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# Development of the Immunomagnetic Enrichment Method Selective for *Vibrio parahaemolyticus* Serotype K and Its Application to Food Poisoning Study

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**A method using immunomagnetic separation was developed to isolate the specified K serotype of *Vibrio parahaemolyticus* from a mixture of a large number of bacteria with other K serotypes. This method was applied to food poisoning studies and could recover the *V. parahaemolyticus* serotype found in the patient from the incriminated foods.**

*Vibrio parahaemolyticus* is the major causative organism of food poisoning during the summer season in Japan. Despite extensive studies, the same serotypes isolated from the stools of patients were not usually recovered from the incriminated foods. This observed inconsistency between the patient and the vehicle food isolates is one of the most enigmatic aspects of *V. parahaemolyticus* infection (1, 2).

Immunomagnetic particles are currently commercially available, and a cell-sorting technique using these particles has been developed for the fractionation of lymphocyte populations (7, 8, 18, 19) and pathogenic bacteria (9, 12, 17).

The present study was carried out to find in the suspected foods the strains isolated from the patients by the application of this cell-sorting technique. The immunomagnetic enrichment culture was established as follows. *V. parahaemolyticus* P52 $Nal^r$ (O1:K38) and F7/$R_{ms419}$ (O4:K13) were from a collection at our laboratory. P52 $Nal^r$ was a spontaneously isolated nalidixic-acid-resistant mutant. The R plasmid, $R_{ms419}$, encodes resistance to chloramphenicol ($Cm^r$), tetracycline, and sulfanilamide (5). *V. parahaemolyticus* was cultured in heart infusion broth (Difco Laboratories) supplemented with 3% NaCl.

A commercially available rabbit antiserum kit for *V. parahaemolyticus* (titers, 1:64 to 1:128 against K antigens; Denka Seiken Co., Tokyo) and Dynabeads M-280 sheep anti-rabbit immunoglobulin G (Dynabeads) were employed. Dynabeads are uniform superparamagnetic polystyrene beads with diameters of 2.8 μm which are coated with sheep anti-rabbit immunoglobulin G (Dynal A.S., Oslo, Norway).

A small number of the F7/$R_{ms419}$ cells ($1.6 \times 10^3$ cells per ml) to be selected were mixed with a $2.5 \times 10^6$-times-larger number of P52 $Nal^r$ cells ($4.0 \times 10^9$ cells per ml). To 2 ml of this cell mixture, 20 μl of anti-K13 antibody was added. After 15 to 20 min of incubation with occasional agitation, the cells were pelleted two times by centrifugation in phosphate-buffered saline (PBS) (8 ml, 10 min, 3,500 rpm) to remove the free antibody and then resuspended in 0.2 ml of PBS (pH 7.2) by vortexing. Ten microliters of Dynabeads, containing $10^6$ particles, was added to the suspension, and then the suspension was incubated for 15 to 20 min with occasional shaking. Four milliliters of PBS was added to the mixture. The nonbinding cells were separated from the Dynabead-bound cells by placing the tube on a magnetic particle concentrator (model MPC-1, Dynal A.S.) which has high permanent magnetic properties. The nonbinding cell suspension was decanted, and the Dynabead-bound cell fraction was washed four times by using the same magnet procedure. Four milliliters of salt polymyxin broth (Nissui Seiyaku Co., Tokyo) was added to this washed fraction to permit multiplication of cells possessing the specific K-type antigen. After overnight culture, the cultures were plated onto thiosulfate citrate bile salt (TCBS) agar plates (Nissui Seiyaku Co.) containing nalidixic acid (75 μg/ml) or chloramphenicol (6.25 μg/ml). The magnitude of the enrichment for F7/$R_{ms419}$ was measured by the number of $Cm^r$ colonies that resulted. F7/$R_{ms419}$ cells were successfully enriched, and they outnumbered P52 $Nal^r$ cells more than 3,000-fold (Table 1).

The serotype-specific selection in reverse was also examined. P52 $Nal^r$ cells ($7.3 \times 10^2$ cells per ml) were selected from a $10^7$-times-larger number of F7/$R_{ms419}$ cells ($8.3 \times 10^9$ cells per ml). In contrast to the enrichment for F7/$R_{ms419}$, the magnitude of the enrichment for P52 $Nal^r$ fell far short of outnumbering the F7/$R_{ms419}$ cells (Table 1).

*V. parahaemolyticus* has been known to be adsorbed onto chitin particles (3, 6). Previous experience was that the ability to adsorb onto chitin particles differed among the strains. This differing adsorption ability might influence the results of immunomagnetic enrichment. Therefore, eight *V. parahaemolyticus* strains were examined for their ability to adsorb onto Dynabeads. These strains were arbitrarily chosen from a collection. Seven strains had $Nal^r$ selective markers. F7/$R_{ms419}$ was used as the tentative standard to measure the adsorption ability of the other strains. The F7/$R_{ms419}$ culture was mixed with each of seven strain cultures. Dynabeads were mixed into these bacterial suspensions. After adsorption onto Dynabeads and multiplication of adsorbed bacteria in the liquid medium, the adsorption ability of each strain was determined by assessing the differences between the relative ratios of $Nal^r$ to $Cm^r$ colonies before and after adsorption. F7/$R_{ms419}$ belonged to a group of strains with greater adsorption ability; P52 $Nal^r$ belonged to a group of lower-ability strains. F7/$R_{ms419}$ was 256 times more efficient than P52 $Nal^r$ in adsorbing onto Dynabeads (data not shown).

Two methods were developed to overcome the differences in adsorption onto Dynabeads among the strains of *V. parahaemolyticus*, namely cell density reduction and the use of *Escherichia coli*-treated Dynabeads. In both experiments, a small number of P52 $Nal^r$ cells with low adsorption ability was enriched among a large number of cells of a strain with high ability, F7/$R_{ms419}$.

TABLE 1. Immunomagnetic enrichment culture selective for specified K-antigenic type of *V. parahaemolyticus* from the P52 Nal$^r$ (O1:K38) and F7/R$_{ms419}$ (O4:K13) mixed-cell suspension

| Culture conditions | Cells/ml (±SD) | | | |
|---|---|---|---|---|
| | Before enrichment | | After enrichment | |
| | Nalidixic acid selection | Chloramphenicol selection | Nalidixic acid selection | Chloramphenicol selection |
| Untreated Dynabeads; anti-K13 antibody added | $(4.0 \pm 0.1) \times 10^9$ | $(1.6 \pm 0.5) \times 10^3$ | $(2.4 \pm 0.2) \times 10^5$ | $(8.1 \pm 1.7) \times 10^8$ |
| Untreated Dynabeads; anti-K38 antibody added | $(7.3 \pm 3.5) \times 10^2$<br>$(4.0 \pm 1.4) \times 10^4$ | $(8.3 \pm 1.6) \times 10^9$<br>$(6.0 \pm 3.6) \times 10^6$ | $(7.5 \pm 1.6) \times 10^4$<br>$(4.2 \pm 0.8) \times 10^8$ | $(4.7 \pm 1.4) \times 10^8$<br>$(4.4 \pm 2.6) \times 10^6$ |
| *E. coli*-treated Dynabeads; anti-K38 antibody added | $(3.9 \pm 1.6) \times 10^5$ | $(1.2 \pm 0.1) \times 10^9$ | $(2.4 \pm 0.6) \times 10^7$ | $(2.1 \pm 1.3) \times 10^5$ |

The bacterial mixture was diluted to a point that allowed antibody-aided specific binding onto Dynabeads to surpass the effects of nonspecific adsorption. The mixed-cell suspension was diluted to a concentration of approximately $10^6$ cells per ml. After the immunomagnetic enrichment culture method was used, effective enrichment of P52 Nal$^r$ was observed (Table 1).

In the second method, *E. coli*-treated Dynabeads were employed. Ten microliters of Dynabeads was mixed with 0.5 ml of *E. coli* K-12 ML4905 nutrient broth culture (about $10^9$ cells per ml) and incubated for 15 min. After the addition of 4 ml of PBS, the mixture was placed on a magnetic particle concentrator, nonbinding cells were removed by decantation, and Dynabeads were recovered. *E. coli* associated with Dynabeads showed little growth in salt polymyxin broth and could not grow on TCBS agar. A small number of P52 Nal$^r$ cells ($3.9 \times 10^5$ cells per ml) was mixed with a large number of F7/R$_{ms419}$ cells ($1.2 \times 10^9$ cells per ml). After an immunomagnetic enrichment culture method selective for the K38 serotype was used, strain P52 Nal$^r$ was successfully enriched (Table 1).

Various volumes of antibody (10, 20, and 30 μl) were examined to determine what volume was necessary to sensitize bacteria. A volume of 20 μl was large enough for either of the methods described.

Both enrichment methods were applied to research on common-source outbreaks of food poisoning. In June 1990, outbreak 1 occurred within a single family in Kobe, Japan. The four family members developed the typical symptoms of *V. parahaemolyticus* infection. The stools of two of the patients and the suspected leftover food were examined. Since the leftover food was the only food that all members of the family had shared, this food (which had been carried out from a restaurant), i.e., sushi or sea vegetable rolls, was highly suspected as the vehicle food.

The stools of the patients were placed in alkaline peptone water (Nissui Seiyaku Co.) and the leftover food was placed in salt polymyxin broth for enrichment culture. After 15 to 16 h of incubation, these cultures were subcultured onto TCBS agar, and a portion of these cultures were stored at −80°C for further studies. The typical bluish-green colonies were isolated and examined further for the minimal characteristics for identifying *V. parahaemolyticus* proposed by Hugh and Sakazaki (14). Serological characterization was performed by using an antiserum kit (Denka Seiken Co.) which contains 65 K types and 11 O groups. These strains were also examined for their production of the thermostable direct hemolysin (TDH), also known as the Kanagawa phenome-

TABLE 2. *V. parahaemolyticus* serotypes recovered from patient stools of patients and leftover food in outbreak 1

| Serotype | No. of K-antigenic-type isolates | O group determined among the isolates | | No. of TDH-positive and -negative strains among the isolates[a] | |
|---|---|---|---|---|---|
| | | O group | No. with O group/ total no.[b] | + | − |
| Patient A | | | | | |
| O4:K63 | 74 | O4 | 5/5 | 5/5[c] | |
| O3:K37 | 12 | O3 | 5/5 | | 5/5[c] |
| O4:K55 | 7 | O4 | 5/5 | 5/5 | |
| O10:K71 | 5 | O10 | 5/5 | | 4/4 |
| O4:K64[d] | 4 | O4 | 4/4 | | 3/3 |
| O3:K5 | 1 | O3 | 1/1 | | 1/1 |
| O6:K46 | 1 | O6 | 1/1 | | 1/1 |
| O1:K56[d] | 1 | O1 | 1/1 | | 1/1 |
| O4:K?[e] | 2 | O4 | 2/2 | 2/2 | |
| Total | 107 | | | | |
| Patient B | | | | | |
| O4:K63 | 3 | O4 | 1/1 | 1/1 | |
| O4:K8 | 1 | O4 | 1/1 | 1/1 | |
| Total | 4 | | | | |
| Leftover food | | | | | |
| O4:K42 | 68 | O4 | 5/5 | | 5/5 |
| O3:K4 | 18 | O3 | 5/5 | | 5/5 |
| O1:K56[d] | 6 | O1 | 5/5 | | 5/5 |
| O9:K23 | 2 | O9 | 1/1 | | 1/1 |
| O4:K64[d] | 2 | O4 | 1/1 | | 1/1 |
| O3:K6 | 1 | O3 | 1/1 | | 1/1 |
| O3:K29 | 1 | O3 | 1/1 | 1/1 | |
| O3:K30 | 1 | O3 | 1/1 | | 1/1 |
| O3:K57 | 1 | O3 | 1/1 | | 1/1 |
| O3:K? | 2 | O3 | 2/2 | | 2/2 |
| O1:K? | 1 | O1 | 1/1 | | 1/1 |
| Total | 103 | | | | |

[a] TDH production was measured: +, titer was more than 16; −, titer was less than 2.

[b] The numerator is the number of the isolates possessing the indicated O group, and the denominator is the total number of isolates examined among the isolated K type.

[c] The numerator is the number of TDH-positive or -negative strains. The denominator is the total number of isolates whose titers were measured.

[d] The same serotype was recovered from both patient A and the leftover food.

[e] ?, K-antigenic type was untypeable.

TABLE 3. Search for *V. parahaemolyticus* strains of patient serotype in the leftover food in outbreak 1 by the immunomagnetic enrichment method selective for the specified K antigenic type

| Dynabeads used | Antibody added | Serotype screened | No. of K antigen-positive isolates[a] | Serotype of the isolates[b] | No. of TDH-positive and -negative strains among the isolates[c] | |
|---|---|---|---|---|---|---|
| | | | | | + | − |
| *E. coli*-treated Dynabeads | Anti-K63 | K63 | 13/33 | O4:K63 | 4/4 | |
| Untreated Dynabeads | Anti-K63 | K63 | 15/19 | O4:K63 | 7/7 | |
| | Anti-K37 | K37 | 7/30 | O3:K37 | | 5/5 |
| | Anti-K55 | K55 | 0/45 | | | |
| | Anti-K71 | K71 | 0/50 | | | |
| | Anti-K64 | K64 | 35/35 | O4:K64 | | 5/5 |
| | Anti-K5 | K5 | 28/34 | O3:K5 | | 5/5 |
| | Anti-K46 | K46 | 0/34 | | | |
| | Anti-K56 | K56 | 35/35 | O1:K56 | | 5/5 |
| | Anti-K8 | K8 | 0/40 | | | |

[a] The numerator is the number of seropositive colonies selected by the specified K antiserum, and the denominator is the number of colonies examined.
[b] O typing was performed with each of the 5 K-type-positive isolates. All of them belonged to the O group shown in the table.
[c] For details, see Table 2, footnotes *a* and *c*.

non hemolysin, because the ability to produce TDH is a very important marker in clinical and epidemiological studies (10, 15, 16, 20). For the detection of TDH, a KAP-RPLA kit (Denka Seiken Co.) was employed, which is a reversed passive latex agglutination test using anti-TDH rabbit antibody-conjugated latex (11). *V. parahaemolyticus* isolates were cultured in mannitol peptone water supplemented with 5% NaCl, and the culture supernatants were applied to a TDH assay. Titers were expressed as reciprocals of the highest dilution resulting in positive agglutination.

Since it was usual to find more than one serotype of *V. parahaemolyticus* within a single stool or food sample (1, 4), more than 100 colonies from the stool of patient A and the leftover food were examined (Table 2). A serotype O4:K63 strain was predominant in both patients A and B, but the sample for patient B was accidentally lost during storage. Therefore, only primarily isolated strains and the record were available.

In the leftover food culture in which the conventional method was used, the predominant serotype was O4:K42; however, the predominant patient serotype, O4:K63, was not recovered.

The immunomagnetic enrichment method was applied to this food culture. This culture showed the presence of a substantial number of *Vibrio alginolyticus* cells on the TCBS agar plates. *V. parahaemolyticus* colonies were estimated to be only 4 or 5% of the total number of colonies. The efficiencies of enrichment using *E. coli*-treated and untreated Dynabeads were compared by using the patient-predominant O4:K63 strain (Table 3). This K-antigenic type of *V. parahaemolyticus* was successfully enriched by both methods. The percentages of recovery of the strains of this serotype were 39.4% when *E. coli*-treated Dynabeads were used and 78.9% when untreated Dynabeads were used. The efficiency of enrichment improved when untreated Dynabeads were employed, only when a low cell concentration was present. Therefore, immunomagnetic enrichment cultures of the remaining patient serotypes were performed by using untreated Dynabeads. Strains of serotypes O4:K63, O3:K37, O4:K64, O3:K5, and O1:K56 were recovered, but others were not (Table 3).

In July 1991, outbreak 2 occurred in a Chinese restaurant in Kobe. Of 235 customers, 141 developed a diarrheal illness. The stool samples of 30 patients and 31 suspected food samples related to this outbreak were examined. *V. parahaemolyticus* was recovered from 13 patient specimens and 14 food samples. Serotype O1:K56 was recovered from 11 of 13 *V. parahaemolyticus*-positive patients. The other two patients yielded untypeable strains. Ten colonies isolated from each of these food cultures by using the conventional method were serotyped. None of them yielded patient serotype K56.

The immunomagnetic enrichment methods were applied to the 14 *V. parahaemolyticus*-positive food cultures. These cultures showed various numbers of *V. parahaemolyticus* colonies, along with other bacterial colonies, on TCBS agar plates. The *V. parahaemolyticus* colonies were estimated to constitute 10 to 90% of the total number of bacterial colonies. One strain of type K56 was recovered from only one food sample when untreated Dynabeads were used. However, this serotype could be recovered from five food samples when *E. coli*-treated Dynabeads were employed. Of 50 colonies examined in these food samples, 24 identified strains were of serotype O1:K56. In this case, the cell concentration of *V. parahaemolyticus* was high and *E. coli*-treated Dynabeads were effective. This method using *E. coli*-treated Dynabeads was proven to be very effective in identifying the causative foods in cases of food poisoning. Thus, the use of *E. coli*-treated or untreated Dynabeads is recommended, depending upon the cell concentration of *V. parahaemolyticus* in the culture.

These food poisoning studies strongly suggest that the same causative serotypes could not be found in the food specimens as were found in the patient isolates because of the presence of many other nonpathogenic *V. parahaemolyticus* strains (13).

In outbreak 2, strains of serotype O1:K56, which were isolated from all 11 patients, were TDH positive. Of the food samples from which strains of the same serotypes were recovered, four of five showed the presence of both TDH-positive and -negative strains. There were 13 TDH-positive and 11 TDH-negative strains. The one other food sample yielded one TDH-negative serotype O1:K56 strain.

The immunomagnetic enrichment method described in this article may contribute to the further understanding of

the role of TDH in the enteropathogenicity of *V. parahaemolyticus*.

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