Presence of Hemolysin Genes (*vmh*, *tdh* and *hlx*) in Isolates of *Vibrio mimicus* Determined by Polymerase Chain Reaction

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A total of 120 strains of *Vibrio mimicus*, 51 clinical and 69 environmental, were examined for the presence of three types of hemolysin genes (*vmh*, *tdh* and *hlx*) by PCR. Ninety-six percent (115) of the strains contained at least one of these hemolysin genes. Only 5 strains from the environment were missing all three hemolysin genes. The *tdh* was only found in 20 of the 51 clinical isolates of *V. mimicus*. This may indicate that the *tdh* gene is a virulence determinant of *V. mimicus*. More than 90% of the strains isolated from both the environment and patients possessed the *vmh* gene. Two clinical isolates possessed the *hlx* gene alone and had no other enterotoxic factors.

Key words —— *Vibrio mimicus*, hemolysin gene, PCR

INTRODUCTION

Vibrio mimicus, which was formerly classified as a sucrose non-fermentating strain of *Vibrio* cholerae, was characterized as a new species by Davis *et al.*[1] Since then, *V. mimicus* has become a pathogenic organism in public health.[2–4] The symptoms of acute gastroenteritis caused by *V. mimicus* are similar to that of *V. cholerae*, such as stomachache, watery to dysentery-like diarrhea, vomiting and nausea. Most studies of the virulence factors of this species have mainly been focused on cholera toxin-like (CT-like) enterotoxin because this species is closely related to *V. cholerae*.[5,6] Nevertheless, CT non-producing strains of *V. mimicus* have been frequently isolated from diarrheal patients,[7] suggesting that enterotoxin(s) other than CT may be involved in the pathogenesis of this species. Recently, we reported that this species also carried the genes encoding zonula occludens toxin (ZOT), accessory cholera enterotoxin (ACE) and heat-stable enterotoxin (Vm-ST) in addition to CT.[8] However, there was low incidence of these toxin genes among *V. mimicus* strains, suggesting that other pathogenic factor(s) than the above toxins also play a role in this species. Some investigators have demonstrated that purified hemolysin induced fluid accumulation in the rabbit ileal loop, the infant rabbit and the suckling mouse models.[9,10] Therefore, hemolysins may be important enterotoxic factors in *V. mimicus*.

It has been known that *V. mimicus* can produce three types of hemolysins. The *V. mimicus* hemolysin (VMH) is heat labile and immunologically similar to *V. cholerae* El Tor hemolysin, the second, Vm-TDH, is heat stable and closely related to the thermostable direct hemolysin (TDH) produced by *V. parahaemolyticus*,[11] and the third is a novel hemolysin designated HLX.[12] In the present study, we report for the first time the distribution of these hemolysin genes (*vmh*, *tdh* and *hlx*) in environmental and clinical isolates of *V. mimicus* determined by polymerase chain reaction (PCR).

MATERIALS AND METHODS

Bacterial Strains —— A total of 120 strains of *V. mimicus*, 51 and 69 strains isolated from human and environmental origins respectively, were used in this study.

Genomic DNA Extraction —— Chromosomal DNA of *V. mimicus* strains was extracted by the method described by Ausubel *et al.*[13] The method consists of lysis of the cells with proteinase K and 10% sodium dodecyl sulfate at 37℃ for 60 min, DNA extraction with phenol/chloroform/isoamyl alcohol, ethanol precipitation, drying under vacuum, and resuspension of DNA in TE buffer (10 mM Tris–HCl–1 mM EDTA, pH 8.0). The DNA concentration was determined by measuring the optical density at 260 nm with a spectrophotometer (Gene Quant RNA/DNA

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Calutator, Pharmacia Biotech). The DNA preparations thus obtained were stored at −20℃.

Primer Selection —— The oligonucleotides used to amplify a 289 bp region of *vmh*, VMH-2 (5′-GGTAGCCATCAGTCTTATCACG-3′) and VMH-3 (5′-ATCGTGTCCCAATACTTCACCG-3′) have been previously reported.[14] The TDH gene was detected as described previously,[6] using primers D5 (5′-GGTACTAAATGGCTGACATC-3′) and D3 (5′-CCACTACCACTCTCATATGC-3′), which amplify a 251 bp fragment. Primers, HLX-1 (5′-CTGCCCATTAGAAACACCCT-3′) and HLX-2 (5′-GTTGCTCATTCTCTGTCACC-3′), were used to amplify a 382 bp fragment of *hlx*.[12]

PCR Amplification Conditions —— The amplification reaction mixture contained 10 μl of template DNA (about 50 ng), 10μl of the buffer solution (100 mM Tris−HCl, 500 mM KCl, 15 mM $MgCl_2$, pH 8.3), 2 μl of each primer, 8 μl of dNTPs mixture (2.5 mM of each dNTP), and 0.5 μl (2.5 U) of *Taq* DNA polymerase (Takara Shuzo Co., Ltd., Japan). Sterilized distilled water was then added to make a final volume of 100 μl. This reaction mixture was overlaid with a drop of mineral oil. The conditions used for amplification of the *vmh*, *tdh* and *hlx* genes were as follows: initial denaturation at 94℃ for 1 min, followed by 29 amplification cycles including denaturation at 94℃ for 30 sec, annealing at 55℃ for 30 sec, and extension at 72℃ for 30 sec. The amplification products were analyzed by electrophoresis on a 3% agarose gel and visualized by UV fluorescence after being stained with ethidium bromide.

RESULTS AND DISCUSSION

Among the *V. mimicus* clinical isolates, all strains examined in this study had at least one kind of hemolysin genes (Table 1). One strain (Vm89-2) was found to carry all three hemolysin genes. Of the 51 clinical isolates of *V. mimicus*, 46 (92%) strains were found to contain the *vmh* gene, and 27 strains contained only this hemolysin gene. Twenty strains were positive for *tdh*, and 5 were positive for *hlx* . Among the tdh^+ or hlx^+ strains, 3 and 2 strains respectively carried only one hemolysin gene.

None of the 69 strains isolated from environmental sources was shown to contain *tdh* (Table 1), whereas 63 (91%) of these strains were found to carry *vmh* . One strain was positive for *hlx* . Only 5 strains from environment were negative for all of the hemolysin genes.

Table 1. Distribution of Hemolysin Genes (*vmh*, *tdh* and *hlx*) in Clinical and Environmental Isolates of *V. mimicus*

Gene			No. strain from	
vmh	*tdh*	*hlx*	Clinical	Environment
+	+	+	1	0
+	+	−	16	0
+	−	+	2	0
+	−	−	27	63
−	+	−	3	0
−	−	+	2	1
−	−	−	0	5
Total			51	69

Of the 120 strains of *V. mimicus* isolated from both clinical and environmental origins, 115 (96%) strains contained at least one kind of hemolysin gene, and 109 (91%) possessed *vmh*, whereas a few strains carried only *tdh* or *hlx* . All 5 strains lacking hemolysin genes were isolated from environmental sources. The *tdh* gene was only found in clinical isolates of *V. mimicus*.

Genetically engineered *V. cholerae* vaccines strains have been created by deleting *ctx*, *zot*, *ace* and *hlyA*. The vaccine strains, however, have still produce a low level of diarrhea in volunteers.[15] The novel hemolysin gene (*hlx*),[12] which encoded a polypeptide having a molecular mass of 10451 Da, was thought to be involved in production of this residual diarrhea. Our data show that the proportion of isolates carrying *hlx* in *V. mimicus* is quite low, with only 5 out of 51 clinical and 1 of 69 environmental isolates carrying the gene. However, 2 clinical isolates possessed only the *hlx* gene. These two strains were previously shown to not to possess other enterotoxic factors such as CT-like toxin, ZOT, ACE or Vm-ST.[8] These data suggest that this gene may play an important role in the pathogenesis of some strains of this species.

TDH has been considered to be a major virulence factor of *V. parahaemolyticus*.[16] TDH-like toxin (Vm-TDH) was previously found to be produced only by clinical isolates of *V. mimicus*, but not environmental isolates.[17] Terai *et al.*[18] have reported that *tdh* could be transferred as a transposon-like unit among some *Vibrio* species. In the present study, 20 strains of *V. mimicus*, all clinical isolates, were found to contain *tdh*. VMH is a pore-forming hemolysin which causes hemolysis in a colloid osmotic manner.[9,19] The

VMH gene has been cloned and the nucleotide sequence is 76% homologous with the El Tor hemolysin gene.[14] We showed that more than 90% of *V. mimicus* strains isolated from either the environment or the clinical possessed *vmh*, demonstrating that it is relatively conserved.

Previously, we reported that *V. mimicus* strains carry the genes encoding CT, ZOT, ACE and ST.[8] In the present study, we showed that most strains of this species also possess at least one of the hemolysin genes. It is obvious that this pathogenic bacterium is capable of producing several toxins. These results may have implications for the diversity of the symptoms caused by *V. mimicus*.

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