Comparative Analysis of Environmental Isolates of *Vibrio* spp. from Marine, Estuarine, and Freshwater Environments in Georgia

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ABSTRACT

Members of the genus *Vibrio* are autochthonous to marine, estuarine, and freshwater habitats. Temperature, salt requirement and tolerance drives the distribution of *Vibrio* spp. in natural waters. Abundance and biodiversity of 12 clinically important *Vibrio* species, including *V. cholerae* were investigated. Water bodies containing these pathogens with potential human interaction and risk of causing disease in Georgia, such as the freshwater lakes near Tbilisi and the Adjara coastal zone of the Black Sea, were included in the study. A total of 195 presumptive *Vibrio* isolates were selected from hundreds of primary isolates collected from water and plankton samples during 2005-2006 and characterized by phenotypic (conventional biochemical and API) and polymerase chain reaction tests From this study 119 isolates of *V. cholerae,* 44 *V. parahaemolyticus*, 10 *V. vulnificus*, 11 *V, alginolyticus*, 4 *V. metschnikovii*, 5 *V. mimicus*, and 2 *V. harvei* were collected. Other clinically important vibrios, such as *Vibrio cincinnatiensis, V. fluvialis, V. furnissii, V.* (*Photobacterium*) *damsela*, and *V. hollisiae* were not detected. As expected, *V. cholerae, V. mimicus,* and *V. metschnikovii* dominated the *Vibrio* population in the freshwater lakes; whereas, *V. parahaemolyticus*, *V. alginolyticus*, and *V. vulnificus* dominated the *Vibrio* population in the Black Sea.

Biotyping of the isolates was performed, including assessment of hemolytic activity, antibiotic resistance, and susceptibility to 29 *Vibrio*-specific phages isolated during the current study. Environmental *V. cholerae* strains demonstrated variability in antibiotic resistance with resistance to the tetracycline group being most frequent. The highest resistance rate for the V*ibrio* isolates was observed in sulfanilamids (47.8%), followed by polymyxin B (44.4%), and ampicillin (42.4%).
Isolates of *V. cholerae* were grouped by hemolytic activity and susceptibility to *Vibrio*-specific phages. Seventy-seven isolates (79.4%) were lysed by at least one of the phages and 50 strains were lysed by 2-5 of the phages (51%). A total of 24 phage variants were identified among 68 *V. cholerae* strains in preliminary experiments using a new phage typing scheme. Genetic relatedness among 26 *V. cholerae* strains isolated from Lisi Lake during the summer of 2006 was determined using pulse-field gel electrophoresis (PFGE). Little to no similarity was obsered among strains from the same reservoir, even among strains isolated from the same site and same day of sampling. Furthermore, 11 isolates of *V. cholerae* from Lisi Lake collected during a 2-month period were examined by ERIC-PCR; all except two had differences in more than one profile.

Thirty-four Black Sea isolates identified as *V. parahaemolyticus* from water and plankton samples were tested for the Kanagawa phenomenon, susceptibility to antibiotics, and to specific bacteriophages, tuned out to be all Kanagawa-negative. Profiles of antibiotic susceptibility shared variability. At least four clusters of strains were observed, based on phage susceptibility. Preliminary scheme for phage-typing of *V. parahaemolyticus* was designed using specific phages with different spectra of lytic activity. The genetic relatedness of 30 isolates of *V. parahaemolyticus* was also studied by PFGE, with significant variability observed, suggesting the presence of several subtypes among the Black Sea strains.

In conclusion, the data indicate significant biodiversity among the *V. cholerae* and *V. parahaemolyticus* in the aquatic environments of Georgia.

INTRODUCTION

The members of the family V*ibrionaceae* are natural inhabitants of the aquatic environment. Among them *V. cholerae* is the causative agent of cholera occurring mostly in developing countries as a result of consumption of contaminated water and food. There are over 60 species within the *Vibrio* genus, with 12 being considered clinically important (Farmer et al, 2003). Although *V. parahaemolyticus* is the most common non-cholera *Vibrio* species reported, *Vibrio vulnificus* is associated with 94% of reported deaths (Ho & Amin, 2002). Clinical manifestations of non-cholera *Vibrio* infections are usually gastroenteritis, diarrhea, vomiting, and septicemia.

A variety of pathogenic vibrios, including *V. cholerae*, are routinely isolated from estuarine waters and freshwater sources worldwide. In the early 1970's 8 cases of cholera were reported from the Georgian territory (Burgasov, 1976; Onishenko et al, 2001). Interestingly, diarrhea-causing non-cholera vibrios ("cholera accompaniers") were regularly isolated from regions of the Black sea bordering the former Republics of the Soviet Union (Onishenko et al., 2001; Bugorskaya et al., 1974; Libinson et al., 1974). Later, in the 1980's, different non-cholera *Vibrio* strains were isolated in the Choroxi and Machaxela estuaries and Batumi aquatoria of the Georgian coast (Pkhaladze et al., 1984; Letuchaya et al., 1988). After the collapse of the Soviet Union, although the routine monitoring of water- and food-borne infections were carried out in various regions of the country, their link to *Vibrio*-associated infections could not be established due to the lack of epidemiological data and effective bacteriological investigations.

An extensive study has been undertaken as a collaborative project involving multiple institutes in Georgia and the United States to determine the presence and diversity of clinically important vibrios in aquatic environments throughout Georgia, including in fresh water and seawater environments.

MATERIALS AND METHODS

Water and plankton sampling and processing

Samples were taken from 2 sites from each lake biweekly from May to October 2006. One hundred liters of water was filtered through 64 µm, followed by filtration through 200 µm plankton nets to collect two different size fractions of plankton. One liter of plankton-free water (PFW) was also collected. Determination of physical and chemical parameters, including air temperature, pH, and salinity was performed on-site using portable instruments. Whole water sample was collected and fixed on-site for the estimation of chlorophyll-a.

Bacteriological analysis

One hundred microliters of PFW was concentrated on 0.45 µm membrane filters, and the filters were incubated at 30ºC for 18 h in 1% APW. For total vibrio count (TVC), 10-, 20-, and 50-ml aliquots PFW were concentrated on 0.45 µm membrane filters, then filters were transferred onto TCBS plates. After 18h of incubation, green and yellow colonies were enumerated. For determination of fecal coli forms, filters were placed onto m=FC plates, and, after incubation at 44.5ºC, blue colonies were counted. In parallel, fecal coliform counts were determined using 3M Petrifilm™ plates (3M Company). Samples were processed for the estimation of chlorophyll-a using a spectrophotometric method (Hansen and Reiman,1978). Because *Vibrio* spp. can be found in the environment in a viable but nonculturable state, direct detection methods, such as PCR were used.

Biochemical analysis of Vibrio spp. isolates

Presumptive *Vibrio* spp. from TCBS plates were picked and subcultured onto T1N1 plates. Selected isolates were tested for gelatinase and oxidase activity, salt requirement, ability for glucose oxidation/fermentation (Hugh-Leifson test),carbohydrate (sucrose, arabinose, lactose, mannose) utilization, arginine dehydrolase and lysine decarboxylase activity. The API 20E and API 20 NE systems (bioMerieux) were used for the identification of selected vibrios to the species level.

Electron microscopy

Bacterial cell morphology was examined by transmission electron microscopy (TEM). Samples of bacterial cultures were prepared on collodium gold grids, negatively contrasted with 2% uranyl acetate, and examined by using a M10 Zeiss electron microscope (Opton - Carl Zeiss, Germany).

Molecular analysis

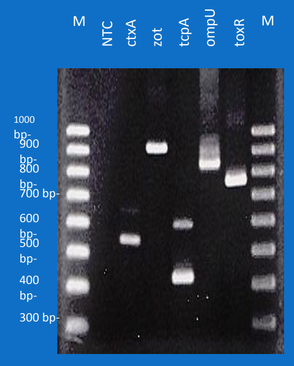
For direct molecular detection of *V. cholerae*, total DNA was extracted from concentrated water samples using the QIAamp DNA extraction kit (Qiagen). *V. cholerae*-specific PCR was performed as described previously (Chun, et al. 1999). For direct molecular detection of the V. cholerae virulence factor genes, *ctxA*, *zot*, *tcpA*, *ompU*, and *toxR*, PCR was performed as described (Rivera et al., 2001). To examine the genetic diversity among *V. cholerae* isolates, PFGE was performed according to Yeung et al. (2002) with minor modifications, and ERIC-PCR were performed according to Jersek (1999). PFGE of *Not I* digests was performed using a Gene Navigator System (Amersham Biosciences) for 18 h at 200V at 14˚C with switching times ramped from 5 to 60s.

RESULTS AND CONCLUSIONS

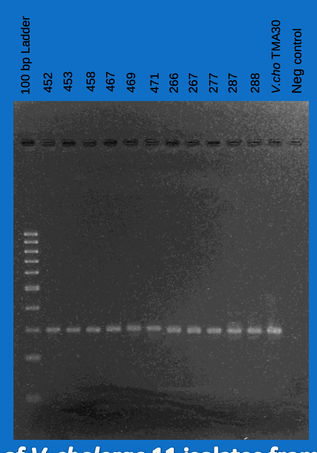
- Since May 2006, approximately 420 isolates from three freshwater lake (Kumisi, Lisi, and Tbilisi Sea) around Tbilisi and up to 300 strains of presumptive *Vibrio spp*. from the marine and estuarine waters of the Black Sea, Georgia have been collected.
- Based on biochemical tests, API and genotypical properties PCR 119 isolates of *V. cholerae,* 44 *V. parahaemolyticus*, 10 *V. vulnificus*, 11 *V. alginolyticus*, 4 *V. metschnikovii*, 5 *V. mimicus*, and 2 *V. Harvey* were collected.
- Using the newly isolated vibrio specific phages, *V. cholerae* and *V.parahaemolyticus* phagetyping scheme has been developed.
- Since Lisi Lake is often used for recreational activities, our detection of toxigenic *V. cholerae* in the lake is significant and represents a potential public health hazard. To our knowledge, this is the first report of the detection of *V. cholerae* O1 in the freshwater lakes in Georgia.
- The obtained data indicate significant biodiversity among the *V. cholerae* and *V. parahaemolyticus* in the aquatic environments of Georgia.

Vibrio-selective media (TCBS agar) – V. cholerae *is yellow.*

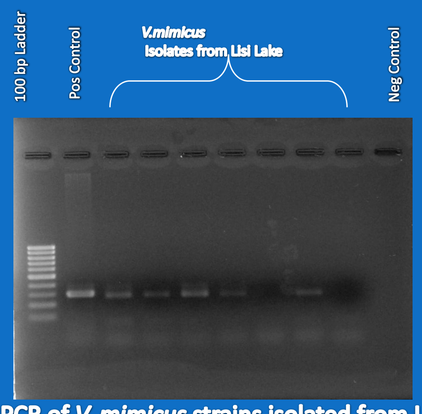
Electron micrographs showing the cell Morphology of Vibrio spp. isolated from Lisi Lake



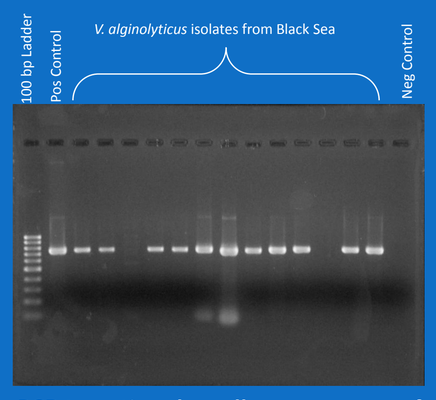
Direct Molecular Detection of *Vibrio cholerae* Virulence Factor Genes from Water Samples from Lisi Lake



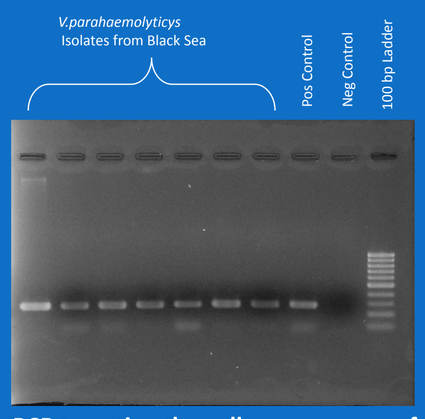
ITS-PCR of *V. cholerae* 11 isolates from Lisi Lake



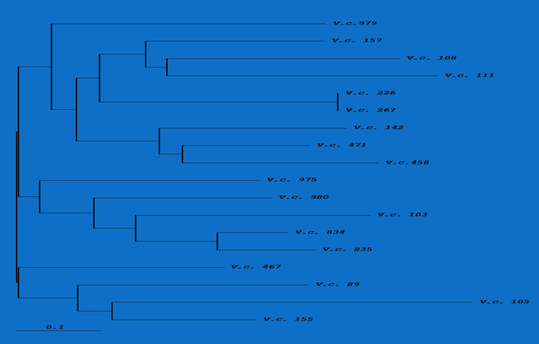
ITS- PCR of *V. mimicus* strains isolated from Lisi Lake



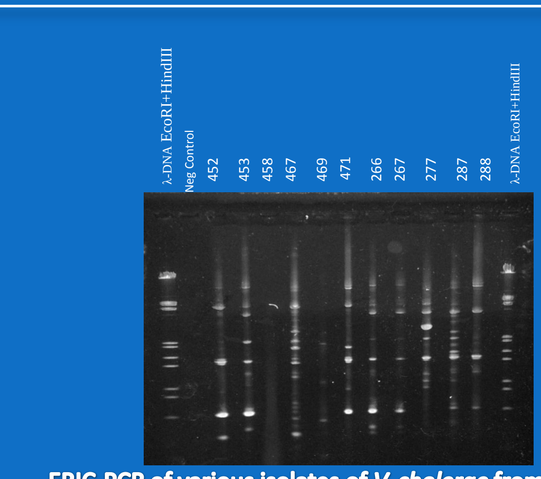
PCR targeting the collagenase gene of *V. alginolyticus* isolates from the Black Sea coastal zones, Georgia



PCR targeting the collagenase gene of *V. parahaemolyticus* isolates from the Black Sea coastal zones, Georgia



Neighbor- Joining Dendrogram Generated from a Freetree and Treerien-based Genetic Distance Data Tree of V. *cholerae*

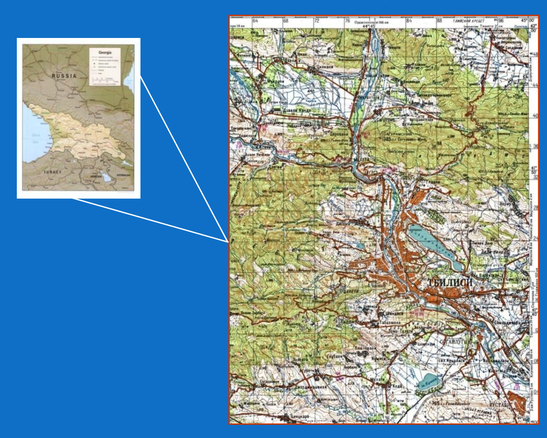


ERIC-PCR of various isolates of *V. cholerae* from Lisi Lake

1.**Marker**, 2. **L89**, 3. **103**, 4. **105**, 5.**111**, 6. **226**, 7.**267**, 8. **467**, 9. **834**, 10. **835**, 11. **975**, 12. **980**, 13. **108**, 14. **142**, 15. **155**, 16. **157**, 17. **458**, 18. **467**, 19. **471**, 20. **979**, 21. **980**

Genetic diversity among *V. cholerae* isolates from Lisi Lake as demonstrated by pulse-field gel electrophoresis analysis

Map of Georgia showing Lisi Lake and other sampling sites and their relationship to the capital city of Tbilisi



Sampling, 2006-2007

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