Preliminary Analyses on Persistence of *Vibrio Parahaemolyticus* in a Brackish Water Clam, *Corbicula Japonica*

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ABSTRACT. *Vibrio parahaemolyticus* strains with different abilities to produce Kanagawa hemolysin were observed for persistence in a brackish water clam, *Corbicula japonica*, held in aquariums with artificial seawater diluted to 60% (60% seawater) and sand bed at 25°C for 28 days. The hemolysin producer persisted at a level of 10^3 cfu/g in the molluscs held in 30–60% seawater, while it was the highest in 60% seawater followed by 50, 40 and 30% seawater in order at the 14th day. The producer was detected from digestive organs in the highest level since the second day. Three *V. parahaemolyticus* strains with different productivities for Kanagawa hemolysin persisted in a level of 10^3 cfu/g while an *Escherichia coli* strain decreased to non-detectable level in 60% seawater.—**KEY WORDS:** brackish water clam, colonization, *Vibrio parahaemolyticus*.

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Vibrio parahaemolyticus is an estuarine organism implicated as a causative agent in gastroenteritis resulting from the consumption of raw or partially cooked seafoods [6]. Most organisms isolated from clinical cases were positive in Kanagawa phenomenon [7]. Though Kanagawa-positive organism has also been detected from incriminated foods including bivalve molluscs associated with the outbreaks of gastroenteritis [9, 10], most strains isolated from the molluscs inhabiting in aquatic environments were Kanagawa-negative [1, 2, 6–8, 10]. Kumazawa and Kato [5] found high accumulations of *V. parahaemolyticus* producing a thermostable direct hemolysin (Kanagawa hemolysin), an active agent of Kanagawa phenomenon, into a gastropod mollusc, *Clithon retropictus*, up to a level of 10^3 viable units per gram in summer, but not into a brackish water clam, *Corbicula japonica*, at Hashizu Creek in Japan. In the present study, levels of *V. parahaemolyticus* strains persisting in *C. japonica* held in aquariums were compared among those with different productivities for Kanagawa hemolysin to elucidate the ecological conditions for

the hemolysin producer to persist in the commercial bivalves.

MATERIALS AND METHODS

C. japonica, 7.0 to 8.0 g weight, collected at Togo Pond in Japan was held in aquariums with 28 liters of artificial seawater diluted to 25% (25% seawater) and sand bed at 5 cm in depth at 25°C for at least one week before examinations. Artificial seawater was originally composed of NaCl 28.15 g, $MgCl_2 \cdot 6H_2O$ 5.51 g, KCl 0.67 g, $MgSO_4 \cdot 7H_2O$ 6.92 g and $CaCl_2 \cdot H_2O$ 1.45 g in 1 liter of tap water with a salinity of 35 g/l. The 25% seawater was circulated through glass wool and irradiated with ultraviolet ray at 254 nm on the way of circulation to aerate, to remove particulates and to kill organisms throughout the study. *Chlorella* sp. was given every other day at an amount of approximately 10^5 cell/ml for foods of the mollusc. The mollusc was confirmed non-detectable for *V. parahaemolyticus* before examinations.

V. parahaemolyticus strains D-3, H-45 and N-18 and *Escherichia coli* strain YS-2 were

used in this study. All strains are nalidixic acid-resistant mutants obtained in our laboratory. Strain D-3 is a Kanagawa phenomenon-positive strain originally isolated from a case of gastroenteritis. Strain H-45 is a Kanagawa hemolysin-producing strain originally isolated from *C. retropictus*, which is positive weakly in hemolysis on Wagatsuma agar [5]. Strain N-18 is a non-producer of Kanagawa hemolysin originally isolated from *C. japonica*. *E. coli* strain YS-2 was isolated originally from a case of enteritis. *V. parahaemolyticus* strains were grown at 37°C for 15 hr with shaking in Trypticase Soy Broth (Difco) with 1% NaCl, washed and suspended in 25% seawater at a concentration of about 10^8 viable units per ml. *E. coli* strain YS-2 was grown in Trypticase Soy Broth with 0.5% NaCl and suspended as *V. parahaemolyticus* strains.

Fifty molluscs were incubated in 400 ml of the bacterial suspensions in 25% seawater at 25°C for 24 hr with aeration and transferred into the aquariums. Every 5 molluscs were sacrificed at appropriated times to obtain homogenates of soft tissues and mantle fluids. Viable units of *V. parahaemolyticus* strains in the homogenates were estimated by plating on Bromothymol Blue-Lactose Agar (Nissui) supplemented with 5% NaCl, 0.5% sucrose and 5 µg/ml nalidixic acid after incubation at 37°C for 18 hr and expressed as the number of colony forming units per gram (wet weight) (cfu/g) of the homogenates. Those of *E. coli* were estimated on the Bromothymol Blue-Lactose Agar supplemented with 25 µg/ml of nalidixic acid. Viable units in water were estimated using membrane filter cultivation method [5].

RESULTS

Molluscs ingested 1.9×10^6 cfu/g of *V. parahaemolyticus* strain D-3 were held in aquariums filled with 30, 40, 50 and 60% seawater at 25°C for 14 days (Fig. 1). The

Fig. 1. Persistence of *V. parahaemolyticus* strain D-3 in *C. japonica* held in aquariums paved with sand beds and filled with artificial seawater diluted to 60% (●), 50% (■), 40% (○) and 30% (□). *C. japonica* ingested the organism in 1.0×10^8 cfu/ml of the bacterial suspension at 25°C for 24 hr with an aeration was transferred to the aquariums. *Chlorella* sp. was given every other day at about 10^5 cells/ml. Persistences of the organism in water were described in corresponding small symbols; 60% (●), 50% (■), 40% (○) and 30% (□) seawaters.

organism detected from the molluscan homogenates decreased to approximately 10^3 cfu/g at the 4th day in any salinity groups. The organism was similar in level among the 4 groups at the 7th day while it was the highest in 60% seawater at 2.2×10^3 cfu/g followed by 50, 40 and 30% seawater in order at the 14th day. The organism in water was at a level of 10^2 cfu per 100 ml at the second day and decreased rapidly in any salinity groups.

Molluscs ingested *V. parahaemolyticus* strain D-3 were held in an aquarium filled with 60% seawater to estimate the levels of the organism in digestive gland and digestive tract (digestive organs), gill, mantle fluid and

Fig. 2. Persistence of *V. parahaemolyticus* strain D-3 in digestive gland and digestive tract (●), mantle fluid (■), gill (○) and other organs (□) of *C. japonica* held in an aquarium paved with a sand bed and filled by 60% seawater. *C. japonica* ingested the organism in 4.9×10^9 cfu/ml of the bacterial suspension at 25°C for 24 hr with aeration was transferred to the aquarium. *Chlorella* sp. was given every other day at about 10^5 cells/ml.

others separately (Fig. 2). Levels of the organism detected just after ingestion were the highest in mantle fluid. Since the second day, however, the levels were the highest in digestive organs, where 6.2×10^3 cfu/g of the organism were detected at the 14th day.

Molluscs ingested approximately 10^6 cfu/g of *V. parahaemolyticus* strains D-3, H-45 and N-18 and *E. coli* strain YS-2 respectively were held in aquariums filled with 60% seawater (Fig. 3). Three *V. parahaemolyticus* strains fluctuated simultaneously in the molluscs and persisted in levels of 10^2 cfu/g at

Fig. 3. Persistence of *V. parahaemolyticus* strains D-3 (●), H-45 (■) and N-18 (○) and *E. coli* strain YS-2 (○) in *C. japonica* held in aquariums paved with sand beds and filled by 60% seawater. Concentrations of the bacterial suspensions ingested by *C. japonica* were 9.9×10^8 cfu/ml for strain D-3, 7.7×10^8 cfu/ml for strain H-45, 1.6×10^9 cfu/ml for strain N-18 and 4.4×10^8 cfu/ml for strain YS-2. *C. japonica* ingested the organisms at 25°C for 24 hr with aeration was transferred to the aquariums. *Chlorella* sp. was given every other day at about 10^5 cells/ml.

the 28th day in which strain N-18 was the highest, while *E. coli* strain YS-2 was eliminated within 21 days. All organisms were detected from water in levels of less than 10 cfu/ml only at the second day.

DISCUSSION

Three *V. parahaemolyticus* strains with different productivities for Kanagawa hemolysin persisted in *C. japonica* for 28 days at a similar level as the maximum of non-producer of the hemolysin detected from *C. japonica* at Hashizu Creek [5], while *E. coli* strain YS-2 was eliminated from the mollusc. *V. parahaemolyticus* strain D-3 was detected from digestive organs in the highest level

since the second day. Greenberg *et al.* [3] showed that Kanagawa-positive *V. parahaemolyticus* and *Vibrio harveyi* persisted in hardshell clam, *Mercenaria mercenaria*, at a level of 10^2 cfu/g for 72 hr after ingestions while *E. coli* was eliminated within 50 hr. Hartland and Timoney [4] showed that *E. coli*, *Salmonella typhimurium* and *Shigella flexneri* were eliminated from *M. mercenaria* and American oyster, *Crassostrea virginica*, within 24 hr at 20°C. Therefore, *V. parahaemolyticus* and some other *Vibrio* species, but not some enteric bacteria, would colonize in digestive organs of *C. japonica* and some other bivalve molluscs. Slow elimination of *E. coli* in our study might be attributed to the use of sand bed in the aquariums.

Level of the organism persisting in the mollusc seems to be dependent upon salinity of the water. The molluscs held in 60 to 30‰ seawaters might have developed different enteric flora competing with *V. parahaemolyticus*, or otherwise, the molluscs held in different salinity conditions might have different protective reactions. In any case, the organism might be unstable in the molluscs held in low salinity conditions.

Successive persistences of the producers of Kanagawa hemolysin in the present study seem to be inconsistent with evidences obtained in our field study as that the producer was detected from *C. retropictus* and sediments but not from *C. japonica* at Hashizu Creek [5]. In the present study, three *V. parahaemolyticus* strains fluctuated simultaneously in the molluscs. As the producer was not detected from the molluscs inhabiting in sediments with the producers in the field study [5], the producers might be eliminated from the molluscs if non-producers would be ingested to those with the producers.

These evidences suggest the routes of transmissions of the organism to cause food poisonings. The hemolysin producer was shown to be distributed in sediments and *C. retropictus* at Hashizu Creek [5]. If some

commercial bivalve molluscs including oyster and clam inhabiting at the coastal areas would be contaminated with the hemolysin producer migrated from the estuaries at the floods or other ecological disturbances, the producer would persist in these molluscs at least in some periods.

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要 約

ヤマトシジミへの腸炎ビブリオの定着: 熊澤教眞・加藤英一・中川幸夫(鳥取大学農学部獣医公衆衛生学教室)
——腸炎ビブリオを経口摂取したヤマトシジミを砂床と人工海水を入れた曝流式水槽内で飼育し、経時的に貝の軟体部乳剤に含まれる摂取菌の生菌数を測定することにより本菌がヤマトシジミに定着する条件を検討した。神奈川県溶血毒産生菌 D-3 株を 10^6 cfu/g の濃度に摂取した貝を60~30%濃度の人工海水中で飼育した結果、貝から検出される菌数は摂取後4日目に 10^3 cfu/g 前後まで減少した後に安定化し、14日目までほぼ同じ濃度で検出されたが、摂取後7~14日目の菌数は貝の飼育水の塩分濃度が高いほど多い傾向を示した。60%濃度の人工海水中では摂取後2日目以降、貝の腸管と中腸腺を含む乳剤から最も多くの菌が検出された。神奈川県溶血毒の産生量の異なる腸炎ビブリオ3株と大腸菌1株を各々 10^6 cfu/g の濃度に摂取した貝を60%濃度の人工海水中で飼育し、貝の体内の菌数の推移を比較した結果、腸炎ビブリオを摂取した貝からはいずれの菌株についても摂取後7日目から28日目まで 10^3 cfu/g 前後の菌が検出されたが、大腸菌は次第に減少し、21日目には検出されなくなった。以上の成績から、腸炎ビブリオは本実験に用いた条件下では神奈川県溶血毒産生能の有無にかかわらずヤマトシジミの体内で少なくとも28日間生残することが明らかになった。