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Vibrio cholerae and *Aeromonas*: do they share a mutual host?

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Species of the genus *Aeromonas* are native inhabitants of aquatic environments and have recently been considered as an emergent human pathogen. It is estimated that aeromonads cause up to 13% of reported gastroenteritis cases in the United States. Although the autochthonous existence of *Aeromonas* in the aquatic environment has been established, its natural reservoir is as yet unknown. Chironomids are closely related to mosquitoes except they do not bite and they are the most widely distributed insects in freshwater. They infest drinking water systems in Israel and all over the world. *Vibrio cholerae* inhabit chironomids and are able to degrade their egg masses. The degradation of the egg masses is followed by failure of the eggs to hatch. In the current study, egg masses from a waste stabilization pond and a river in northern Israel were collected and cultured during a five-month period. Bacterial colonies were randomly chosen and checked for their egg mass degradation abilities. In addition to *V. cholerae*, most of the other isolates that had the ability to degrade the egg masses were identified as *Aeromonas* species, thus, demonstrating that *Aeromonas* species are natural inhabitants of chironomid egg masses. The following virulence-associated genes were detected in *Aeromonas* species that were isolated from chironomid egg masses: *alt* (78%); *ahpB* (76%); *act/aerA/hlyA* (65%); *fla* (59%); *plalipH3/apl-1/lip* (43%); and *ast* (2%). These findings indicate that the *Aeromonas* species inhabiting chironomid egg masses pose a potential health risk. Understanding the natural reservoir of *Aeromonas* will help to develop methods to monitor and control the bacteria in fresh and drinking water reservoirs and to better understand the relationships between chironomids, *V. cholerae* and *Aeromonas* populations.

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Introduction

The genus *Aeromonas* are waterborne Gram-negative bacteria that are ubiquitous in water, including groundwater and chlorinated drinking water (Le-Chevallier *et al.*, 1982; Kühn *et al.*, 1997a, b; Gavriel *et al.*, 1998). Worldwide studies have demonstrated that *Aeromonas* spp are universally distributed and widely isolated from clinical and environmental samples (Nakano *et al.*, 1990; Huys *et al.*, 1996). *Aeromonas* comprise important human pathogens, including *A. hydrophila*, *A. veronii* and *A. caviae* (also known as *A. punctata*), which cause gastroenteritis, primary and secondary septicemia, and wound infections. *Aeromonas* strains isolated from water have been shown to possess virulence traits, such as adhesins, hemolysins and cytotoxic

enterotoxins, presumably involved with human pathogenicity (Handfield *et al.*, 1996; Albert *et al.*, 2000; Schubert, 2000). The prevalence of *Aeromonas* species in the aquatic environment has been recognized as a potential health risk, and some countries have adopted aeromonad counts as an additional indicator of water quality. For example, *A. hydrophila* has been placed on the US Environmental Protection Agency's Contaminant Candidate List of emerging pathogens in drinking water (Borchardt *et al.*, 2003). Although it is clear that members of *Aeromonas* are primarily aquatic organisms, their natural host in the aquatic habitat is as yet unknown.

Among aquatic invertebrates, the nonbiting midges (Chironomidae, Diptera) are globally distributed, and they are the most abundant group of insects found in freshwater ecosystems (Armitage *et al.*, 1995). They undergo four life stages, three of which are aquatic—egg, larva and pupa—and the last of which is the adult stage, when the insect emerges from the water into the air. After mating, the adult female lays a gelatin egg mass on the water's edge, with each mass containing hundreds of

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embedded eggs. Egg masses of *Chironomus* sp were found to harbor *Vibrio cholerae*, and they provide a natural reservoir for this bacterium (Broza and Halpern, 2001; Halpern *et al.*, 2004, 2006). It was found that chironomid egg masses were destroyed in the presence of *V. cholerae*, and degradation of the egg masses was followed by failure of the eggs to hatch (Broza and Halpern, 2001). Hemagglutinin/protease, an extracellular enzyme of *V. cholerae*, was purified from *V. cholerae* O9 and from the pathogenic O1 and O139 serogroups as the enzyme responsible for the degradation of chironomid egg masses (Halpern *et al.*, 2003). Furthermore, it was found that all *V. cholerae* strains, which were isolated from chironomid egg masses were able to degrade chironomid egg masses (Halpern *et al.*, 2003, 2006, 2007). An interaction of host–pathogen population dynamics was found between the chironomid population and *V. cholerae* in their egg masses, and a peak in the chironomid population was followed by a peak in the *V. cholerae* population (Halpern *et al.*, 2006).

The primary objective of this study was to explore bacteria other than *V. cholerae* that inhabit chironomid egg masses and are able to degrade the egg masses. Most of the isolates that have shown the ability to degrade the egg masses have been identified as *Aeromonas* species, demonstrating that *Aeromonas* species are natural inhabitants of chironomid egg masses. This study will allow us to understand the relationships between chironomids, *V. cholerae* and *Aeromonas* populations better. Moreover, since the prevalence of *Aeromonas* species in the aquatic environment has been recognized as a potential health risk, understanding the natural reservoir of *Aeromonas* will help in developing methods to monitor and control the bacteria in fresh and drinking water reservoirs.

Materials and methods

Sampling sites and environmental parameters

The study was performed in northern Israel near Haifa at two sampling sites: the Tivon waste stabilization pond (WSP; longitude 699, latitude 618) and the Kishon River (near Kiryat Haroshet; longitude 697, latitude 620). The sites were within a radius of 2 miles. Water temperature, pH (Scan Model2, Rajah Crescent, Singapore) and conductivity (El-Hamma Model TH-250, Mevo Hamma, Israel) were routinely monitored. Fluctuations of 11.3 and 8.1 °C in water temperature were measured across the sampling months in the Tivon WSP and the Kishon River, respectively. Maximal temperature (33.5 °C) was measured on June 23 in the Tivon WSP. The water temperatures were always higher in the Tivon WSP than the water temperatures in the Kishon River. A basic pH was measured in both the Tivon WSP (7.8 ± 0.36) and the Kishon River (8.7 ± 0.24). The conductivity that was measured in

the Tivon WSP was between 1.5 and 1.8 mmoh cm⁻¹ and it was three times lower than that of Kishon River (between 4.0 and 5.9 mmoh cm⁻¹).

Egg mass sampling

Egg masses were sampled twice each month between May and September 2005. Styrofoam boards (25 × 25 cm) were used as artificial oviposition sites for adult females. Egg masses along the side of the board were collected 24 h after introducing the board into the water habitat.

Isolation of V. cholerae and chironomid egg mass-degrading bacteria

Chironomid egg masses from both sampling sites were brought to the laboratory immediately after collection. Three egg masses from each sampling site were chosen randomly for isolation of the bacteria. Each freshly collected egg mass was treated separately to remove all the bacteria that were not firmly attached to the egg mass. After washing five times and vortexing for 1 min in sterile saline water, each egg mass was crushed in 1 ml saline, using a sterile glass homogenizer. The homogenate was diluted, and 0.1 ml from each dilution was then spread onto thiosulfate–citrate–bile salts (TCBS; Becton, Dickson and Company, Sparks, MD, USA) for isolation of *V. cholerae* strains. To screen for other bacterial species with the potential ability of degrading the egg masses, samples from each dilution were also spread onto the following media: (i) cetrimide agar base (Himedia, Mumbai, Maharashtra, India); (ii) modified cetrimide agar base (no glycerol; gelatin—15 g l⁻¹); (iii) modified minimal broth Davis without dextrose (Becton, Dickson and Company)—gelatin and yeast extract were added, 15 g l⁻¹ and 0.5 g l⁻¹, respectively; and (iv) LB agar (Luria) (Himedia). Yellow colonies from the TCBS medium that were suspected to be *V. cholerae* were subcultured on LB agar and further examined as described below. Individual colonies from all the other four media were randomly picked and streaked onto LB agar to obtain single colonies. All isolated colonies were subcultured at least four times before further examination. Bacterial isolates were kept in LB with 30% glycerol (–80 °C).

Identification of V. cholerae

Yellow colonies from the TCBS medium that were suspected to be *V. cholerae* were subcultured on LB agar and then tested for oxidase (1% tetramethyl-*p*-phenylenediamine; Sigma, Saint Louis, MO, USA), as well as for the string test (0.5% sodium deoxycholate; Sigma). The identity of the isolates with positive results in the above tests was further verified by using the multiplex PCR assay in accordance with Nandi *et al.* (2000). The presence of toxin genes (in addition to *ctxA*, as described in

Nandi *et al.*, 2000) *stn/sto*, *hlyA*, *hapA* and *ompU* were determined in all *V. cholerae* strains. The primers used and the PCR procedure is described in Halpern *et al.* (2006) (Table 1).

Egg mass-degradation activity

All the isolates (ca. 1100) were tested for their ability to degrade chironomid egg masses. Bacterial isolates were grown in 5 ml LB medium in a shaker incubator at 30 °C (or 37 °C for *V. cholerae* isolates) for 48 h. Culture supernatants were obtained by centrifugation (12 000 r.p.m. for 7 min) and then were filtered through a 0.45-µm pore size filter (Corning; Corning, New York, NY, USA), which resulted in a clear supernatant. Degradation activity was determined by using freshly collected or ethanol (70%)-rinsed chironomid egg masses. Degradation assay was performed by incubating one egg mass in 1.0 ml clear supernatant at 30 °C for up to 72 h. Each test was carried out at least three times. A reaction was considered positive when the egg mass structure disintegrated and a large excess of free eggs was found separated from the egg mass, as compared with the control LB medium (see Figure 1). Positive results were obtained between 10 and 40 h.

Identification of egg mass-degrading bacteria

Universal bacterial primers: 8F (5'-CACGGATCCAGAC TTTGAT(C/T)(A/C)TGGCTCAG-3') and 1512R (5'-GTG AAGCTTACGG(C/T)TAGCTTGTTACGACTT-3'), based on *Escherichia coli* positions, were used to amplify internal fragments of 16S rRNA gene (Felske *et al.*, 1997). Eight microliters of a bacterial suspension was transferred to a sterile thin-walled PCR tube. One microliter of each primer (20 pmole µl⁻¹) and 10 µl of PCR master mixture (ReddyMix, ABgene,

Epsom, UK) were added to the tube to a final reaction volume of 20 µl. Initial DNA denaturation was performed at 94 °C for 4 min followed by 33 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 50 s, and elongation at 72 °C for 2 min, and then a final elongation step at 72 °C for 10 min. The amplified PCR products were purified with the AccuPrep PCR purification kit (BIONEER, Daedeok-gu, Deajeon, South Korea). Purified PCR products were sequenced at Technion Medical School (Haifa, Israel) directly by the dideoxy-nucleotide chain-termination method using a DNA sequencer (ABI PRISM 3100) with BigDye

Figure 1 Chironomid egg masses before (left) and after (right) 10 h incubation with the supernatant of a 48-h culture of *Vibrio cholerae*. The eggs were freed from the degraded gelatinous matrix.

Table 1 The presence of *V. cholerae* or *Aeromonas* species among the bacteria that were cultured from chironomid egg masses on all the sampling dates

Sampling (month, 2005)	Tivon WSP		Kishon River	
	<i>V. cholerae</i>	<i>Aeromonas</i>	<i>V. cholerae</i>	<i>Aeromonas</i>
1 (4 May)	+	+ ^a	ND	ND
2 (18 May)	+	+	ND	ND
3 (8 June)	+	+	+	+
4 (23 June)	—	—	+	—
5 (7 July)	+	+ ^a	+	—
6 (21 July)	—	+ ^a	—	—
7 (1 August)	+	+	ND	ND
8 (10 August)	+	+	+	—
9 (24 August)	+	+	+	—
10 (7 September)	+	+	+	+
11 (19 September)	+	+	—	+

Abbreviation: ND, no egg masses were sampled on this sampling date.

Aeromonas was also detected in DNA extracted from egg masses that were sampled from the Tivon WSP by *aero*-specific primers for *Aeromonas*. DNA was not extracted from egg masses that were sampled from the Kishon River.

^a*Aeromonas* was not cultured but was detected in the egg mass by using *aero* primers for the detection of *Aeromonas* sp.

terminator reagents (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. The sequencing was performed by using 8F primer and for several isolates—the reverse 1512 primer was used as well. This resulted in data for approximately 750 or 1504 bp, respectively. Newly determined sequences were compared to those available in the GenBank (<http://www.ncbi.nlm.nih.gov>) databases, using the standard nucleotide–nucleotide BLAST program (BLASTN; <http://www.ncbi.nlm.nih.gov>), to ascertain their closest relatives. These sequence data have been submitted to the GenBank database under accession numbers EF634206–EF634234 (see also Supplementary Table S1). A phylogenetic tree was generated by using the neighbor-joining method with NJPlot (MEGA 3), based on alignments from CLUSTAL W. The bootstrap values obtained were from 1000 iterations.

Identification of isolates using specific primers for *Aeromonas*

Since most isolates were identified as *Aeromonas* species while using 16S rRNA gene sequencing, the identification of the remaining isolates was done by using specific primers for the genus *Aeromonas* and for four species in this genus: *A. hydrophila*, *A. punctata*, *A. veronii* and *A. schubertii*. All nucleotide primers were targeted to the 16S–23S intergenic spacers, in accordance with Kong *et al.* (1999). To confirm amplicon production, the mixture was analyzed by electrophoresis on 1.5% agarose gel, followed by staining with ethidium bromide and visualization under ultraviolet light.

Direct identification of *Aeromonas* spp presence in egg masses using PCR

DNA was extracted from individual egg masses that were sampled in Tivon WSP as follows: Each egg mass was first incubated with proteinase K and then DNA was extracted by using Invisorb Spin Plant Mini Kit (Invitec, Berlin, Germany), according to the manufacturer's instructions. The presence of *Aeromonas* was detected in the extracted DNA by using *aero*-F and *aero*-R primers (Kong *et al.*, 1999), specific for the detection of members of the *Aeromonas* genus.

Genotypes associated with virulence in isolates of *Aeromonas* species

The presence of the following genes coding for the virulence factors, was determined in all *Aeromonas* isolates: *act/aerA/hlyA* by using one set of primers AHCF1/AHCR1; *alt* and *ast* genes for cytotonic enterotoxins; *ahyB* genes for elastase; *pla/lipH3/apl-1/lip* genes for phospholipase; and *fla* gene for flagellin (Kingombe *et al.*, 1999; Sen and Rodgers, 2004). The presence of the genes *act/aerA/hlyA* and

ast; *fla* and *alt*; *ahyB* and *pla/lipH3/apl-1/lip* was tested simultaneously in the same reaction mixture, according to Sen and Rodgers (2004).

Results

Chironomid egg mass-degrading bacteria

A total number of approximately 1100 bacterial colonies were isolated from chironomid egg masses between May and September 2005. All isolates were examined for their ability to degrade chironomid egg masses. The supernatants of all the isolates that were identified as *V. cholerae* (82 isolates) were able to degrade the egg masses within 10 h at the most (Figure 1). Only 45 out of all the rest of the isolates (1018), which were randomly picked from the different culture media and were not identified as *V. cholerae*, were able to degrade the egg masses. These degrading bacteria were identified either by the 16S rRNA gene or by using specific primers for *Aeromonas* species. Thirty-three isolates were identified as *A. punctata*, nine as *A. veronii*, one as *A. hydrophila*, one as *Pseudomonas otitidis* and one as *P. pseudoalcaligenes* (see Figure 2 and Supplementary Tables S1 and S2). The time required for the egg mass degradation by the 48-h supernatant ranged between 15 and 40 h, and there was no clear association with particular bacterial species (Supplementary Tables S1 and S2). The phylogenetic relationships between the egg mass-degrading isolates and their closest relatives from public databases are shown in Figure 2.

During the 5 month period, 11 samplings were performed at the two sampling sites. Table 1 summarizes the presence of *V. cholerae* and/or *Aeromonas* species among the bacteria that were cultured from chironomid egg masses on all the sampling dates. DNA was extracted from egg masses that were collected on all the sampling dates from the Tivon WSP, but not from the Kishon River. The primers *aero*-F and *aero*-R were used to amplify a 550-bp amplicon, demonstrating the presence of *Aeromonas* sp in all the sampled egg masses from the Tivon WSP (except for sampling 4, see Table 1). All the egg masses were washed and vortexed several times before they were cultured or before DNA was extracted. Thus, the detected or isolated bacteria were attached to the egg masses. No enrichment procedures were used to isolate *V. cholerae*. Likewise, no enrichment or selective media were used for the isolation of *Aeromonas* species.

Genotype of *V. cholerae* isolates

None of the strains ($n=82$) that were isolated from the egg masses and examined in this study contained the genes for the cholera toxin (*ctxA*) or for non-O1 heat-stable enterotoxin (*stn/sto*). The genes for El Tor-like hemolysin (*hlyA*) and outer

Figure 2 Phylogenetic tree of chironomid egg mass-degrading bacteria isolated from the egg masses. The tree shows the relationships based on partial sequences of the 16S ribosomal RNA gene. Strains in bold were previously isolated from chironomid egg masses (Halpern *et al.*, 2007). The accession numbers of the isolates and the time required for egg mass degradation are listed in Supplementary Table S1. The sequences alignments were performed using the CLUSTAL W program, and the tree was generated using the neighbor-joining method with Kimura 2 parameter distances in MEGA 3 software. Bootstrap values (from 1000 replicates) greater than 50% are shown at the branch points. The bar indicates 2% sequence divergence.

membrane protein (*ompU*), which have been associated with enteropathogenicity, were found in 70% and 16% of the strains, respectively. The genotypes were found in various combinations (see Table 2). All isolates possessed the structural gene *hapA*, which is responsible for soluble hemagglutinin/protease production (see Table 2). The supernatants of all the strains examined in this study

demonstrated the ability to degrade chironomid egg masses.

Virulence factors in *Aeromonas* species

Aeromonas bacteria that were isolated from the chironomid egg masses were screened for the presence of genes for six virulence factors: *act/aerA/hlyA* genes

Table 2 Genotypic traits of representative isolates of *V. cholerae* non-O1/non-O139 examined in this study (n = 82)

Genotype	Presence (%)
<i>ompW</i> ⁺ <i>hapA</i> ⁺ <i>hlyA</i> ⁺ <i>ompU</i> ⁺	15
<i>ompW</i> ⁺ <i>hapA</i> ⁺ <i>hlyA</i> ⁺ <i>ompU</i> ⁻	55
<i>ompW</i> ⁺ <i>hapA</i> ⁺ <i>hlyA</i> ⁻ <i>ompU</i> ⁺	1
<i>ompW</i> ⁺ <i>hapA</i> ⁺ <i>hlyA</i> ⁻ <i>ompU</i> ⁻	29

All the examined strains had the genotype of *ctxA*⁻ and *stn/sto*⁻.

Table 3 Genotypic traits of *Aeromonas* spp isolated from chironomid egg masses (n = 43)

Genotype	Presence (%)
<i>pla/lipH3/apl-1/lip</i> ⁺ <i>ahpB</i> ⁺ <i>ast</i> ⁻ <i>act</i> ⁺ <i>fla</i> ⁺ <i>alt</i> ⁺	26
<i>pla/lipH3/apl-1/lip</i> ⁻ <i>ahpB</i> ⁺ <i>ast</i> ⁻ <i>act</i> ⁺ <i>fla</i> ⁺ <i>alt</i> ⁺	12
<i>pla/lipH3/apl-1/lip</i> ⁺ <i>ahpB</i> ⁺ <i>ast</i> ⁻ <i>act</i> ⁻ <i>fla</i> ⁺ <i>alt</i> ⁺	7
<i>pla/lipH3/apl-1/lip</i> ⁻ <i>ahpB</i> ⁻ <i>ast</i> ⁻ <i>act</i> ⁺ <i>fla</i> ⁺ <i>alt</i> ⁺	9
<i>pla/lipH3/apl-1/lip</i> ⁺ <i>ahpB</i> ⁺ <i>ast</i> ⁻ <i>act</i> ⁻ <i>fla</i> ⁻ <i>alt</i> ⁺	7
<i>pla/lipH3/apl-1/lip</i> ⁺ <i>ahpB</i> ⁺ <i>ast</i> ⁻ <i>act</i> ⁺ <i>fla</i> ⁺ <i>alt</i> ⁻	2
<i>pla/lipH3/apl-1/lip</i> ⁻ <i>ahpB</i> ⁺ <i>ast</i> ⁻ <i>act</i> ⁺ <i>fla</i> ⁻ <i>alt</i> ⁺	2
<i>pla/lipH3/apl-1/lip</i> ⁻ <i>ahpB</i> ⁺ <i>ast</i> ⁻ <i>act</i> ⁺ <i>fla</i> ⁺ <i>alt</i> ⁻	2
<i>pla/lipH3/apl-1/lip</i> ⁺ <i>ahpB</i> ⁺ <i>ast</i> ⁻ <i>act</i> ⁺ <i>fla</i> ⁻ <i>alt</i> ⁻	2
<i>pla/lipH3/apl-1/lip</i> ⁻ <i>ahpB</i> ⁺ <i>ast</i> ⁻ <i>act</i> ⁻ <i>fla</i> ⁻ <i>alt</i> ⁺	9
<i>pla/lipH3/apl-1/lip</i> ⁻ <i>ahpB</i> ⁻ <i>ast</i> ⁻ <i>act</i> ⁺ <i>fla</i> ⁻ <i>alt</i> ⁺	7
<i>pla/lipH3/apl-1/lip</i> ⁻ <i>ahpB</i> ⁺ <i>ast</i> ⁻ <i>act</i> ⁺ <i>fla</i> ⁻ <i>alt</i> ⁻	2
<i>pla/lipH3/apl-1/lip</i> ⁻ <i>ahpB</i> ⁺ <i>ast</i> ⁺ <i>act</i> ⁺ <i>fla</i> ⁻ <i>alt</i> ⁻	2
<i>pla/lipH3/apl-1/lip</i> ⁻ <i>ahpB</i> ⁺ <i>ast</i> ⁻ <i>act</i> ⁻ <i>fla</i> ⁻ <i>alt</i> ⁻	5
<i>pla/lipH3/apl-1/lip</i> ⁻ <i>ahpB</i> ⁻ <i>ast</i> ⁻ <i>act</i> ⁺ <i>fla</i> ⁻ <i>alt</i> ⁻	2
<i>pla/lipH3/apl-1/lip</i> ⁻ <i>ahpB</i> ⁻ <i>ast</i> ⁻ <i>act</i> ⁻ <i>fla</i> ⁻ <i>alt</i> ⁻	2

Figure 3 The frequency of the virulence genes among *A. veronii* (n = 9) and *A. caviae* (n = 33) isolated from chironomid egg masses.

for a cytotoxic enterotoxin and hemolytic activity; *ast* and *alt* genes for cytotoxic enterotoxins; *ahpB* gene for elastase; *pla/lipH3/apl-1/lip* genes for lipase; and *fla* gene for flagella. The most prevalent genes were *alt* and *ahpB*, which were found in 78% and 76% of the isolates, respectively (see Table 3,

Figure 3). The genes *act/aerA/hlyA*, *fla* and *pla/lipH3/apl-1/lip* were also quite prevalent among the isolates (65%, 59% and 43%, respectively). The most infrequent gene was *ast* (see Table 3, Figure 3). It was found only in one isolate, which was identified as *A. hydrophila* (isolate 10T1D19) (see Figure 2 and Supplementary Table S1). The isolate 9T1LB56 was the only one that did not possess any of the six examined virulence factors. Although the virulence genotypes were found in different combinations, five of the six examined virulence factors were positive (*ast* was negative) in the most widespread genotype (26% of all isolates) (see Table 3).

Discussion

Chironomid egg masses are known to be a natural reservoir of *V. cholerae* (Broza and Halpern, 2001; Halpern et al., 2004, 2006, 2007). While exploring for bacteria other than *V. cholerae* that can degrade the egg masses, we demonstrated that chironomid egg masses harbor *Aeromonas* species along with *V. cholerae*. *Aeromonas* species were isolated from the egg masses throughout most of the samplings and were further shown to be inhabitants of the egg masses by using specific primers for *Aeromonas* sp (Table 1).

In a recently published study, *Aeromonas* species were isolated from chironomid egg masses when the bacterial diversity of the egg masses was studied (Halpern et al., 2007). Both in Halpern et al. (2007) and in the current study, egg masses were thoroughly washed to obtain only bacteria that were attached, probably as a biofilm, to the egg mass and no selective or enrichment methods were used for the isolation of *Aeromonas* species. Thus, *Aeromonas* species seem to be inhabitants of chironomid egg masses.

The following virulence-associated genes were detected in *V. cholerae* isolates from chironomid egg masses: hemagglutinin/protease (*hapA*, 100%); hemolysin (*hlyA*, 70%); and outer membrane protein (*ompU*, 16%) (Table 2). Cholera toxin (*ctxA*) and the non-O1 heat-stable enterotoxin (*stn/sto*) were not detected. A very similar pattern (*hapA* 100%, *hlyA* 72% and *ompU* 28%) was observed in a previous study examining *V. cholerae* isolates from egg masses (Halpern et al., 2006).

The following virulence-associated genes were detected in *Aeromonas* in the current study: 78% (*alt*), 76% (*ahpB*), 65% (*act/aerA/hlyA*), 59% (*fla*), 43% (*pla/lipH3/apl-1/lip*) and 2% (*ast*) (see Table 3, Figure 3). These results (except for the *ast* frequencies) are quite similar to those obtained by Sen and Rodgers (2004): 88% (*ahyB*), 88% (*lip*), 59% (*fla*), 43% (*alt*), 70% (*act*) and 30% (*ast*). The genes were found in various combinations in both studies. The results show that *Aeromonas* bacteria found in chironomid egg masses possess a wide variety of virulence-related genes, as was shown by Sen and

Rodgers (2004) for bacteria that were isolated from drinking water.

Chironomid infestation in drinking water supply systems is an existing problem in Israel (Halpern *et al.*, 1999, 2002) and in other countries in the world, for example, the United Kingdom (Williams, 1974), the United States (Bay 1993; Alexander *et al.*, 1997), China (Sun *et al.*, 2007) and Japan (Tanaka *et al.*, 2004). In Japan, the source of chironomids in tap water contamination was suggested to be the larvae, which live in the water purification plant (Tanaka *et al.*, 2004). Chironomids may disseminate pathogenic species of *Aeromonas* between drinking water reservoirs, as was suggested for *V. cholerae* (Broza *et al.*, 2005). Furthermore, the gelatinous egg mass matrix may protect *Aeromonas* that inhabit the egg masses from the disinfecting agents, which are used in the drinking water systems.

No correlation was found between the concentration of *Aeromonas* spp and fecal indicator organisms (Legnani *et al.*, 1998). However, *Aeromonas* species showed a seasonal distribution, as was found for chironomids (Holmes *et al.*, 1996; Halpern *et al.*, 2004, 2006). In surface water, *Aeromonas* abundance peaks in the warm summer and fall months (Burke *et al.*, 1984; Chauret *et al.*, 2001). The seasonal increase in *Aeromonas* detection in the drinking water supply was found to match the peak occurrence of clinical isolates (Burke *et al.*, 1984).

Since the prevalence of *Aeromonas* species in the aquatic environment has been recognized as a potential health risk (Borchardt *et al.*, 2003), understanding the natural reservoir of *Aeromonas* will help in developing methods to monitor and control the bacteria in fresh and drinking water reservoirs. More work is needed to survey *Aeromonas* abundance in chironomid egg masses in different freshwater habitats, including drinking water reservoirs, so as to identify the secreted factor that degrades the egg mass and to monitor the population dynamics of chironomids, *Aeromonas* and *V. cholerae*, and their dependency.

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