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Gene Encoding Zonula Occludens Toxin (*zot*) Does Not Occur Independently from Cholera Enterotoxin Genes (*ctx*) in *Vibrio cholerae*

JUDITH A. JOHNSON,[1,2]* J. GLENN MORRIS, JR.,[1,2] AND JAMES B. KAPER[1]

Division of Geographic Medicine, Department of Medicine, and Center for Vaccine Development, University of Maryland School of Medicine, Baltimore, Maryland 21201,[1] and Department of Veterans Affairs Medical Center, Baltimore, Maryland 21218[2]

Received 20 November 1992/Accepted 9 December 1992

Of 167 *Vibrio cholerae* isolates screened for sequences homologous with zonula occludens toxin (*zot*) or cholera toxin (*ctx*) genes, 3.0% of non-O1, 100.0% of clinical O1, and 0.0% of environmental O1 strains contained both *zot* and *ctx*. *zot* was present only in strains that were *ctx* positive; all *ctx*-positive strains carried *zot*. The absence of *zot*-positive, *ctx*-negative strains suggests ZOT is not an independent virulence factor for *V. cholerae*, although ZOT may play a role in the pathogenesis of toxigenic strains.

Vibrio cholerae is a major gastrointestinal pathogen causing significant morbidity and mortality worldwide. The disease cholera is caused by serogroup O1 strains producing cholera enterotoxin (CT) (5). However, non-O1 and nontoxigenic O1 strains also cause diarrheal disease ranging from mild, self-limited infection to severe dehydrating diarrhea and dysentery (1, 3, 14, 16, 17). In one study in Mexico, non-O1 *V. cholerae* was implicated in 16% of cases of diarrheal disease (7). Not all non-O1 *V. cholerae* can cause disease, and the factors responsible for the virulence of pathogenic strains are not well understood. One volunteer study showed that adherence is necessary but not sufficient for diarrheal disease (18). A variety of possible toxins have been identified in non-O1 strains, but most have not been conclusively shown to play a role in pathogenesis (4). Non-O1 strains may carry *ctx*-like genes, but these strains account for only a small fraction of isolates (8, 10, 23). Another 2.3 to 4% of non-O1 strains have the gene for a heat-stable enterotoxin (NAG-ST) (9, 20), but toxins responsible for the virulence of the majority of non-O1 strains have not been identified.

The recent description of the zonula occludens toxin (ZOT) in *V. cholerae* (2, 6) and the suggestion that it may play a role in the pathogenesis of cholera raised the possibility that ZOT may be important in the virulence of non-O1 and nontoxigenic O1 *V. cholerae* strains. ZOT disrupts tight junctions, resulting in increased permeability of rabbit small-bowel mucosa (6). Volunteers fed CVD101, a *ctxA* deletion mutant retaining *zot*, still experienced mild to moderate diarrhea (2, 12).

To determine the frequency of *zot* genes in non-O1 *V. cholerae*, 100 non-O1 strains (49 environmental and 51 clinical) were examined for the presence of *zot*. The frequency of *zot* in O1 strains was also determined for 59 clinical and 8 environmental isolates. To examine the frequency with which *zot* and *ctx* genes occur together, strains were also probed for *ctxA*. Colony blots of clinical and environmental strains from Africa, South America, Guam, Bangladesh, Bahrain, United States, Mexico, and Japan were prepared on Whatman 541 filters (19, 22). Cholera toxin genes were identified with a 23-nucleotide alkaline phosphatase-labelled oligonucleotide (CTAP) derived from the sequence of *ctxA* as previously described (21). A 575-bp *Acc*I-*Stu*I fragment internal to *zot* was isolated from pBB24 (2) and labelled with [α-^{32}P]ATP (Amersham, Arlington Heights, Ill.) by using a random priming kit (BRL, Bethesda, Md.). Hybridization was done at 37°C in 40% formamide–5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate (SDS)–1 mM EDTA–1× Denhardt's solution–100 μg salmon sperm DNA per ml, followed by a high-stringency wash at 65°C in 5× SSC–0.1% SDS (13). Duplicate sets of blots were tested with each probe.

Evaluation of colony blots (Table 1) demonstrated only three (3.0%) of the non-O1 strains had *zot*, and these three strains also were positive with CTAP. None of the environmental isolates had *zot* or *ctx*, but all 59 (100.0%) of the clinical O1 strains were both *zot* positive and *ctx* positive. In no strain did either *zot* or *ctx* genes occur alone.

With the increasing sophistication of genetic studies, we are becoming aware of the complexity of mechanisms by which a pathogen such as *V. cholerae* can cause disease. Although CT is the major virulence factor for most O1 strains, a series of cholera vaccine candidate strains constructed by abolishing their ability to produce CT did not have a total loss of virulence (12). For example, CVD101 and 395-N1 are both *ctxA* deletion mutants of classical Ogawa strain 395. However, CVD101, which has a high level of ZOT activity, caused mild to moderate diarrhea in 54% of recipients when administered to healthy North American volunteers at a dose of 10^6 CFU, while 395-N1, having reduced ZOT activity, produced only mild diarrhea in 1 of 21 volunteers given 395-N1 (6, 12). This observation, as well as the observation that some but not all strains of non-O1 and nontoxigenic O1 *V. cholerae* cause diarrhea, has led to a search for other possible toxins produced by these strains (11, 17, 18). ZOT is one such candidate toxin.

In *V. cholerae* O1 strains that have been examined (395 and 569B), *zot* and *ctx* genes are closely linked, with *zot* located immediately upstream of ctxA (2). What has not been known is how consistent this linkage is: i.e., whether *zot* is ever present in the absence of *ctx* or whether *ctx* occurs without *zot*. This has particular relevance for non-O1 *V. cholerae* strains and environmental O1 strains, both of

* Corresponding author.

TABLE 1. Results of colony hybridization with *zot* or *ctx* probes

Serogroup and source	No. (%) of strains with genotype[a]:			
	ctx⁺ *zot*⁺	*ctx*⁻ *zot*⁻	*ctx*⁺ *zot*⁻	*ctx*⁻ *zot*⁺
Non-O1				
Clinical	2 (3.9)	49 (96.1)	0	0
Environmental	1 (2.0)	48 (97.9)	0	0
O1				
Clinical	59 (100.0)	0	0	0
Environmental	0	8 (100)	0	0

[a] Plus or minus signs indicate presence or absence of the gene.

which have been associated with human illness but which often lack *ctx* genes. Our data indicate that *zot* does not occur independently of *ctx* genes and, as such, cannot be used to explain the ability of some *V. cholerae* strains to cause illness in the absence of CT. *zot* and *ctx* genes were rare in non-O1 or environmental O1 isolates. Only 3% of non-O1 strains were *zot* and *ctx* positive, which is not significantly different from the frequency seen in the toxigenic environmental O1 strains. In addition, the frequencies of *ctx* and *zot* genes in clinical and environmental non-O1 isolates in this study were not significantly different, although *ctx*-positive non-O1 strains have been reported to be associated with clinical isolation in Thailand (8). In contrast, both *zot* and *ctx* genes were present in all of the O1 clinical isolates, suggesting that *V. cholerae* strains that cause cholera are strikingly different from *V. cholerae* strains in the environment.

It is interesting that there has been such striking conservation of the relationship between *zot* and *ctx*. Mekalanos et al. have shown that the core region in El Tor strains is flanked by RS1 elements and can undergo deletion or amplification in these strains (15). This suggests not only that *zot* is conserved in toxigenic strains, but that copy numbers of *zot* and *ctx* genes may be maintained at equal levels. It is attractive to speculate that the action of ZOT from toxigenic *V. cholerae* may also contribute to the greater severity of cholera compared with disease due to *Escherichia coli* strains producing LT (heat-labile toxin showing striking homology to CT).

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