>–</td>
<td>Non-O1</td>
<td>Yes</td>
</tr>
<tr>
<td>0469&lt;sup&gt;‡&lt;/sup&gt;</td>
<td>–</td>
<td>O5</td>
<td>No</td>
</tr>
<tr>
<td>SG2&lt;sup&gt;‡&lt;/sup&gt;</td>
<td>–</td>
<td>O60</td>
<td>No</td>
</tr>
</tbody>
</table>

<sup>a</sup> With courtesy of the Centers for Disease Control and Prevention, Atlanta, GA. ATCC = American Type Culture Collection.
<sup>†</sup> With courtesy of the South African Institute for Medical Research, Johannesburg, South Africa.
<sup>‡</sup> With courtesy of Dr. G. B. Nair, National Institute of Cholera and Enteric Diseases World Health Organization, Collaborating Centre for Diarrhoeal Disease Research and Training, Calcutta, India.

The results of increasing iron concentrations on the toxigenicity of <i>V. cholerae</i> are summarized in Figure 1. Probability values were calculated separately for each bacterial strain by means of a computerized multiple regression analysis using the iron concentrations as variables and the toxin OD readings as responses. In each case, iron was found to be the significant factor in the toxin production of the strain concerned. The data were further analyzed to identify the iron concentration(s) of significance in this context. The results of this analysis showed that when iron levels were increased to 1 g/L and higher, significant (<i>P</i> < 0.05–<i>P</i> < 0.01) enhancement of toxigenicity occurred with all the toxigenic strains except the El Tor isolate (no. 20856). The OD readings for the nontoxigenic strains remained below the cut-off point of 0.2 and were not increased by increased iron concentrations in their growth environment.

**Results of the Y<sub>i</sub> cell line technique.** A greater than four-fold increase in titre was obtained with 87.5% of the cultures exposed to 10 g/L of iron. The remainder showed a doubling of the titre.

**DISCUSSION**

All of the known toxigenic strains (O1 and non-O1) used in this study showed increased enterotoxin production on exposure to high concentrations of ferric oxide. In keeping with the findings of other investigators, the El Tor isolates studied produced much lower levels of toxin than the classical strains.12,13

Of great interest was the finding that moderate increases in the concentration of iron (up to 0.1 g/L) had little effect on toxin production. However, iron concentrations greater than 0.1 g/L markedly stimulated the toxigenicity of some strains, notably the classical strains and the clinical isolate of El Tor Ogawa (Figure 1). In this regard, it is also of interest to note that during the current epidemic in South America, the initial cholera waves in many epidemic regions were caused by the Inaba serotype, but an Inaba-to-Ogawa shift occurred and gave rise to later outbreaks. Such shifts can be caused by several genetic mechanisms.14

The causative Inaba strain involved in the Inaba-to-Ogawa shift during the recent Latin American cholera epidemic has been shown by biochemical analyses and RNA restriction fragment length polymorphism to be unique to Latin America.15 The emerging Ogawa strain in that region was shown to be identical to the local Inaba strain in all other respects, and it was suggested that a serotype conversion had occurred, possibly as a result of immune pressure. Not surprisingly, in view of the electrophoretic findings that grouped the O139 (Bengal) and the O1 El Tor strains of <i>V. cholerae</i> into the same zymovar, the responses of these strains to iron in this study were also similar.16

Another question that arises from these studies, although not addressed, concerns the possibility that environmental factors (in addition to host factors such as serotype-specific immunity) may be instrumental in the serotype shifting observed during the course of cholera epidemics.

Chemical water monitoring data collected over many years on numerous surface waters were kindly made available for this study by Rand Water in Vereeniging, one of the major water suppliers in South Africa. These data, with rare exceptions, showed that in water that is not subject to sig-
Figure 1. Cholera toxin production by *Vibrio cholerae* in response to increasing concentrations of iron. For the classical strain 25870, which had very high toxin levels, the optical densities are plotted at a 1:8 dilution. Error bars represent the standard deviations.
significant industrial pollution, total iron concentrations were less than 1 g/L.

Iron, which constitutes some 5% of the earth’s crust, is an essential requirement for most living organisms. It is, for example, a vital component in biological electron transport mechanisms. Accumulation of iron in the natural environment where it may persist and exert toxic effects on local ecosystems has received much attention, especially in recent years. Several studies in different parts of the world showed that industrial and other pollution of water sources was associated with increased levels of metals such as lead, nickel, copper, cadmium, manganese, zinc, and iron. Iron concentrations are usually not very high in surface waters (in the order of 0.01–5.00 mg/L), but accumulation of the metal occurs to very high levels in their sediments (80–164000 mg/kg), aquatic vegetation, and zooplankton. We believe that these findings and conclusions have important implications in the interpretation of our toxigenicity findings since the iron levels reported in these studies overlap with those that enhanced CT production in our study. In contrast, the possible impact of excess iron on CT production by *V. cholerae*, which was the main objective of this study, was not an objective of investigations by these workers.

Tiwary and Dhar and Yurukova and Kochev observed that iron tended to be taken up and concentrated more heavily in sediments than the other metals included in their studies. Several workers also pointed out that although industrial and other forms of human-made pollution are important sources of the high iron levels in certain aquatic environments, the abundant presence of this metal in the earth’s crust is an additional, albeit, natural source.

Evidence accumulated over the past two decades indicates that *V. cholerae* is an autochthonous inhabitant of brackish water and estuarine systems. These studies also showed that a close association between plankton and *V. cholerae* affects the survival of these bacteria. The survival of this organism between cholera epidemics and its isolation from such waters, even in nonepidemic regions, can be explained on the basis of its persistence in a viable, though often non-culturable, state.

The largest concentrations of water hyacinths (Eichhornia crassipes) are found in waters enriched by sewage and industrial effluent or by run-off from fertilized farm lands. Attachment of *V. cholerae* to fresh-water plants, such as water hyacinth, phytoplankton and zooplankton, and green algae, has been established. Previous work by Patel and Isaacs has shown that iron contributed to the enhancement of both the survival and multiplication of cholera bacilli.

Several factors, therefore, render the aquatic environment ideal for the survival and growth of *V. cholerae*, which, in their turn, are important factors in the epidemiology and pathogenesis of cholera. Other investigators also addressed *V. cholerae* toxigenicity, an important virulence factor. Tamplin and Colwell used microcosm cultures to show that a salinity of 2–2.5% was optimal for CT production. Also in microcosm, Miller and others showed that toxigenicity was a stable property. On the other hand, Islam demonstrated increased production of CT by *V. cholerae* O1 when it was associated with *Rhizoclonium fontanum*, a green alga. Therefore, the findings of Islam suggest that CT production can be affected by environmental factors.

Our findings that iron concentrations of 1–10 g/L in an artificial aquatic environment can significantly enhance CT production may have important epidemiologic and pathogenic implications. From the pathogenesis point of view, the infectious dose of *V. cholerae* needed for development of clinical disease is usually quite high (≥ 10⁸ organisms). In healthy volunteers, the infectious dose was found to be in excess of 10⁸ cholera bacilli. The infectious dose for cholera due to *V. cholerae* with enhanced virulence, in this case iron-mediated, may be considerably lower than 10⁸ organisms. Other potential clinical consequences of enhanced toxigenicity could include a shorter incubation period and/or more severe illness. All of these sequelae can affect the epidemiology of the causative agent in that a larger number of sick individuals with more rapid onset of illness and more severe illness are likely to result in heavier contamination of the environment by *V. cholerae*.

Several clinically important questions, requiring further research, arise from our findings. For example, might the incorporation of an iron-chelating substance in oral rehydration fluids be therapeutically useful? Such substances have been used in the treatment of patients with hemochromatosis resulting from massive iron overload. On the other hand, the possibility of a bacterial pathogen using such a therapeutically administered substance for its own ends, as has been the case with *Yersinia enterocolitica*, and thereby aggravating such an infection, would need to be considered. In a similar context, could the protective effect of tea against *V. cholerae* be a function of the iron-binding properties of this plant?

Another question concerns the relationship, if any, between in vivo toxin production by different *V. cholerae* strains and their hemolytic, iron-releasing, properties. With regard to the well-documented tendency of cholera to cause more severe illness in persons belonging to blood group O, could this phenomenon be related to an association between iron levels and CT production? Furthermore, could increased intestinal availability of iron play a role in the pathogenesis of cholera? Sources of such iron could be endogenous, e.g., from hemolyzed erythrocytes, or exogenous, following the ingestion *per se* of chemically polluted water with high iron levels. The latter seems more likely because although *V. cholerae* has been shown capable of using hemin and hemoglobin as sole iron sources, bloody diarrhea is not a feature of cholera. However, bloody diarrhea is more common with many other intestinal pathogens, including non-O1, non-choleragenic strains of *V. cholerae*. Finally, does in vivo toxin production correlate with the hemolytic properties of *V. cholerae* strains?

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Authors’ address: Mrudula Patel and Margaretha Isaacs, Department of Clinical Microbiology and Infectious Diseases, South African Institute for Medical Research and The University of the Witwatersrand, PO Box 1038, Johannesburg 2000, South Africa.

Reprint requests: Mrudula Patel, Department of Clinical Microbiology and Infectious Diseases, South African Institute for Medical Research and The University of the Witwatersrand, PO Box 1038, Johannesburg 2000, South Africa.
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