

Chironomid Egg Masses as a Natural Reservoir of *Vibrio cholerae* Non-O1 and Non-O139 in Freshwater Habitats

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Abstract

Cholera is a diarrheal disease caused by the gram-negative bacterium *Vibrio cholerae*, and an estimated 120,000 deaths from cholera occur globally every year. The natural reservoir of the bacterium is environmental. A recent report indicated an association between *V. cholerae* and chironomid egg masses. Chironomids, the “non-biting midges” (Diptera; Chironomidae), are the most widely distributed and frequently the most abundant insects in freshwater. Females attach egg masses, each containing hundreds of eggs encased in a layer of gelatin, to the water’s edge where bacteria are abundant and may encounter the nutrient-rich substrate. Here we report the isolation of non-O1 and non-O139 *V. cholerae* from chironomid egg masses from different freshwater bodies in Israel, India, and Africa. In a yearly survey in Israel, chironomid populations were found to peak biannually, and it seemed that those peaks were followed by subsequent bacterial growth and disappearance during the winter in the Mediterranean region. The bacterial population rose as water temperature surpassed 25°C. Thirty-five different serogroups of *V. cholerae* were identified among the bacteria isolated from chironomids, demonstrating population heterogeneity. Two strains of *V. cholerae* O37 and O201 that were isolated from chironomid egg masses in Zanzibar Island were NAG-ST positive. Our findings support the hypothesis that the association found between chironomids and the cholera bacteria is not a rare coincidence, indicating that chironomid egg masses may serve as yet another potential reservoir for *V. cholerae*.

Introduction

Cholera is an epidemic and life-threatening disease. There have been seven cholera pandemics since 1817. The seventh and current pandemic began in 1961 and is the longest one to date. It continues to be an important cause of morbidity and mortality in many areas of Asia, Africa, and Latin America. The fatal effects of the disease are mainly due to the toxin produced by specific serogroups (O1 and O139) of *V. cholerae* [11].

The natural reservoir of *V. cholerae* has long been assumed to be humans, but it appears that there is no chronic state of the disease. Evidence to date suggests that the natural reservoir is in the aquatic environment. Colwell and collaborators [14] showed an association between *V. cholerae* and zooplankton, notably copepods, as viable and as “viable but nonculturable” (VBNC) forms. Propagules may be carried by marine zooplankton along the continental seashore, associated with warmer sea-surface temperatures and aided by major climatic events such as the El Niño Southern Oscillation [9, 10]. Our recent observations suggested that *V. cholerae* is also associated with *Chironomus* sp. (non-biting midge) egg masses in freshwater ecosystems [5].

Chironomids, the “non-biting midges” (Diptera; Chironomidae), are the most abundant macroinvertebrate group, in numbers of species and individuals, encountered in the majority of freshwater aquatic habitats [2]. They undergo a complete metamorphosis of a four-stage life cycle: eggs, larvae, and pupae, which are aquatic, and the adults, which emerge into air. Females of the genus *Chironomus* deposit egg masses at the water’s edge, each egg mass may contain 400–1000 eggs embedded in a thick, gelatinous matrix (Fig. 1a). The presence of several thousand egg masses at one site is not unusual, and in extreme cases, gelatinous layers several centimeters thick are formed along the edge of the freshwater bodies (Fig. 1b) [4, 20]. Chironomids are closely related to mosqui-

toes (Culicidae), yet, unlike their mosquito relatives, female chironomids do not bite. Chironomids have invaded the sea; they are one of only few free-living insects to do so, being found along coastlines worldwide. They are also found living from heights of 5600 m on the mountains of Nepal down to depths of over 1000 m in Lake Baikal [2, 12].

The recent discovery that *Chironomus* sp. egg masses harbor *V. cholerae* and act as its carbon source [5] highlight the possibility that *Chironomus* egg masses may provide an additional reservoir for the cholera bacterium. This finding was observed when more than 1000 chironomid egg masses collected from a waste stabilization pond settled out overnight as thousands of individual eggs, most of which did not hatch. *Vibrio cholerae* 09 was isolated and found to be the cause of the egg mass destruction.

The aim of this study was to further support the observation done by Broza and Halpern [5] with long-term monitoring of the *Vibrio*–chironomids association.

Methods

Sampling Sites. A field study was conducted between 20 February 2001 and 4 March 2002, in northern Israel near Haifa at three sampling sites: Tivon waste stabilization pond (WSP), Elroi spring (two sampling sites), and Kishon River (near Kiryat Haroshet). All sites were within a radius of 2.5 miles. Samples were taken every other week. A short field survey of *V. cholerae* in chironomid egg masses was also performed in India in Uttar Pradesh (Ganga River; Rishikesh, Varana River, Varanasi), West Bengal (Calcutta), and Maharashtra (Pune) in February 2001 and in Africa (Zanzibar Island and Malawi) in February 2002. In Zanzibar we sampled five freshwater bodies on the northeast suburb of Zanzibar City and the only two villages that exist on Uzi Island. Both areas suffered from a cholera epidemic during Dec 2001–Jan 2002. In Malawi we sampled water bodies near Cape McTear close to the southwest shore of Lake Malawi.

Chironomid Sampling. Egg mass sampling was used to monitor chironomid populations [3, 5]. Styrofoam boards (25 × 25 cm) were used as artificial oviposition sites for female midges. They were placed in the pond for 24 h and then transferred to a basin full of water to count the egg masses along the side of the board (Fig. 1a, b).

In all the sampling sites in India and Africa both styrofoam board (20 × 20) and direct hand picking of egg masses were carried out.

Isolation of *V. cholerae*. Ten egg masses from each sampling site on each sampling date were enriched in alkaline peptone water (APW, 1% peptone, 1% NaCl; pH

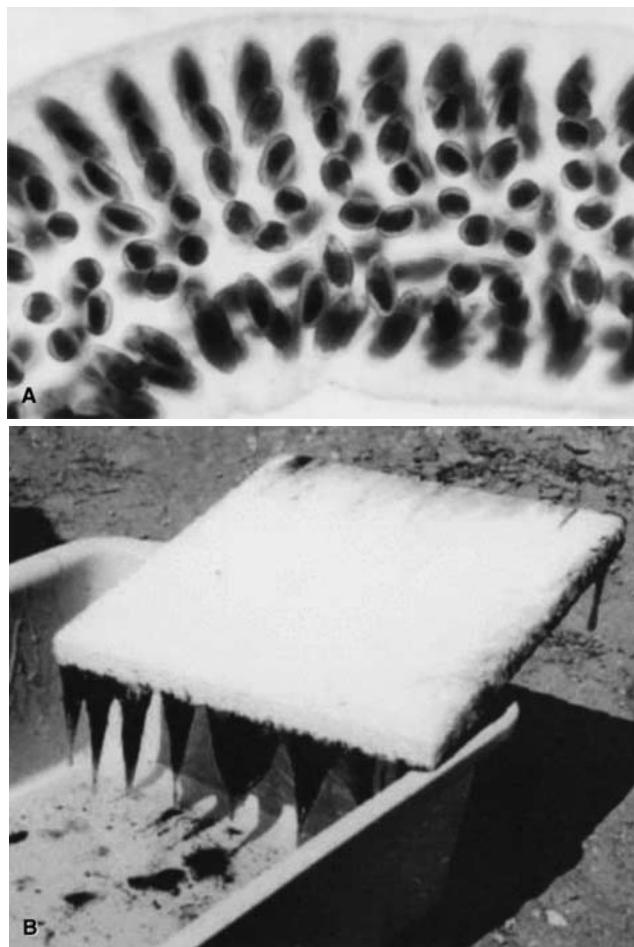


Figure 1. (a) *Chironomus* sp. egg mass. The eggs are arranged in a row, folded into loops to form a spiral, and embedded in a thick layer of gelatinous cylinder. Enlarged part of the mass showing the string-shaped mass and the gelatinous edge. Original magnifications, 40× (egg mass size ca. 20 × 5 mm; egg size ca. 250 × 80 μm). (b) Egg-mass sampling. Styrofoam boards placed for 24 h serve as an attractive oviposition site for female chironomids. During high female density, the edges of the plate were covered with thousands of egg masses glued together, which formed a typical triangular shape while being removed from the water.

8.4 to 8.6) for 6 h at 37°C. When <10 egg masses were collected, the number of the inoculated egg mass was <10. Bacterial colonies were isolated from the enrichment cultures by using thiosulfate–citrate–bile salts (TCBS) agar (Difco). The colonies on TCBS were confirmed to be *V. cholerae* by biochemical testing. Yellow colonies from TCBS were subcultured onto a nonselective medium such as Luria agar, and then tested for oxidase (1% tetramethyl *p*-phenylenediamine, Sigma), string test (0.5% sodium deoxycholate, sigma) and api 20e (BioMérieux, Missouri, USA). Somatic antigen serogrouping identification [23] was employed on most of the *V. cholerae* isolates. The presence of heat-stable enterotoxin NAG-ST in the iso-

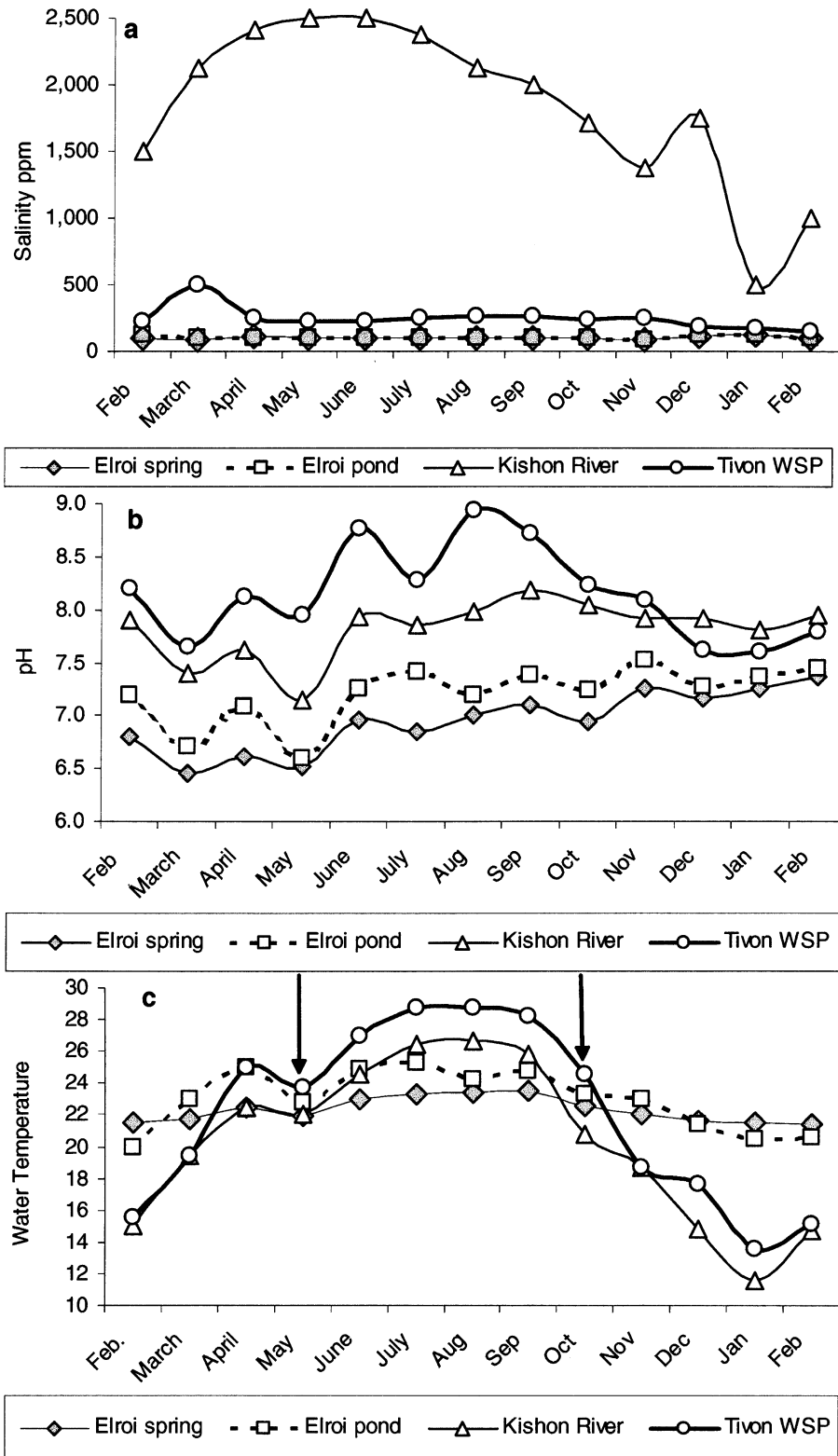


Figure 2. Determination of environmental parameters. Water salinity, pH and temperature were measured at Elroi spring, Elroi pond, Kishon River, and Tivon waste stabilization pond (WSP) throughout 2001. (a) Salinity (mg/L). (b) pH. (c) Water temperature ($^{\circ}\text{C}$); the arrows indicate the timing of chironomid population peaks in WSP. See also Fig. 3.

lates from Zanzibar was examined by PCR according to [22].

Egg masses that were collected in India and Africa were placed into plastic tubes containing Carry Blair

semisolid agar (Difco). The isolation procedure for *V. cholerae* was carried out in the laboratory.

Bacterial isolates were kept in Carry Blair agar and in LB with 30% glycerol (-80°C).

Table 1. Chemical composition of the water (concentrations in mg/L) at each sampling site

Parameter	Date of sampling ^a	Elroi spring	Elroi pond	Kishon River	Tivon WSP
BOD	1	4	—	19	62
	2	0.0	9	13	128
COD	1	14	6	103	181
	2	0.0	57	146	227
TSS-105	1	58	—	130	96
	2	4	68	1,260	48
N from ammonia	1	0.0	0.0	0.5	6.2
	2	0.0	0.0	5.9	25.8
P	1	0.1	—	0.7	6.9
	2	0.0	0.0	2.2	6.1

^aWater was sampled on 15 October 2001 (date 1) and 20 January 2002 (date 2).

Determination of Environmental Parameters. The following parameters were measured throughout the experimental period: water temperature, pH (Scan Model2, Rajah Crescent, Singapore), conductivity (El-Hamma Model TH-250, Mevo Hamma, Israel), and salinity (Chloride Test, Merk, Darmstadt, Germany). BOD, COD, TSS, N from ammonia, N from nitrate, P, and K^+ were analyzed twice (in October and January) by the "Neve Ya'ar" extension laboratory (Ministry of Agriculture, Israel). This laboratory also analyzed metal content. Total coliform and fecal coliform count was performed twice by the MPN method [1].

Results

Field Study in Israel

Environmental Parameters. Three freshwater habitats were sampled: a spring, a polluted river, and an anaerobic waste stabilization pond. In the spring two sites were sampled each time (the stream source and a pond along the running water, about 0.3 km downstream). Seasonal fluctuation of salinity in the waste stabilization pond and Kishon River is shown in Fig. 2a. Conductivity data showed a similar trend (not shown). The water pH of Elroi spring origin and its pond were 6.9 ± 0.32 and 7.2 ± 0.37 , respectively. A basic pH was measured in Kishon River (7.8 ± 0.3) and in the WSP (8.2 ± 0.5) (Fig. 2b). Water temperature changed seasonally in the WSP and in Kishon River, but remained almost constant in the spring groundwater throughout the year (21.5 – 23.5°C) (Fig. 2c). Fluctuations of 15°C were measured across the annual seasons with a maximum of 30°C in Tivon WSP in August. No trace of metal was detected in any of the sampling water sites. The BOD and COD in the WSP was the highest. In the Kishon River and the spring, much lower values were measured (Table 1), except for January 2002 in the Kishon River.

Fecal coliforms were counted in October 2001 and January 2002. In January 2002, 2.5×10^6 bacteria per 100 mL were counted in Tivon WSP. This number was 100 times higher than in Kishon River and 2.7×10^4 times

higher than in the spring. Nevertheless, fecal coliform in springwater indicates fecal contamination of that water.

Chironomid Population. Chironomid population peaked biannually (Fig. 3). The major population peak was in early summer (May), and the minor peak in the autumn (Sept–Oct). After peaking in May, there was a one-third decrease in the chironomid population in July. A significant correlation (Pearson) was found between the dynamics of chironomid populations in the three sampling sites: between the spring pond and Kishon River ($r = 0.91$, $n = 28$, $P = 0.0000$), between the spring pond and Tivon WSP ($r = 0.58$, $n = 28$, $P = 0.001$), and between Tivon WSP and Kishon River ($r = 0.58$, $n = 28$, $P = 0.001$). Note that the chironomid population in Tivon WSP was 2 magnitudes higher than that observed in the other sampling sites (Fig. 3).

The chironomid population peaked when the water temperature was about 25°C (the arrows in Fig. 2c point to the temperature of the chironomid population peak). Despite the continuous favorable temperature in July there was a decline in chironomid population. When water temperature rises, the chironomid population decreases. A significant correlation was found between water temperature in Tivon WSP and its chironomid population ($r = 0.61$, $n = 28$, $P = 0.0005$) (Figs. 2c and 3).

Isolation of *V. cholerae*. *Vibrio cholerae* non-O1 and non-O139 was isolated from chironomid egg masses during the field survey from all three sampling sites. Egg masses were collected in all the above sampling sites and in all seasons, except for December–January, when water temperatures dropped below 18°C and chironomid egg masses were not found (or were <3 per sample site). The bacteria were isolated almost every time chironomid egg masses were sampled (Table 2).

The occurrence of vibrios in egg masses was scored from 0 to 4 depending upon the number of sites in which bacteria were successfully isolated (Fig. 4). Bacterial isolation success line was drawn according to the rank

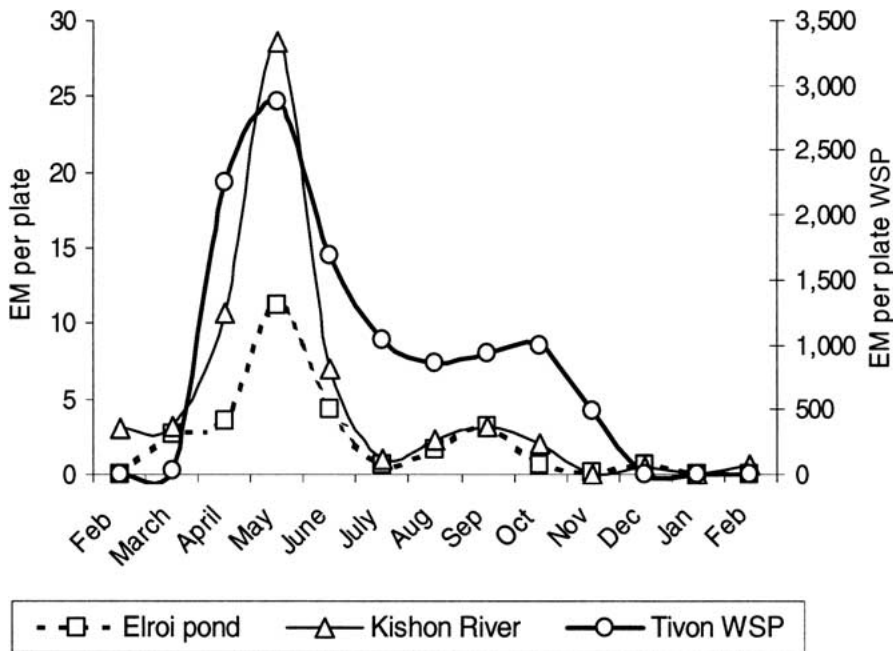


Figure 3. *Chironomus* sp. population dynamics, as was monitored by egg-mass (EM) sampling in Elroi pond, Kishon River, and Tivon WSP throughout 2001. A significant correlation was found between the population dynamics in Elroi pond, Kishon River, and Tivon WSP. Note that the population peaked biannually in spring and autumn.

numbers from Table 2, and chironomid population dynamic was shown by the represented line of chironomid sampling in Tivon WSP site. The two lines may demonstrate the relationships between the bacteria and the chironomids. A peak in the chironomid population is seen in May, followed by a bacterial population peak a month later. The decline in the chironomid population to one-third was followed by a decline in the bacterial level. Note that during June–September when there was a bacterial peak, the water temperature was the highest.

Sampling in India and Africa. *Vibrio cholerae* non-O1 and non-O139 was also isolated from egg masses that were collected from Ganga River and nearby ponds in some other sites in India in 2001 and in Africa (Zanzibar and Malawi) in 2002 (Tables 3, 4). We found chironomid egg masses infected by *V. cholerae* in all but two sites we visited, in both India and Africa. Chironomid egg masses were not found in rice fields near Calcutta, and on the sandy lakeshore of Lake Malawi.

The first two sites in Zanzibar City (Table 4) were the natural water source within the heart of the area where a cholera epidemic occurred a few weeks prior to our visit. Although egg masses were not common, all of them (two dozen) were infected. Three isolates (serogroups O37, O37, O201, Table 4) were characterized as producing the NAG-ST toxin.

Isolation of *Vibrio cholerae* Serogroups. In our study, ca. 200 isolates of *V. cholerae* were obtained from chironomid egg masses as well as from its other life-cycle stages (not all data shown). None of the isolates belonged to the pathogenic O1 or O139 serogroups. Out of 90

isolates that were identified as to serogroup, the following 35 non-O1, non-O139 serogroups were identified: O2, O3, O9, O8, O10, O12, O13, O23, O27, O34, O37, O39, O41, O43, O49, O51, O62, O70, O78, O79, O83, O94, O97, O99, O109, O120, O122, O123, O124, O128, O140, O166, O179, O184, O201.

Discussion

The aim of this study was to further confirm the previous findings that chironomid egg masses may serve as a natural reservoir of *V. cholerae* non-O1 and non-O139 serogroups [5]. Broza and Halpern [5] isolated *V. cholerae* O9 as the cause of chironomid egg mass degradation and egg hatching prevention. *Vibrio cholerae* was isolated using minimal medium with egg masses as sole carbon source. The egg mass degrading factor was purified from the bacterial supernatant and was found to be a 32–34 kDa protein that was identified as the secreted hemagglutinin protease of *V. cholerae* [13].

The association of *V. cholerae* with chironomid egg masses was observed in all three freshwater habitats sampled in Israel. The habitats differed from each other in their water quality: salinity, pH, and parameters of organic concentrations (Fig. 2a, b; Table 1). In Kishon River and in Tivon waste stabilization pond (WSP), a basic pH was measured (7.8 and 8.2, respectively) and water temperature changed seasonally with fluctuations, whereas in Elroi spring the pH was neutral (6.9) and the temperature was constant throughout the year (21.5–23.5°C) (Fig. 2b, c). A constant salinity of 100 mg/L was measured in Elroi spring, whereas higher salinities with

Table 2. A list of *V. cholerae* strains that were isolated from chironomid egg masses at four sampling points near Tivon, Israel throughout the year 2001^a

Date	Waste stabilization pond	Kishon River	Elroi pond	Elroi spring
20 Feb	–	+	+	–
5 March	+	+	+	–
18 March	+	(K183) O2	+	+
2 April	(T24)	(K24)	+	+
16 April	(T164)	(K164)	+	+
30 April	(T304)	(K304)	+	+
14 May	(T145) O79	(K145)	+	+
29 May	(T295) O23	(K295) O179	(E295) O184	+
11 June	(T116) O27	(K116) O12	(E116) O2	(M116) O39
25 June	(T256) O10	(K256) O27	(E256) O70	–
10 July	(T107) O8	(K107) O34	(E107) O109	(M107) O34
24 July	(T257) O23	–	–	(M257) O23
8 Aug	(T88)	–	(E88)	–
21 Aug	(T218) O122	(K218) O109	(E218) O120	(M218)
3 Sep	(T39) O124	(K39)	(E39)	–
17 Sep	(T179) O9	(K179) O9	(E179)	–
1 Oct	(T110)	(K110)	+	–
15 Oct	(T1510) O94	(K1510) O124	–	–
28 Oct	(T2810)	–	–	+
12 Nov	+	–	–	+
26 Nov	(T2611) O99	–	+	–
10 Dec	+	–	+	–
24 Dec	–	+	+	–
9 Jan 2002	–	–	–	–
28 Jan 2002	–	–	–	–
11 Feb 2002	–	–	–	–
4 March 2002	–	–	+	–

(+) Egg masses were collected and *V. cholerae* was not isolated; (–) no egg masses were collected.

^aTo the right of the serial number of isolates is its serogroup classification. When there is no serogroup identity, the identification of the bacteria is *V. cholerae* non-O1/O139.

fluctuations due to winter rains were measured in Tivon WSP and Kishon River (with an average of 250 mg/L and 1900 mg/L, respectively; Fig. 2a). BOD was the highest in the WSP. Although the habitats differed from each other in their water quality, significant correlation was found in chironomid population dynamics sampled in WSP, Kishon River, and Elroi stream. The population size in the WSP was two magnitudes higher (Fig. 3). The populations peaked biannually in May–June and in September–October. The early summer peak was much higher than

the autumn peak. Significant correlation between water temperature and chironomid population was found only in Tivon WSP, although a decline in chironomid population was observed when temperature surpassed 25°C (Figs. 2c, 3). We demonstrated that the chironomid population peak was followed by a bacterial growth according to host-pathogen population dynamic curve (Fig. 4). It seems that bacterial population rose as water temperature surpassed 25°C. The role of temperature level as a limiting factor of cholera epidemics has already

Table 3. List of *V. cholerae* isolated from chironomid egg-masses that were collected from freshwater in India during February and the beginning of March 2001

Place of isolation	Region of isolation	Strain no.	Serogroup
Calcutta	Small freshwater pond	H24	O39
		H3	O39
		113B	O39
Varanasi	Ganga river	209	Non-O1/O139
	Varana River, fish market	H12, H17, H19	Non-O1/O139
	Freshwater pond outside the city	H11, H14, H16, H18, H22	Non-O1/O139
Rishikesh	Ganga River	103A	O78
		104	O3
		H4	O166
Pune	Ganga River	H20	O78
		H1	O13

Table 4. List of *V. cholerae* isolated from chironomid egg masses that were collected in Africa during February/March 2002 (except for K2 from Kenya which was isolated from the adult stage, at June 2000)^a

Place of isolation	Region and source of isolation	Strain no.	Serogroup	NAG-ST
Kenya	Shore of Lake Victoria	K2	O2	–
Zanzibar	Zanzibar City N.E. suburb	ZC24	O49	–
	a. Small running stream	ZC21B	O201	+
		ZC22B	O10	–
		ZC22	O10	–
		ZC22AP	O49	–
		ZC23	O49	–
		ZC23B	O49	–
	b. Amani Reservoir	ZC32	O49	–
		ZC32A	O37	+
		ZC33	O140	–
		ZC5	O37	+
Malawi	Cap Mc. Tear—running water near Lake Malawi	MC42	O128	–

^aAll the strains in this list were *ctx* negative.

been shown [9, 11]. We suggest exploring the hypothesis that an increase in the number of egg masses is followed by an increase in the bacterial numbers, up to a level at which the bacteria control the chironomid population density by destroying their gelatinous egg masses and preventing their eggs from hatching. This process may explain the decline of chironomid population in July–August despite the favored temperature in this period.

Seasonality of the chironomid population was shown in the current research, in another WSP plant in central Israel [4] as well as in an oligomesotrophic lake in the Netherlands [25, 26] and in shallow salt pans in Austria [27]. In many areas of endemic infection, cholera epidemics occur seasonally as well [8, 11]. In Bangladesh the disease peaks twice: a high peak in November–January when temperatures are 20–24°C, and a low peak in April–May (29°C) [11, 15]. In South America cholera appeared seasonally but with only one peak in January–February

[21, 24]. The last and very serious cholera outbreak in Peru and Ecuador occurred at that season from 1990 to June 1995 and was related to the El Niño event, an unusual warming in the central Pacific Ocean that creates storms and disrupts wind patterns [9].

In the current study, ~200 isolates of *V. cholerae* non-O1 and non-O139 were isolated from chironomid egg masses. However, the pathogenic strains were not isolated, since most of this study was conducted in Israel in which the last cholera outbreak was recorded in 1970 (a total of 186 cases). The 90 isolates that were identified to its serogroup belonged to 35 serogroups. It seems that environmental *Vibriosis* in a site are highly variable. Moreover, three different serogroups (O70, O79, and O123) were isolated from egg masses that were collected at the same time and at the same place in June 2002 (data not shown). The heterogeneity found in the bacteria isolates may imply that there may be similarity in the

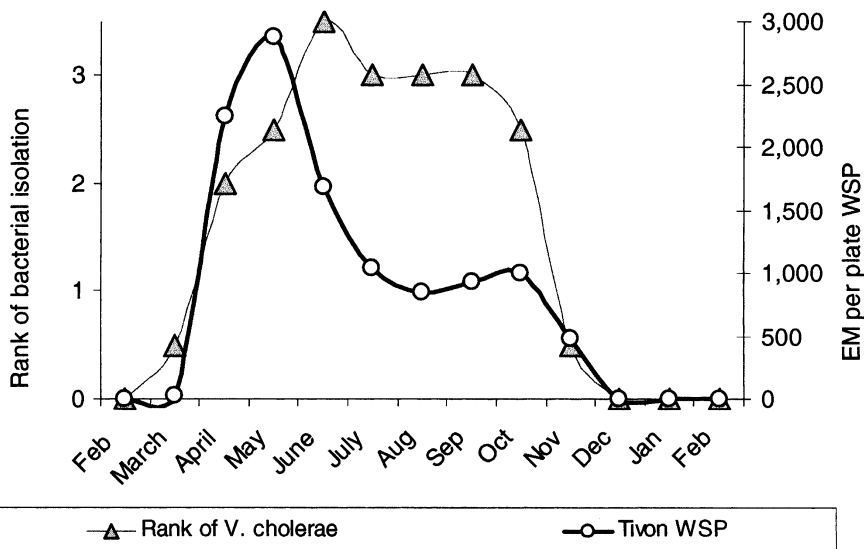


Figure 4. Population dynamics of *Chironomus* sp. (Tivon WSP) and of *V. cholerae* rank values calculated as the monthly average sum of the bacterial isolation success from chironomid egg masses in all four sampling sites (see Figs. 2c and 3).

ecological niche of all *V. cholerae* serogroups, non-O1, non-O139, and O1, O139 [17]. Until now, the detection of *V. cholerae* O1 and O139 in egg masses has remained a challenge.

There are indications that toxigenic strains may arise from environmental, nontoxigenic progenitors in coastal areas [7, 15, 18]. It is important to monitor the presence of *V. cholerae*, irrespective of serotype or serogroup, pathogenicity, or virulence, because of the discovery that lateral transfer of genes can readily occur in the aquatic environment [7]. Virulence gene expression in non-O1 strains of *V. cholerae* has been demonstrated. In addition, studies have shown that *V. cholerae* non-O1 cells can convert to the O1 epidemic cholera serotype and vice versa [11]. Some serogroups from the above list were involved in a cholera-like diarrhea disease (O2, O8, O9, O10, O13, O23, O27, O37, O39) and were isolated from patients hospitalized with acute secretory diarrhea [19]. Of special interest is the finding of O37 and O201 in the heart of the last cholera epidemics in Zanzibar City (Table 4), and the fact that NAG-ST toxin was found in these specific isolates. The heat-stable enterotoxin NAG-ST is a virulence factor that has been associated with *V. cholerae* non-O1. An epidemic of diarrhea that was caused by *V. cholerae* non-O1 NAG-ST positive was reported in a refugee camp in Thailand. The clinical features of the epidemic were indistinguishable from those of cholera [6]. The O37 serogroup has already been recorded as responsible for localized outbreaks in 1968 in Sudan [16]. Because the O1 and O139 serogroups were never isolated from patients during the epidemic that took place just before our visit to Zanzibar (Dr. Mario Mariani, Zanzibar, pers. comm), we may assume that serogroups O37 or O201 or both could be the source of this epidemic.

The isolation of various *V. cholerae* non-O1 and non-O139 serogroups from chironomid egg masses from different freshwater bodies in Israel, India, and Africa supports the hypothesis that the association found between chironomids and the cholera bacteria is not a rare coincidence. Our findings indicate that chironomid egg masses may serve as a reservoir for *V. cholerae* non-O1 and non-O139. If such a connection will be further substantiated for the O1 and O139 serogroups, sampling the bacterial population in egg masses may lead to a new and easy technique for monitoring and predicting cholera epidemics.

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