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Current Topics on *Vibrio vulnificus* Infection

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Vibrio vulnificus is a halophilic estuarine bacterium that causes fatal primary septicemia and necrotizing wound infections. The gram negative bacterium was first identified in 1976 and clinical syndromes associated with the organism were described in 1980. In Korea, clinical cases were first reported in 1979 and the bacterium was first isolated in 1982. The primary septicemia occurs following ingestion of raw seafood. *V. vulnificus* preferentially affects subjects with hepatic diseases, heavy alcohol drinking habit, diabetes mellitus, hemochromatosis, and immunosuppression from corticosteroid therapy, AIDS, and malignancy. The primary septicemia progresses very rapidly and results in high fatality, 50 to 80% in a day or two.

Researches about *V. vulnificus* have been focused on the ecological distribution, virulence factors, diversities among clinical and environmental isolates, nonculturability under adverse conditions, role of iron in the pathogenesis, and early detection. Since 1984, our laboratory has been studying *V. vulnificus* concerning bacteriological characterization, preventive measures, antimicrobial therapies, early detection, and pathogenesis. Among the topics, I want to restrict this review to the early detection by nested PCR and the molecular biological studies on the virulence regulaton that have been performed in our laboratory during last two or three years.

Direct Identification of *V. vulnificus* in Clinical Specimens.

The genus *Vibrio* contains more than 30 species, and 12 of these are human pathogens or have been isolated from human clinical specimens. Eight of the 12 human-associated *Vibrio* species have been isolated from extraintestinal clinical specimens. For definitive diagnosis, *V. vulnificus* should be differentiated from at least 7 other extraintestinal *Vibrio* species. For those patients who die from *V. vulnificus* septicemia, most do so within 2 days of hospital admission. Even with most sophisticated and high-tech equipment or rapid presumptive detection methods employing differential media such as MacConkey and thiosulfate-citrate-bile salts-sucrose (TCBS) agar, it takes more than two days for the definitive identification of *V. vulnificus* from blood or tissue samples. Clinicians usually start multiple antibiotic therapy based on their 'best guesses' without waiting culture reports. However, the choices of antimicrobial agents against *V. vulnificus* and other gram-negative septicemia are dichotomous. The most effective antibiotics recommended for *V. vulnificus* infections are tetracyclines, especially doxycycline. Tetracycline, well known as a bacteriostatic antibiotic, uniquely showed bactericidal activity against *V. vulnificus* while third generation cephalosporins and aminoglycosides showed minimal antibacterial activities in broth-dilution antimicrobial susceptibility testing. In an animal experiment, tetracycline also showed superior protective activity over aminoglycosides and cephalosporins. Tetracyclines are seldom prescribed for the treatment of life threatening gram-negative septicemias. How early definitive antibiotic therapy can be started for *V. vulnificus* septicemia based on the identification of the causative organism is the most crucial determinant of the therapeutic outcome. The importance of developing rapid diagnostic measures that can identify the bacterium within hours cannot be overemphasized.

We performed this study under the aim of establishing a nested PCR protocol that gives highly sensitive and specific results within several hours. Nested PCR provides improved sensitivity and specificity in comparison to ordinary PCR. We designed two sets of primers targeting the cytolysin gene vvh and developed an effective DNA extraction method. Morris et al. proved that the gene was specific for *V. vulnificus*, and all clinical and environmental isolates of *V. vulnificus* possess the gene by DNA-DNA hybridization. By employing these methods, we established a nested PCR protocol that could effectively detect *V. vulnificus* from clinical specimens such as sera or bullae aspirates.

We designed two sets of primers targeting the *V. vulnificus* hemolysin/cytolysin gene. The target of the first primer set (P1 and P2: sense 5'-GAC-TAT-CGC-TC- AAC-AAC-CG-3' and antisense 5'-AGG-TAG-CGA-GTA- TA-CTG-CC-3') is a 704 bp DNA fragment. The second set (P3 and P4: sense 5'-GCT-ATT-TCA-CCG-CCG-CTC- AC-3' and antisense 5'-CCG-CAG-AGC-CGT-AAA-CCG- AA-3') amplifies internal 222 bp DNA fragment. We developed a direct DNA extraction method boiling specimen pellet in a 1 mM EDTA-0.5% Triton X-100 solution. The new DNA extraction method was more sensitive and reproducible than other conventional methods. The DNA extraction method guaranteed sensitivity as well even when *V. vulnificus* cells were mixed with other bacteria such as Escherichia coli or

Staphylococcus aureus. The nested PCR method could detect as little as 1 fg of chromosomal DNA and single CFU of *V. vulnificus.* We applied the nested PCR protocol to a total of 39 sera and bullae aspirates from septicemic patients. Seventeen (94.4%) out of the eighteen *V. vulnificus* culture-positive specimens resulted in positivity by the nested PCR. Eight (42.1%) out of the nineteen culture-negative samples gave positive nested PCR results.

Cloning and Characterization of the Transmembrane Virulence Regulator ToxRS of *Vibrio vulnificus*

The most optimal natural habitat of *V. vulnificus* is estuary. The bacterium normally flourish in esturine sea water, shellfish, and planktons in warm months. *V. vulnificus* is concentrated in oysters and probably in other shell fish. *V. vulnificus* opportunistically infects human when the contaminated shellfish was eaten raw by susceptible patients. This opportunist should experience a very dramatic change in the environment parameters during the infection process. Successful infection by pathogenic bacteria, in general, is established by coordinate expression of various virulence factors in vivo. Exp ression of the virulence factors is controlled by environmental cues. Pathogenic bacteria possess elegant regulatory systems that sense and react to the fluctuations of environmental parameters such as temperature, osmolarity, pH, iron concentration, or CO_2 concentration etc.

Many pathogens employ the 'two-component signal transduction systems' in regulating the virulence gene expression. Toxigenic *V. cholerae* has toxRS system for that purpose. Several virulence factors of *V. cholerae* are coordinately regulated by the gene products ToxR and ToxS, which are anchored in the cytoplasmic membrane. The genes *toxR* and *toxS* are clustered as an operon and encode transmembrane proteins ToxR and ToxS respectively. ToxR regulates expression of multiple *V. cholerae* virulence factors such as cholera toxin (ctx), toxin coregulated pilus (tcp), and accessory colonization factor (acf) genes. The activity of ToxR is further enhanced by ToxS, which interacts with the former protein in the periplasmic space and stabilizes it. There is a possibility that toxRS system plays universally important roles for the survival and multiplication of other *Vibrio*s than *V. cholerae. V. parahaemolyticus* (Vp) and *V. fischeri* (Vf) were reported to have homologs of the *V. cholerae* toxRS (Vc-toxRS) system.

Several bacterial components or products have been suggested as the virulence factors of *V. vulnificus* through in vitro or in vivo experiments. Suggested virulence factors are as follows: an extracellular hemolysin/cytolysin and an elastolytic protease, a phospholipase A2, polysaccharide capsule, resistance to the serum bactericidal activity and phagocytosis, and the iron utilization system that can acquire transferrin-bound iron. Among the exotoxins, hemolysin (HS) proved to be the most potent one. Intravenously administered HS kills mice at very low dosages (LD_{50} for mice is less than 3 hemolytic units). The HS lyses red blood cells from various animals by forming small pores in the cytoplasmic membrane and shows cytolytic activity against cultured cell lines. In animal models, locally and systemically administered HS reproduces the same clinical and pathological manifestations of the septicemia as caused by the administration of live bacteria. The HS was detected in serum and skin lesion of mice infected with live *V. vulnificus.* Recently, our laboratory found that the hemolysin causes vasodilatation at far lower dosage than that required for cytotoxicity, and that the hemolysin might play an important role in the pathogenesis of hypotensive septic shock. We came to hypothesize, on the basis of our and other groups' experimental data, that the hemolysin should play an auxiliary role in the pathogenesis of hypotensive shock though it is not a decisive virulence factor involved in the establishment of infection at the earlier stage.

All *V. vulnificus* strains tested have been shown to carry the gene(s) for HS. From a standpoint of evolution, *V. vulnificus* should have maintained the gene because it is essential for survival. We recently found that *V. vulnificus* produces more HS when the cultures were shifted from 25℃/2.5% NaCl reflecting natural habitat to 37℃/0.9% NaCl reflecting human host environment). This finding suggests that *V. vulnificus* hemolysin gene (vvh) expression can be modulated by a signal transduction system that monitors changes in environmental parameters.

In an attempt to dissect the molecular biology of virulence regulatory system in *V. vulnificus,* we examined the chromosomal DNA of *V. vulnificus* ATCC 29307 for

sequences homologous to those of *V. cholerae* toxRS (Vc-*toxRS*)genes that produce transmembrane virulence regulator proteins. By comparing the sequences of *toxRS* of *V. cholerae* and *V. parahaemolyticus* (Vp), we designed a set of degenerate primers targeting well- conserved sequences. The PCR product of 864 bp encompassing parts of both putative *toxR* and *toxS* was cloned, mapped, and sequenced. After confirmed of homologies with Vc-*toxRS* and Vp-*toxRS*, the cloned insert was used as the authentic probe. A 2.8 kbp DNA fragment hybridizing with the probe was cloned and sequenced. They contained two open reading frames attributable to Vv-*toxR* and Vv-*toxS*. The intact Vv-*toxRS* fragment in the chromosome was cloned by PCR. The Vv-*toxR* shared sequence homology of 51% and 61% with Vc-*toxR* and Vp-*toxR*, respectively. Vv-*toxS* was 64% and 66% homologous to Vc-*toxS* and Vp-*toxS*, respectively. The deduced amino acid sequences of the Vv-*toxRS* gene product (ToxRS) showed regions similar to the proposed transmembrane and activity domains of Vc-*ToxRS* and Vp-*ToxRS*. Expression of Vv-*ToxR* and Vv-*ToxS* could be observed as glutathione S-transferase (GST) fusion proteins. The fusion proteins expressed as the sizes expected from the DNA sequences of each open reading frames. The GST-Vv-ToxR fusion protein was used to immunize a rabbit to make anti-Vv-ToxR antiserum. By Western blot analysis, expression of the Vv-*ToxR* in *V. vulnificus* was confirmed. In order to test the functional homology of Vv-ToxR with Vc-*ToxR*, we examined whether Vv-ToxR could cross-activate the cholera toxin promoter incorporated in an *E. coli* strain. Vv-ToxR could activate the ctx promoter with high efficiency comparable to Vc-ToxR. As one of the candidate downstream genes regulated by Vv-toxRS, hemolysin gene (vvh) was tested whether it could be modulated by Vv-toxRS in an *E. coli* background. In an *E. coli* background, induction of the Vv-toxRS operon resulted in the increase of hemolysin production by 5- to 10-fold. To verify the up-regulating role of Vv-*ToxR* on the hemolysin production in *V. vulnificus*, Vv-*toxR* in the chromosome was interrupted by incorporating a suicide vector in the middle of the gene through homologous recombination. The knockout mutant produced less hemolysin than the parent wild strain. In conclusion, Vv-ToxR was functionally expressed in *V. vulnificus* and up-regulated the production of hemolysin.

References

1. Kook H, Lee SE, Baik YH, Chung SS, Rhee JH. *Vibrio vulnificus hemolysin dilates thoracic aorta by activating guanylate cyclase. Life Sci. 1996;59:41-7.*
2. Miller VL, Taylor RK, Mekalanos JJ. 1987. *Cholera toxin transcriptional activator ToxR is a transmembrane DNA binding protein. Cell 1987;48:271-9.*
3. Miller VL, DiRita VJ, Mekalanos JJ. *Identification of toxS, a regulatory gene whose product enhances ToxR-mediated activaton of the cholera toxin promoter. J Bacteriol 1989;171:1288-93.*
4. Lin Z, Kumagai K, Baba K, Mekalanos JJ, Nishibuch M. *Vibrio parahaemolyticus has a homolog of the Vibrio cholerae tosRS operon that mediates environmentally induced regulation of the thermostable direct hemolysin gene. J Bacteriol 1993;175:3844-55.*
5. Yamamoto K, Wright AC, Kaper JB, Morris JG. *The cytolysin gene of Vibrio vulnificus: Sequence and relationship to V. cholerae El Tor hemolysin gene. Infect Immun 1990;58:2706-9.*