Sixty Years of *Vibrio parahaemolyticus* Research

Despite decades of research following a severe foodborne outbreak in Japan, this pathogen remains mysterious at the molecular level

Takeshi Honda, Tetsuya Iida, Yukihiro Akeda, and Toshio Kodama

A severe foodborne disease outbreak late in 1950 in Osaka, Japan, led to 20 fatalities among 272 patients, who suffered from acute gastroenteritis, with symptoms that included severe abdominal pain, described by many as a “burning sensation in the stomach,” vomiting, and severe diarrhea. The onset of symptoms was very rapid, within 2 to 6 h in most cases, and autopsies revealed extensive damage to the stomach, other components of the gastrointestinal tract, and other internal organs. Local experts quickly ruled out chemical agents as the cause of this extraordinarily potent, foodborne illness.

Experts soon identified the common food source for this outbreak—namely, a small, semi-dried fish, called shirasu. Although available shirasu samples were heavily laden with bacterial contaminants, microbiologist Tsunesaburo Fujino (1907–1992) of nearby Osaka University quickly excluded a series of well-known enteropathogens. However, he also grew frustrated when his early efforts to isolate the mysterious culprit proved unsuccessful.

**Animal Passage Technique Proves Key for Identifying Novel Pathogen**

When conventional plating techniques failed to identify the culprit pathogen, Fujino resorted to another approach, animal passaging, that earlier proved useful to him and his colleagues when they used it to isolate the mysterious virulent plague. The concept is relatively simple: virulent bacteria will grow when injected into the intraperitoneal cavity of animals such as guinea pigs or mice, whereas nonpathogens do not.

“We injected a suspension of colonies containing the two kinds of gram-negative bacteria intraperitoneally into mice,” Fujino and his collaborators reported in 1953. “Several hours later, when symptoms appeared, we took samples of ascites from the mice and inoculated them intraperitoneally into other mice and we repeated this procedure. . .” Although these repeated passages uncovered a then-familiar pathogen, *Proteus morganii*, Fujino and his collaborators found another microorganism that did not correspond to any then-known pathogen.

When Fujino isolated the new organism, he

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Takeshi Honda is Professor in the Department of Bacterial Infections, Tetsuya Iida is Professor in the Laboratory of Genomic Research of Pathogenic Bacteria, Yukihiro Akeda is Assistant Professor in the Department of Bacterial Infections, and Toshio Kodama is Assistant Professor in the Department of Bacterial Infections at the Research Institute for Microbial Diseases, Osaka University, Japan.

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**Summary**

- Animal passaging experiments in the 1950s led Japanese researchers to identify a novel, foodborne, halophilic vibrio that can cause severe gastroenteritis.
- During the 1990s, a clonal serotype of *V. parahaemolyticus* emerged from Southeast Asia and soon became pandemic.
- In the late 1960s, researchers recognized that virulent strains are hemolytic, later attributed to the thermostable direct hemolysin (TDH) in some strains and TDH-related hemolysin in others.
- Genomic analysis indicates that this pathogen also carries genes encoding two distinct sets of type III secretion systems and perhaps other virulence factors.
called it *Pasteurella parahaemolytica*. Subsequently, it was renamed *Vibrio parahaemolyticus*. The Osaka microbiologists described the pathogen as “a rod-shaped organism, 1 to 3 μm in length, with rounded ends, which is slightly pleomorphic on blood agar. It shows a tendency to bipolar staining and is monotrichal. A very few organisms can be seen to move like a *Vibrio* comma.”

Several years later, following another foodborne outbreak of gastroenteritis in Yokohama, Japan, other microbiologists uncovered another critical property of this microorganism, i.e., its halophilic character. Indeed, *V. parahaemolyticus* grows particularly well in the presence of 3% NaCl. Accumulating evidence of such properties proved instrumental in reassigning this bacterium from the genus *Pasteurella* to the genus *Vibrio* in 1958.

**Pandemic Serotype of *V. parahaemolyticus* Emerges**

Since Fujino first isolated and identified *V. parahaemolyticus*, microbiologists elsewhere in Japan and in other countries have isolated this microorganism during widespread outbreaks and in sporadic cases of gastroenteritis. In Japan, it is isolated almost as frequently as *Salmonella* spp., another bacterial pathogen causing foodborne gastroenteritis.

Although *V. parahaemolyticus* infections sometimes prove fatal, typically they are self-limiting, and clinical symptoms last from 2 to 7 days. In some cases, infected individuals may develop severe dehydration, collapse, and cyanosis. In other cases, infections lead to cardiovascular abnormalities. Although this microorganism is better known for causing gastroenteritis, it also can cause wound infections and septicemia. The major source of *V. parahaemolyticus* infection is contaminated, undercooked seafood or seafood products.

In 1996, strains of serotype O3:K6 *V. parahaemolyticus* emerged from India and spread throughout the world, including countries not only in Southeast Asian but also the United States. More recently, strains with other specific serotypes, including O4:K68, O1:K25, and O1:K untypeable, all probably derived from a common clonal ancestor, caused a gastroenteritis pandemic. This widespread series of outbreaks, attributable to so few serotypes and serovars of *V. parahaemolyticus*, was unprecedented. The handful of isolates responsible for

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**Serotyping for pathogenic isolates of *V. parahaemolyticus***

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*Under discussion by the Committee on the Serological Typing of *V. parahaemolyticus*. 

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*FIGURE 1*

Presence of two sets of T3SS gene clusters in *V. parahaemolyticus* RIMD2210633
these outbreaks is designated the “pandemic clone.” How this particular clone gave rise to the recent pandemic is not known.

Although serotyping is a time-honored traditional approach to distinguishing bacterial strains, it remains a powerful tool not only for identifying bacteria but also for conducting epidemiological analyses. In terms of V. parahaemolyticus serotyping, there are 13 O antigens (among which, 11 are approved) and 75 K antigens that are recognized by the Committee on the Serological Typing of V. parahaemolyticus. Among human isolates, various combinations of O and K serotypes (Table 1) are considered human pathogens.

The TDH and TRH Exotoxins Are Major but Not the Only Virulence Factors

When grown as colonies on agar plates, some V. parahaemolyticus strains produce a surrounding hemolytic zone, and this finding was termed the “Kanagawa phenomenon,” after the province where it was first observed. In 1968, Saburo Wagatsuma from Kanagawa Prefectural Institute of Public Health formulated a blood agar medium for quickly determining whether such strains were hemolytic or nonhemolytic. Further testing of this phenomenon indicates that hemolytic strains are far more likely than nonhemolytic strains to cause gastrointestinal illnesses. Of 2,720 isolates from human patients, 2,655 (96.5%) were hemolytic, whereas of 650 isolates from fish or seawater, only 7 (1%) were hemolytic.

By the late 1970s, researchers identified a specific protein, called the thermostable direct hemolysin (TDH), as being responsible for the Kanagawa phenomenon. The TDH toxin proves to be a homooligomer containing four identical subunits that retains activity after boiling but loses that activity when held at 55 to 60°C for 10 min. Purified TDH has several activities, including being lethal, hemolytic, cytoxic, cardiotoxic, and enterotoxic.

However, nonhemolytic strains that lack TDH also can cause food-poisoning outbreaks. In 1988 we traced such an outbreak to a V. parahaemolyticus strain that was found in travelers who visited the Republic of Maldives, an archipelago nation in the northern Indian Ocean. These findings suggested that TDH is not the only and might not be the major enterotoxic molecule associated with V. parahaemolyticus.

After extensive study, we determined that these strains produce another toxin that we named TDH-related hemolysin (TRH). The amino acid sequence of TRH is approximately 67% homologous to that of TDH, and both molecules have enterotoxigenic activity. This finding explains how individuals infected with TDH-negative strains (albeit not many) develop gastroenteritis. Further, nonpathogenic, nonvirulent strains produce neither TDH nor TRH, strongly suggesting that TDH and TRH are virulence factors for this microorganism. Moreover, purified TDH and TRH can induce fluid to accumulate in rabbit ileal tubes, a standard test for diarrhea-causing activity. Although anti-TDH/TRH antibodies can block this activity, such antibodies are not effective in neutralizing intact V. parahaemolyticus clinical isolates. Furthermore, mutant strains whose tdh or trh genes were disrupted led to weaker accumulation of
fluid than did parental strains. These results suggest that TDH and TRH are not the sole factors causing diarrhea, but we have not identified other proteins that might act as virulence factors.

Genomic Analysis Could Help Identify Other Factors

The complete sequence for the genome of a pandemic O3:K6 V. parahaemolyticus strain was made public in 2003. Notably, it contains the two sets of genes encoding the type III secretion system (T3SS) (Fig. 1). The T3SS apparatus, which consists of a basal body and needle-like structure, is found widely among gram-negative bacteria, which depend on this system to secrete and translocate virulence factors into the cytosol of eukaryotic cells. T3SS thus plays an important role in virulence for several gram-negative pathogens, including Yersinia, Salmonella, and Shigella. However, there were no previous reports of T3SS in vibrios.

One set of T3SS genes is located on the large and the other on the small chromosome of V. parahaemolyticus. They are named T3SS-1 and T3SS-2, respectively (Fig. 1). T3SS-1 consists of nearly 30 open reading frames (ORFs) and possesses significant homology with T3SS-related genes from other gram-negative bacteria, including Pseudomonas, Salmonella, Shigella, and Yersinia (Fig. 2). The average G+C content in Vibrio T3SS-1 is similar to that found throughout the genome.

The second set of Vibrio T3SS genes falls within the pathogenicity island (PI) on chromosome 2 (Fig. 1, 3). The arrangement of the T3SS-2 genes within the PI resembles that of no other known set of such genes. We also found two copies of genes for TDH and other virulence factors that resemble cytotoxic necrotizing factor from Escherichia coli and exoenzyme T from Pseudomonas aeruginosa. The G+C content of the PI is 39.8% compared to 45.4% for the rest of the genome, suggesting that Vibrio obtained this segment of DNA via horizontal transfer. T3SS-2 is found only in hemolytic strains, whereas T3SS-1 is apparently in all strains.

We constructed a series of mutant strains of T3SS genes using a tdh-deleted strain as a parent to avoid any influence of TDH on bacterial phenotypes. Mutants lacking T3SS-1 components (vscC1, vcrD1, or vscN1) were no longer cytotoxic for HeLa cells, while those missing T3SS-2 genes, including vscC2, vcrD2, or vscN2, continued to be cytotoxic. These results suggest that T3SS-1, but not T3SS-2, is involved in cytotoxicity, although the role of this phenotype is not fully understood. In terms of pathogenicity, however, T3SS-2 plays an important role, particularly in establishing enterotoxicity as determined by fluid-accumulating activity in a rabbit ileal loop test, although the effectors responsible for the enterotoxicity have not yet been identified.

Further, several bacterial proteins, called effectors, are secreted in a T3SS-dependent manner. For instance, T3SS-1 and T3SS-2 recognize distinct effector proteins for secretion. Moreover, the gene responsible for T3SS-1-related cytotoxicity in HeLa cells is VP1680, which appears to encode a protein involved in apoptosis. In general, T3SS-secreted effector proteins are difficult, if not impossible, to detect, and there are no straightforward means
for measuring their activity except when they are injected directly into target cells, making genomic analysis such a powerful tool for detecting evidence for such molecules.

ACKNOWLEDGMENTS
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SUGGESTED READING


