Sequence Based Analysis reveals a polyphyletic origin of *Vibrio vulnificus* biotype 2

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Introduction

Vibrio vulnificus is a bacterial species which inhabits brackish waters from warm and tropical ecosystems distributed worldwide (Jones and Oliver, 2009). *V. vulnificus* is highly heterogeneous and has been subdivided into three biotypes (Bisharat et al., 1999; Tison et al., 1982). Biotype 1 is worldwide distributed and includes most environmental and clinical isolates of the species. This biotype causes sporadic cases of human vibriosis. Biotype 2 is less abundant but also has a worldwide distribution, and it is the only one that harbors the genetic information necessary to infect both fish and humans (Amaro and Biosca, 1996; Lee et al., 2008). Finally, biotype 3, was described in Israel (Bisharat et al., 1999), caused outbreaks of human infections associated to handling tilapia. Several studies based on MLST (Bisharat et al., 2007; Bisharat et al., 2005; Cohen et al., 2007) and on ribotyping (Sanjuan et al., 2009) have shown that the species is subdivided in two main lineages with apparently different human pathogenic potential. Under this scenario, the aim of this study has been to analyze the evolutionary origin of the biotype 2 within *V. vulnificus* species.

Material and methods

Sequence analysis

Sequences from four housekeeping genes (*glp*, *mdh*, *pntA*, and *pyrC*) and three virulence-associated genes (*pilF*, *vvhA*, and *wwz*) were aligned using Vector NTI 9.0.0 software. Descriptive analyses of the genetic variability at the loci studied were obtained using DnaSP4.09 (Rozas et al., 2003). Maximum likelihood phylogenetic trees were obtained with PHYML 2.4.4 (Guindon and Gascuel, 2003) using the most appropriate model for nucleotide substitution assessed using Modeltest V3.7 (Posada and Crandall, 1998) and support for the nodes was evaluated by bootstrapping with 1000 replicates.

Recombination analysis

Concatenated sequences of both chromosomes were tested for putative recombination events using the Recombination Detection Program, version 3 (RDP3) (Martin et al., 2005). To further corroborate the RDP3 results, two different ML trees were constructed for each recombinant locus. The tree obtained from the multiple sequence alignment of each locus was compared with the one obtained from the concatenated sequences of all the other loci. We used TreePuzzle v.5.2 (Schmidt et al., 2002) to compare both phylogenetic trees using the SH and the ELW tests.

Results and discussion

Genetic diversity

We identified 73 different sequences types (ST) among the 115 strains analysed, which indicates a high degree of genotypic diversity in *V. vulnificus*. The number of

haplotypes found at each gene ranged from 32 in *wzz* to 22 in *vvhA*. The number of polymorphic sites observed varied per locus from 23 in *mdh* to 61 in *wzz.* Among the loci studied, *mdh* had the lowest nucleotide diversity (π= 0.008), although this locus was the second in number of haplotypes. The number of polymorphic sites and total nucleotide diversity of the housekeeping genes are similar to that reported for the species in other studies (Bisharat et al., 2005; Cohen et al., 2007). These values are significantly higher for the virulence-related genes *pilF* and *wzz* but not for *vvhA,* which shows the same genetic diversity than the housekeeping genes. These data suggest that the gene *vvhA* is highly conserved within the species and that mutations in this gene could be negatively selected.

Phylogenetic relationships

The phylogeny of the *V. vulnificus* collection was analysed constructing a maximum likelihood (ML) tree from the 3159-bp concatenated sequence of the seven loci. The species is subdivided into three distinct evolutionary lineages according to the results from the concatenated tree. LI contains biotypes 1 and 2 from fish-farm-related environments and isolates from diseased fish and humans infected through fish manipulations or water contact from different geographical localizations. This lineage was enriched in European isolates probably because the fish-farming industry is especially developed in Europe, whose countries apply specific pathogen control programs. LII is uniquely formed by biotype 3 isolates, and LIII included biotype 1 isolates mostly recovered from environmental samples or from human septicaemic cases registered in USA and Asia.

Previous studies on the phylogeny of *V. vulnificus* divided the species in two main lineages (Clinical/Environmental). In our study, the division in two lineages was observed in the trees from virulence-related or from housekeeping genes, but not in the concatenated one, which clearly showed biotype 3 forming an independent lineage (LII), similar to the result obtained by Bisharat *et al*. (2005). In addition, LI (that would correspond to the predefined Environmental lineage) includes human isolates of biotypes 1 and 2, most of them from wound infections and a few ones from secondary septicaemia, while LIII (that would correspond to the previously described Clinical lineage) comprises environmental isolates from seawater and seafood, none from cultured fish, and human isolates, all from blood.

The comparison of the genetic diversity in the three main groups of isolates reveals that human isolates are more diverse than those from an environmental origin and both these are much more diverse than isolates from diseased animals. This result would suggest that multiple environmental clones have the ability to infect humans, which correlates with human cases presented as sporadic infections worldwide, and a few clones are able to infect fish, although they are overrepresented by clone amplification after epizootics in fish-farms. The exception would be the clone formed by biotype 3 isolates that are the only ones able to cause outbreaks of human vibriosis.

Intergenic recombination

There was no strong evidence supporting recombination events in chromosome II. However, recombination was detected in chromosome I by at least 4 of the methods used involving *pilF* and *wzz* in strains from fish farming environments, including biotype 2 isolates. The conditions of aquaculture settings might favour the exchange of genetic material among strains of *V. vulnificus* originating new variants of the species. To further corroborate the RDP3 results, two different ML trees were constructed for each recombinant locus. In both cases, SH and ELW tests revealed significant differences between the two topologies.

Conclusion.

V. vulnificus species is subdivided in three different phylogenetic lineages which do not correspond to the current intraspecific classification in biotypes. LI and LII seem to have evolved in fish-farming-related environments where recombination or/and horizontal transfer phenomena would have favoured the emergence of pathogenic clones for fish or humans, which would have been amplified after outbreaks of fish (biotype 2) or human vibriosis (biotype 3). The putative human virulent clones would be specially adapted to infect wounds and, occasionally, cause secondary septicaemia in humans. In contrast, LIII seems to have evolved associated to filtering organisms and would comprise clones capable of producing septicaemia probably of the primary type after seafood ingestion, although this has not been confirmed yet. Finally, the polyphyletic origin of the so-called biotype 2 supports its reclassification within the species as a pathovar that would group the strains of the species with pathogenic potential to infect and develop vibriosis in fish. The rest of the strains must be classified by using phylogenetic criteria, which would divide them into three subgroups with different potential to infect humans.

References

Amaro, C., and Biosca, E.G. (1996). *Vibrio vulnificus* biotype 2, pathogenic for eels, is also an opportunistic pathogen for humans. Appl Environ Microbiol *62*, 1454-1457.

Bisharat, N., Agmon, V., Finkelstein, R., Raz, R., Ben-Dror, G., Lerner, L., Soboh, S., Colodner, R., Cameron, D.N., Wykstra, D.L., *et al.* (1999). Clinical, epidemiological, and microbiological features of *Vibrio vulnificus* biogroup 3 causing outbreaks of wound infection and bacteraemia in Israel. Lancet *354*, 1421-1424.

Bisharat, N., Amaro, C., Fouz, B., Llorens, A., and Cohen, D.I. (2007). Serological and molecular characteristics of *Vibrio vulnificus* biotype 3: evidence for high clonality. Microbiology *153*, 847-856.

Bisharat, N., Cohen, D.I., Harding, R.M., Falush, D., Crook, D.W., Peto, T., and Maiden, M.C. (2005). Hybrid *Vibrio vulnificus*. Emerg Infect Dis *11*, 30-35.

Cohen, A.L.V., Oliver, J.D., DePaola, A., Feil, E.J., and Fidelma Boyd, E. (2007). Emergence of a Virulent Clade of *Vibrio vulnificus* and Correlation with the Presence of a 33-Kilobase Genomic Island. Appl Environ Microbiol *73*, 5553-5565.

Guindon, S., and Gascuel, O. (2003). A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. Syst Biol *52*, 696-704.

Jones, M.K., and Oliver, J.D. (2009). Vibrio vulnificus: disease and pathogenesis. Infect Immun *77*, 1723-1733.

Lee, C.-T., Amaro, C., Wu, K.-M., Valiente, E., Chang, Y.-F., Tsai, S.-F., Chang, C.-H., and Hor, L.-I. (2008). A common virulence plasmid in biotype 2 *Vibrio vulnificu*s and its dissemination aided by a conjugal plasmid. J Bacteriol *190*, 1638-1648.

Martin, D.P., Williamson, C., and Posada, D. (2005). RDP2: recombination detection and analysis from sequence alignments. Bioinformatics *21*, 260-262.

Posada, D., and Crandall, K.A. (1998). MODELTEST: testing the model of DNA substitution. Bioinformatics *14*, 817-818.

Rozas, J., Sanchez-DelBarrio, J.C., Messeguer, X., and Rozas, R. (2003). DnaSP, DNA polymorphism analyses by the coalescent and other methods. Bioinformatics *19*, 2496-2497.

Sanjuan, E., Fouz, B., Oliver, J.D., and Amaro, C. (2009). Evaluation of Genotypic and Phenotypic Methods To Distinguish Clinical from Environmental Vibrio vulnificus Strains. Appl Environ Microbiol *75*, 1604-1613.

Schmidt, H.A., Strimmer, K., Vingron, M., and von Haeseler, A. (2002). TREE-PUZZLE: maximum likelihood phylogenetic analysis using quartets and parallel computing. Bioinformatics *18*, 502-504.

Tison, D.L., Nishibuchi, M., Greenwood, J.D., and Seidler, R.J. (1982). *Vibrio vulnificus* biogroup 2: new biogroup pathogenic for eels. Appl Environ Microbiol *44*, 640-646.