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Characterization of *Vibrio parahaemolyticus* and Their Bacteriophages Isolated from Fresh Water and the Black Sea in Georgia

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Abstract

Vibrio parahaemolyticus is a naturally occurring bacterium commonly found in coastal waters. When ingested, *V. parahaemolyticus* causes watery diarrhea with abdominal cramping, nausea, vomiting, and fever. Bacteriophages ("phages") can be considered biocontrol agents of pathogenic vibrios. *V. parahaemolyticus* and their phages isolated from the Black Sea during 2006-2007 were studied. *Vibrio spp.* were isolated from water samples employing TCBS agar. *V. parahaemolyticus* was identified using standard biochemical tests and PCR. Strain polymorphisms were studied using Pulsed-Field Gel Electrophoresis (PFGE). Phage genomes were compared by restriction digest analysis, and their morphology was studied by transmission electron microscopy (TEM). More than 50% of the total number of the Black Sea *Vibrio spp.* isolates was identified as *V. parahaemolyticus*. All 65 *V. parahaemolyticus* strains were Kanagawa negative. Profiles of antibiotic susceptibility showed variability. PFGE analyses revealed several subtypes among the Black Sea *V. parahaemolyticus* strains. Phage isolates were grouped into four clusters based on results of host range screening. Ten *V. parahaemolyticus* specific lytic phages were isolated from the Black Sea water samples. Most showed high specificity towards the host bacteria. TEM studies revealed phage morphology consistent with the *Myoviridae* family of viruses. The genomes of selected phages were studied using restriction analysis. Phage-host cell interactions, such as one-step growth cycle and lysis stability in liquid culture, were investigated. Environmental *V. parahaemolyticus* and their phages are abundant and diverse in the Black Sea, representing a major component of the *Vibrio* and vibriophage populations of that body of water.

Introduction

The members of the family *Vibrionaceae* are natural inhabitants of the aquatic environment. *V. parahaemolyticus* and *V. vulnificus* are the most common non-cholera *Vibrio* species causing serious clinical diseases. Infections with these organisms often present with clinical manifestations such as gastroenteritis, diarrhea, vomiting, and septicemia. A variety of pathogenic vibrios, including *V. parahaemolyticus*, are routinely isolated from marine and estuarine waters worldwide. Bacteriophages with the potential to control different species of *Vibrio* can also be isolated from environmental waters (Depaola et al, 1998; Cerveny et al, 2002; M. Tediashvili et al, 2006, unpublished data). Recently, the therapeutic efficacy of naturally occurring *V. vulnificus* phages were demonstrated in animal models (Cerveny et al, 2002). A phage typing scheme for *V. cholerae* O139 was also described, using natural isolates of vibriophages (Chakrabarti et al, 2000). A recent study conducted in Bangladesh demonstrated an inverse correlation with prevalence of phages specific to *V. cholerae* in the environment and the occurrence of cases of cholera (Farouque et al., 2004). Little is known about the spatial-temporal variation of *Vibrio spp.* and corresponding phages in the aquatic environment of the Black Sea basin countries. The goal of the study conducted in 2006-2007 was assessment of the abundance and biodiversity of *Vibrio parahaemolyticus* bacteria and their phages in the Georgian coastal zone of the Black Sea. Isolation and selection of specific bacteriophages to be used for improvement of diagnostics and phage typing scheme for *V. parahaemolyticus* as well as for treatment and prevention of infections caused by *V. parahaemolyticus* was another goal of the work undertaken.

Materials and Methods

Selection and characterization of *Vibrio parahaemolyticus* isolates was done by plating of alkaline peptone water (APW) enrichments followed by plating onto the TCBS agar plates (Fig. 1). After incubation, the olive green colonies were subcultured on gelatin agar with 1% sodium chloride, after enrichment in T1N1 (trypticase 1% and sodium chloride 1%) for gelatinase production. Oxidase activity, oxidation/fermentation of glucose (Hugh-Leifson glucose test), lysine decaboxylase and arginine dehydrolase tests were performed followed by utilization of carbohydrates, sucrose, arabinose, lactose, mannose. Salt requirement was additionally evaluated in tryptone broth containing 0, 1, 4, 6, 8 and 10% of NaCl.

Kanagawa phenomenon was determined by subculturing *V. parahaemolyticus* isolates on the Mannitol-Salt agar with 5% of sheep blood cells and 7.5% salt content.

Antimicrobial activity was determined by disc-diffusion method on Mueller–Hinton Agar using a set of 13 antibiotics. The results were classified according to the commonly accepted method (S, I and R types if susceptibility).

Pulse field gel electrophoresis (PFGE) was performed on overnight bacterial cultures digested at 37°C with 30U of the restriction enzyme *NotI*. PFGE was performed with a Gene Navigator System apparatus (Amersham Biosciences) for 18hours at 200V at 14˚C with switching times ramped from 5 to 60s. After electrophoresis, gels were stained with ethidium bromide and photographed under UV light.

Isolation of bacteriophages from environmental sources was performed standard enrichment techniques using susceptible bacterial hosts, followed by a series of passages for cloning and concentration of phages. For obtaining phages from bacterial strains the filtrates of overnight broth cultures were used. Host range of phages was determined by spot test on solid media.

Transmission electron microscopy (TEM) was used to study the cell morphologies of bacteria and the nuclocapsid ultrastructure of bacteriophages. Samples were prepared on collodium copper grids, negatively contrasted with 2% uranyl acetate, and examined by using M10 electron microscope (Opton - Carl Zeiss, Germany).

Restriction analysis of phage genomes was performed on phage DNA obtained by standard phenol/chloroform extraction. DNA was digested by various restriction endonucleases, and cleavage products were separated by agarose gel electrophoresis.

Sampling area

Collecting water samples in the Batumi area of the Black Sea

Fig. 1. *Vibrio spp.* colonies on TCBS agar
Yellow colonies- *V. cholerae*; olive green colonies - non-cholera Vibrio species(*V. parahaemolyticus* , *V. vulnificus* ,etc.)

Fig. 2. Distribution of *V. paraheamolyticus* isolates by sampling season (a) and site (b)

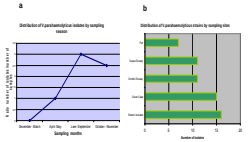
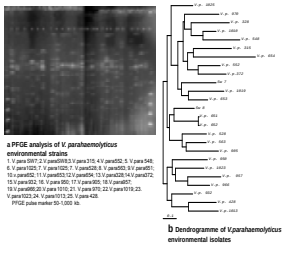


Fig. 3. Electron micrographs of *V. parahaemolyticus* isolates (EM Opton Zeiss M10)

V. parahaemolyticus 372

V . parahaemolyticus 548

Fig. 4. PFGE analysis of V. parahaemolyticus isolates



a PFGE analysis of *V. parahaemolyticus* environmental strains
1. V. para 5997; 2.V para5998;3.V para 315; 4.V para352; 5. V para 548; 6. V para1525; 7. V para1525; 7. V para528; 8. V para563; 9.V para551; 10.v para552; 11.V para553;12.V para554; 13.V para328;14.V para372; 15.V para 832; 16. V para 850; 17.V para 905; 18.V para907; 19.V para908;20.V para 1510; 21. V para 970; 22.V para1019; 23. V para 1023; 24. V para1013; 25. V para 428.
PFGE pulse marker S5-1,000 kb.

b Dendrogramme of V.parahaemolyticus environmental isolates

Fig.5. Resistance to antibiotics of *V. parahaemolyticus* and other *Vibrio* isolates

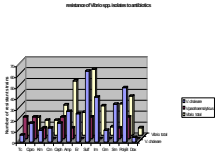
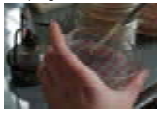
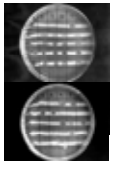


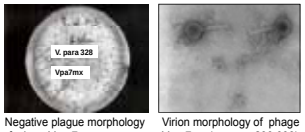
Fig 6. Screening for host range *of V. parahaemolyticus* phages





Screening for phage susceptibility

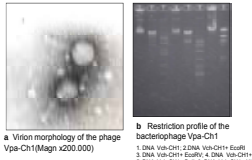
Fig. 7. *V. parahaemolyticus* phage Vpa-7mx: Negative plaque(a) and virion morphology(b)



Negative plague morphology of phageVpa-7mx

Virion morphology of phage Vpa-7mx (magn. x200.000)

Fig. 8. *V. parahaemolyticus* phage *Vpa –Ch1 :* Virion morphology(a) *and DNA restriction profile (b)*



a Virion morphology of the phage Vpa-Ch1(Magn x200.000)

b Restriction profile of the bacteriophage Vpa-Ch1
1. DNA Vch-CH1; 2.DNA Vch-CH1+ EcoRI
3. DNA Vch-CH1+ EcoRV; 4. DNA Vch-CH1+ BamHI
5. DNA Vch-CH1+ BglI; 6. DNA Vch-CH1+ HindIII
7. DNA Vch-CH1+ XhoI; 8. Marker (λ + Hind III)

Table 1. List of Bacteriophages specific to *V. parahaemolyticus*

	Bacteriophage	Isolation source	Isolation date
1	Vpa-Ch1mx	Black Sea water,Chorokhi estuary	Sept.2006
2	Vpa-B28mx	Black Sea water , Batumi Boulvard	Aug.2006
3	Vpa-B282	Black Sea water , Batumi Boulvard	Aug.2006
4	Vpa-B01	Black Sea water , Batumi Boulvard	Aug.2006
5	Vpa-B52	Black Sea water , Batumi Boulvard	Aug.2006
6	Vpa-B53	Black Sea water , Batumi Boulvard	Aug.2006
7	Vpa-L26mx	Lor lake	June 2006
8	Vpa-F1-3mx	Kumisi lake	Sept.2006
9	Vpa-Mi13mx	River Mtkvari	Sept.2006
10	Vpa-5mx	Sea water , Green cape	June 2007
11	Vpa-TS-1mx	Tbilisi Sea	July 2007
12	Vpa-9mx	River Supsa	Aug.2007
13	Vpa-N55	Intelri N55	Oct.2006
14	Vpa-N53	Intelri N53	Oct.2006
15	Vpa-B.S-a	Black Sea water , Batumi Boulvard	Oct.2007
16	Vpa-Sp	N.W. Supsa	Oct.2007
17	Vpa-Np	Nuri lake	Oct.2007
18	Vpa-Lp	Lor lake	Oct.2007
19	Vpa-Ks	Kumisi lake	Oct.2007
20	Vpa-TSc	Tbilisi Sea	Oct.2007
21	Vpa-P7mx	N.W. Poti	Aug.2006

Table 2. Phage susceptibility of *V. parahaemolyticus* isolates*

Bacterial strains	Bacteriophages (Vpa																					
	I					II					III					IV					
	Vpa -Ch mx	Vpa-B28mx	Vpa-B282	Vpa-B52	Vpa-B53	Vpa-B52	Vpa-Sp	Vpa-Np	Vpa-Lp	Vpa-TSc	Vpa-Ks	Vpa-B5-2	Vpa-5mx	Vpa-S55	Vpa-F1-3	Vpa-5-2mx	Vpa-9mx	Vpa-P7mx	Vpa-B53	Vpa-B01
V. parahaemolyticus 1003																				
V. parahaemolyticus 970	+	+	+	+	+							+							+	
V. parahaemolyticus 328						+	+	+	+	+	+							+		
V. parahaemolyticus 1019												+	+			+				
V. parahaemolyticus 549							+							+	+					
V. parahaemolyticus 315													+	+	+				+	
V. parahaemolyticus 554							+			+							+			
V. parahaemolyticus 552																	+			
V. parahaemolyticus 372	+	+	+	+	+							+							+	
V. parahaemolyticus sp1												+	+	+	+	+				
V. parahaemolyticus 1019							+					+	+			+				
V. parahaemolyticus 553								+	+	+			+	+			+			
V. parahaemolyticus 551								+	+	+										
V. parahaemolyticus 552																	+			
V. parahaemolyticus 317													+	+				+		
V. parahaemolyticus 338	+	+	+	+	+	+	+						+	+	+					
V. parahaemolyticus 1023							+	+						+						
V. parahaemolyticus 557											+									
V. parahaemolyticus 932	+					+	+						+		+					
V. parahaemolyticus 438						+	+						+		+					
V. parahaemolyticus 1013	+										+	+			+					
V. parahaemolyticus 870													+	+						
V. parahaemolyticus 887														+						
V. parahaemolyticus 874										+			+							
V. parahaemolyticus 339															+					
V. parahaemolyticus 356	+	+	+	+	+	+							+							
V. parahaemolyticus 436						+	+	+	+	+	+				+					
V. parahaemolyticus																				

Results of the spot test, incubation at 26°C 6 -8 hr ; "+"- positive lytic reaction; blank cells - NO lytic activity

Results and Conclusions

RESULTS

During 16 months of 2006-2007, 20 samplings of the Black sea coastal region were carried out for the assessment of the above mentioned microbiological parameters and for culturing of clinically important *Vibrio spp*, including *V. parahaemolyticus*. The rate of isolation of *V. parahaemolyticus* appeared to be season- and site-dependent (Fig.#1a,b). The majority of isolates were recovered from the marine water and concentrated plankton samples collected from the Green Cape and Batumi Blvd area- the sites with considerably high salinity (14-17‰). The isolation rate increased with the increase of the water temperature (June through October).

Detailed characterization of 65 isolates of *V. parahaemolyticus* was performed by biochemical analysis, hemolytic activity, and antibiotic and phage susceptibility profiles. TEM examinations revealed typical cell morphology (Fig.#3). The identity of 40 V. *parahaemolyticus* isolates was confirmed by IST-PCR. All *V. parahaemolyticus* strains were negative for the Kanagawa phenomenon (β-hemolysis under high salt concentration), which is consistent with other environmental strains of *V. parahaemolyticus*. Comparative studies of PFGE patterns for 25 selected *V. parahaemolyticus* isolates confirmed high variability, suggesting the presence of several *NotI* subtypes (2 clusters) among the studied population (Fig # 4 a,b). Profiles of antibiotic susceptibility revealed obvious variability among different *Vibrio spp.* isolates (Fig. # 5). High efficacy of Doxicycline, Imipenem, Gentamycine and Chloramphenicol towards environmental strains of *V. parahaemolyticus* in comparison with other *Vibrio spp.* should be mentioned.

V. parahaemolyticus specific phages were isolated by enrichment from marine samples collected in warm seasons. Ten *V. parahaemolyticus* specific lytic phages were obtained from the Black Sea water samples, 9 from freshwater sources (lakes and rivers) and 2 from *V. parahaemolyticus* culture supernatants (Table #1). Screening of 21 phage isolates against 58 *V. parahaemolyticus* host strains (Fig. #6) revealed characteristic patterns of phage susceptibility. Some phages showed high specificity towards the host strains, while others were capable of lysing up to 9 different *V. parahaemolyticus* isolates . Phages of *V. parahaemolyticus* were grouped into four clusters (Table #2) based on comparison of host range profiles towards 28 susceptible strains of *V. parahaemolyticus* : Group 1 –phages with high similarity, Group II- medium, Group III- low and Group III no similarity.

TEM studies revealed the main phage morphology consistent with the *Myoviridae* family of viruses, with a hexagonal head and a contractile tail (Fig. # 7b,8a). The genomes of 8 selected phages were examined by DNA restriction analysis (Fig# 8b) resulted in different restriction profiles. Based on the above mentioned results the preliminary selection of phages to be used for phage typing and for the biocontrol of *V. parahaemolyticus* infections was done.

CONCLUSION

Environmental *V. parahaemolyticus* and their phages are abundant and diverse, representing a major component of the Black Sea *Vibrio* and vibriophage population. Specific bacteriophages can be used for characterization and grouping of *V . parahaemolyticus* environmental isolates as well as for biocontrol of the infections caused by these bacteria.

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*Opinions, interpretations, conclusions, and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

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