Occurrence of Vibrionaceae Bacteria in Five Kind Species of Marine Fish Organs in Southern Area of Japan

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Occurrence of Vibrionaceae Bacteria in Five Kind Species of Marine Fish Organs in Southern Area of Japan

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Abstract

A total of 1664 bacterial colony was isolated from different organs of five fish species; *Sillago parvisquamis*, *Sebastes inermis*, *Trachurus japonicus*, *Sardinops melanostictus* and *Nemipterus virgatus* which are living in sea water in southern area of Japan. More than 50% of the isolated bacteria were *Vibrio* sp., which was concentrated in different fish organs depending on the fish species. These results are due to feeding habit and quality of water where fishes are living. In addition, the percentage of bacteria which have hemolytic activity was found to be higher in stomach than in gills, and surface.

INTRODUCTION

Members of family Vibrionaceae are gram-negative bacteria. Their shapes and characters are; straight or curved rods, 0.5－0.8 μm in width and 1.4－2.6 μm in length, chemoorganotrophs and facultative anaerobes, capable of respiratory and fermentative metabolism (Paul and Schubert, 1984). They are a natural inhabitant of estuarine and marine environments (Tada *et al.*, 1992) and are enhanced by warmer water (Trust, 1986). Some members of the genus *Vibrio* causes diarehea or gastroenteritis in human (Lin *et al.*, 1993), others results in a rapidly progressive hemorrhagic septicaemia that can account for high mortalities among marine animals, and especially among Salmonides under conditions of high-density aqua culture (Singer *et al.*, 1992; Valla *et al.*, 1992).

Vibrionaceae bacteria exhibit a remarkable capacity to secrete proteins through their double-membraned envelops into the surrounding medium. In general, bacterial proteases have been suggested to be important virulence factors for a variety of organisms by causing massive tissue damage in the host, which may aid the bacteria in host cell entry. For example *V. vulnificus* secretes a protease that is suggested to be involved in the tissue

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necrosis observed in wound infections, and to aid in the utilization of iron from heme proteins (Milton *et al.*, 1992). These virulence proteases include Cholera enterotoxin, neuraminidases and hemolysins, *etc.* (Leece and Hirst, 1992). One of these hemolysins is the thermostable direct hemolysin (TDH) which is mainly produced by *Vibrio parahaemolyticus*, other *Vibrio* and *Aeromonas* spp. (Lee *et al.*, 1992; Terai *et al.*, 1991).

The classification of some members of family Vibrionaceae is based on their ability to show clear hemolytic zones around colonies on a special blood-agar medium. In marine environment; there are numerous possibilities of the interactions between micro-organisms and fishes, and this is one area of microbiology that requires carefully designed and manipulated procedures (Austin, 1987). Also, since members of family Vibrionaceae are the commonest cause of food poisoning in summer, especially in association with the eating of raw or partially cooked seafood (Lida and Yamamoto, 1990) both medical and food microbiologists should be aware of the potential significance of these organisms.

The aim of this study is to determine the occurrence percentages of Vibrionaceae bacteria in different fish organs, and the relationship between their occurrence percentages and feeding habit and quality of water where fishes are living. Furthermore to detect the percentages of the lethal *Vibrio* sp. (*e. g.* species that have hemolytic activity) among the isolated Vibrionaceae species.

MATERIALS AND METHODS

Examined fishes

This study deals with five kinds of healthy fishes that were bought from a fish market in Kagoshima City southern Japan. The fishes were freshly stored in ice boxes. The fishes were; *Sillago parvisquamis, Sebastes inermis, Trachurus japonicus, Sardinops melanostictus* and *Nemipterus virgatus* which are widely distributed in adjacent seas around Kyushu Island.

Vibrionaceae bacterial isolation from fishes

Fishes were dissected to obtain surface (epidermis and flesh), gills and internal viscera (stomach) that were placed separately into petri-dishes. Approximately 500 mg of each tissue or organ was put in a test tube containing 5 ml sterile saline solution (2.5% NaCl). Subsequently, each tube was vortexed for about 2 minutes. Twenty μl of that saline solution was spread on peptone agar plates (pH 7.2) and incubated overnight at 28℃. Fifty colonies were picked up and inoculated on TCBS (Thiosulfate citrate bile salt sucrose) agar plates and incubated overnight at 28℃. TCBS agar is a selective medium for detecting Vibrionaceae bacteria which is composed of the following in grams per liter: Yeast extract, 5.0; peptone, 10.0; sucrose, 17.0; sodium thiosulfate-pentahydrate, 10.0; sodium citrate, 10.0; sodium cholate, 3.0; iron III citrate, 1.0; beef bile powder, 5.0; bromothymol blue,

0.04; thymol blue, 0.04; agar, 15.0; pH 9.0. The occurrence of Vibrionaceae bacteria was recognized by the appearance of characteristic large, green and yellow colonies of Vibrionaceae bacteria (Barrow, and Miller, 1976).

For the regular culture of the isolated bacteria, peptone agar medium was used which consisted of the following in grams per liter: Polypeptone, 5.0; yeast extract, 2.0; NaCl, 25.0; $MgSO_4$, 1.0; and agar, 15.0; pH 7.2.

Testing of hemolytic activity of the isolated bacteria

One hundred ml of peptone agar medium was prepared in 200 ml Erlenmeyer flask. The medium was autoclaved and cooled to 45–50℃. Five ml of human blood erythrocytes was added using a sterile pipette (final conc. 5%). The medium was well mixed. The green and yellow colonies that appeared on the TCBS agar plates were transferred to the blood agar plates. Hemolysis test for the isolated bacteria was done by using blood agar medium which consisted of the following: Peptone agar medium and 5% (vol./vol.) of human blood (received from Red Cross Blood Center, Kagoshima, Japan), with pH 7.2.

Inoculated blood agar plates were incubated for one or two days at 28℃ and the percentage of hemolysis was calculated. When alpha (α) hemolysis occur an indistinct zone of partial destruction of the erythrocytes surrounds the colonies and is often accompanied by a greenish to brownish discoloration of the medium. When alpha prime (α') hemolysis occur, a small halo of intact or partially lysed erythrocytes is present adjacent to the colony with a zone of complete hemolysis extending farther out into the medium. When beta (β) hemolysis occur, a clear, colorless zone surrounds the colonies and the erythrocytes in the zone are completely lysed (Robert, and Krieg, 1994). Positive test give clear zone of beta-hemolysis. Discoloration and alpha hemolysis should be regarded as negative (Barrow, and Miller, 1976).

Identification of bacterial strains

For identification of *Vibrionaceae* sp. from the isolated bacteria. ID TEST & EB-20 kit (Nissui Pharmaceutical Co., LTD., Tokyo) was used. This kit contains twenty enzyme depending tests which are; Hydrogen sulfide production, Esculin hydrolysis (ESC), Phenyl pyruvic acid (PPA), Indole production (IND), Voges-proskauer (VP), Citrolin utilization (CIT), Lysine decarboxylase (LDC), Arginine dihydrolase (ADH), Ornithine decarboxylase (ODC), O-Nitrophenyl-β-D-galactopyranoside (ONGP), Urea production (URE), Malonate utilization (MALO), Adonitol utilization (ADO), Inositol utilization (INO), Raffinose utilization (RAFF), Rhamnose utilization (RHA), Sorbitol utilization (SOR), Sucrose utilization (SUR), Manitole utilization (MAN), and Arabinose utilization (ARA). This kit has valuable importance, because it takes a short time (18–24h) to identify the isolated bacteria.

First, the bacterial suspension was prepared by inoculating 5 ml of 2.5% NaCl saline solution from the slant agar with an optical density of about 0.2 at 660 nm by using

TAITEC mini (photo 518) photometer. Subsequently, 2.5 ml of nutrient broth tube was inoculated with 250 μl of the previous bacterial suspension. Also this tube was inoculated with 350 μl of 20% NaCl saline solution (final conc. of NaCl is 3% and 3 x 10^9 cells/ml of bacteria). Nutrient broth was used for bacterial suspension in the identification of the isolated bacteria which consisted of the following (μg per 2.5 ml): Sodium thiosulfate, 100 ; iron III citrate, 30; esculin, 100; phenylalanine, 100; tryptophan, 100; pyruvic acid sodium salt, 10; sodium citrate, 1,000; lysine monohydrochloride, 1,000; arginine monohydrochloride, 1,000; ornithine-monohydrochloride, 1,000; 2-nitrophenyl ß-D-galactopyranoside, 100; urea, 2,000; malonic acid disodium salt, 450; ribitol, 1,500; inositol, 1,000; raffinose, 1,000; rhamnose, 1,500; sorbitol, 1,000; saccharose, 1,000; mannitol, 1,000; and arabinose, 1,000. Finally from this inoculated tube, 100 μl was transferred in every slot of the ID test kit which was incubated at 37℃ for 24 h. After incubation, activity of enzymes were scored and the bacteria was identified using a standard code booklet in the kit.

Statistical procedures

Data from three independent experiments were statistically analyzed by one way ANOVA and Dunnet-t test using Macintosh software Stat View SE+Graphics at confidence level 95%. Probabilities of $P<0.05$ were considered to be significant.

RESULTS

Percentage occurrence of *Vibrionaceae* sp. among other bacterial groups

A total of 1664 bacterial colony was isolated from the surface, stomach and gills of five fish species on peptone agar plates. Subsequently, these colonies were transferred to

Table 1 Total number of isolated colonies from the organs of five fish species

Fish organs	Fish speceis				
	Sillago parvisquamis	*Sebastes inermis*	*Trachurus japonicus*	*Sardinops melanostictus*	*Nemipterus virgatus*
	Total number of isolated colonies[a]				
Surface	110	93	77	92	107
Stomach	102	106	113	103	140
Gills	145	150	117	89	120
	Total number of isolated *Vibrio* colonies[b]				
Surface	11	23	8	19	6
Stomach	95	41	104	39	122
Gills	67	90	97	55	94

[a] Each 500 mg from fish organs was suspended in 5 ml saline solution, 20 μl of that saline solution was spread peptone agar plates for colony isolation.
[b] Fifty colony was tested for the occurrence of *Vibrio* colonies.

TCBS agar plates, and incubated overnight. Although not all the transferred colonies grew on TCBS agar plates, 871 colonies (52.3%) of the 1664 isolates were *Vibrio* sp. which could grow. This indicated high occurrence of Vibrionaceae bacteria in these organs (Table 1).

In the present study three independent experiments were conducted in the period from May-August, and the average results of the three experiments were calculated. By following Bergey's manual of systematic bacteriology (Paul *et al.*, 1984), it could be recognized that the less abundant *Vibrionaceae* sp. were *V. cholera, V. alginolyticus, V. anguillarum.* etc. which have yellow color on TCBS agar plates, and *Vibrionaceae* sp. like *V. parahaemolyticus, V. metschnikovii, V. camphellii.* etc. that had green color on TCBS agar plates were the most abundant.

In this study, the average percent of the yellow colonies on TCBS agar plates was 46.1% while the average percent of the green colonies on the same plates was 47.1% i.e. the percent of yellow colonies was lower than that of the green one. An uncounted 6.8% was the result of undetected growth of *Vibrionaceae* sp. in one of the three experiments which were conducted (*e.g.* surface and stomach of *Sardinops melanostictus* and surface of *Nemipterus virgatus*) (Table 2).

Table 2 Descriptions of average percent of isolated colonies from the organs of five fish species[a]

Fish organs	Fish speceis				
	Sillago parvisquamis	*Sebastes inermis*	*Trachurus japonicus*	*Sardinops melanostictus*	*Nemipterus virgatus*
Average % of green *Vibrio* colonies on TCBS medium					
Surface	52.80	5..53	33.30	64.80	44.40
Stomach	44.20	57.60	64.63	47.10	74.96
Gills	59.90	17.76	16.53	44.43	79.44
Average % of yellow *Vibrio* colonies on TCBS medium					
Surface	47.20	94.43	66.6	1.83	22.20
Stomach	55.76	42.33	35.3	19.53	25.00
Gills	40.04	82.14	83.4	55.50	20.46
Average % of α' hemolysis of *Vibrio* colonies[b] on blood agar medium					
Surface	16.60	26.60	–	16.60	–
Stomach	–	6.03	–	1.83	34.80
Gills	27.40	–	–	–	25.90
Average % of α hemolysis of *Vibrio* colonies[b] on blood agar medium					
Surface	50.00	6.60	33.30	–	–
Stomach	10.40	27.20	–	31.50	31.30
Gills	15.00	78.30	36.70	66.60	64.80
Average % of β hemolysis of *Vibrio* colonies[b] on blood agar medium					
Surface	–	–	–	16.60	–
Stomach	64.00	–	11.90	–	–
Gills	22.50	–	–	–	–

[a] These results are the average of the three independent experiments.
[b] The hemolysis percent of yellow and green colonies.
- No hemolytic activity detected.

The occurrence percent of α, α' and β hemolysis

The average percent of α hemolysis was greater than that of α' hemolysis which was also recognized to be greater than that of β hemolysis which were 30%, 10.4% and 7.7% respectively. This characteristic β hemolysis was termed as Kanagawa Phenomenon (KP) (Cherwoncgrodzky *et al.*, 1981; Cherwoncgrodzky *et al.*, 1982; Cherwoncgrodzky *et al.*, 1984; Chun *et al.*, 1975; Kishishita, *et al.*, 1992; Lida, *et al.*, 1990; Lin, *et al.*, 1993; Nishibuchi, *et al.*, 1988 and Tada, *et al.*, 1992), which is caused by TDH (thermostable direct hemolysin) produced by this organisms. Concerning this phenomenon, *Vibrios* are classified into two groups: hemolytic strains (Kanagawa Phenomenon-positive; KP^+) and non hemolytic strains (KP^-). Those colonies with α and α' hemolysis are considered as KP^- strains and with β hemolysis are considered as KP^+ strains.

Since β hemolysis only can be considered as positive result, it can be concluded that only 7.7% of hemolysis was the result of those three experiments (Table 2). In Table 2, colonies showing β hemolysis resulted only from stomach and gills of *Sillago parvisquamis*, stomach of *Trachurus japonicus*, and surface of *Sardinops melanostictus*. Moreover, one of the *Vibrionaceae* sp. which was isolated from *Sillago parvisquamis* stomach was *Aeromonas sobria*. It was observed that two *Aeromonas sobria* strains have a true β hemolytic activity on blood agar plates. Further biochemical tests were performed on a selected colonies which resulted in the identification of these strains.

The percentage occurrence of *Vibrionaceae* sp. in the different organs of five fish species

The percentage occurrence of *Vibrio* sp. in the stomach of *Sillago parvisquamis*, *Trachurus japonicus* and *Nemipterus virgatus* were 92.94, 92.02, and 86.93, respectively which were higher than that of gills and surface of that fishes. From the gills of *Sebastes inermis* and *Sardinops melanostictus* were 60.0 and 61.82, respectively which were higher than those from the stomach and surface of that fishes (Table 3). Moreover, these results were accompanied with the highest percentage of hemolysis in the stomach (75.9%) than in the gills (22.5%) and surface (16.6%) (Table 2).

Table 3 The average occurrence percentages of *Vibrio* sp. in different fish organs of five fish species[a]

Fish organs	Fish speceis				
	Sillago parvisquamis	*Sebastes inermis*	*Trachurus japonicus*	*Sardinops melanostictus*	*Nemipterus virgatus*
Surface	9.80	24.51	10.11	20.52	5.60
Stomach	92.94	38.53	92.02	37.90	86.93
Gills	46.17	60.00	82.82	61.82	78.25

[a] The average is the result of three independent experiments.

Further biochemical tests were done on selected colonies which resulted in the identification of the strains to the species level.

Results of statistical analysis

Statistical analysis using one way ANOVA for the occurrence of *Vibrio* sp. in different fishes organs was calculated. By following Dunnet-t test, it was concluded that the probabilities of Vibrionaceae occurrence was $P>0.05$ which indicate insignificant difference between the number of *Vibrio* sp. in the five fish species.

DISCUSSION

From the previous results it is concluded that the occurrence percentage of *Vibrio* sp. is higher in the case of *Sillago parvisquamis, Trachurus japonicus*, and *Nemipterus virgatus* stomach than in the gills and surface, in contrast the case of *Sebastes inermis* and *Sardinops melanostictus* opposite results were obtained.

Two questions arose our minds. First, why is the occurrence of *Vibrio* sp. higher in the stomach than in the gills and in other cases the reverse occurs? Fishes like *Sillago parvisquamis, Sebastes inermis* and *Nemipterus virgatus* are benthic species that gather on mud or sand bottoms of the sea. They feed on, crustacea, fishes, molluscs, lugwarms, and cephalopods, while fishes like *Trachurus japonicus* and *Sardinops melanostictus* are pelagic, forming large schools, migratory, moving northward in summer and tending also to move more in shore as temperatures begin to drop. They feed not only on zooplankton, especially copepods, but also phytoplankton; i. e. they are filter feeders through their gills (FAO, 1992a; FAO, 1992b; Hajime, *et al.*, 1987; Lagler, 1962).

From a logical point of view, the accumulation of *Vibrio* sp. in the stomach of benthic fishes and in gills of pelagic ones support the previous results in the case of *Sillago parvisquamis, Sardinops melanostictus* and *Nemipterus virgatus*. But in the case of *Trachurus japonicus* (which is pelagic) and *Sebastes inermis* (which is benthic) the reverse results were obtained (Table 3). Also, statistical analysis revealed insignificant differences between that occurrence per fish species.

As a conclusion we can say that the highest occurrence of Vibrionaceae bacteria in fish stomach or gills is due to the food and living habits of only some kinds of fishes, i.e. we can not rule out that all benthic fishes have high occurrence of *Vibrio* sp. in their stomach, or all pelagics have high occurrence of *Vibrio* sp. in their gills. Occasionally some exception happens. This is due to the adherence of the bacteria to the food particles which are eaten by the benthic type, so it will accumulate directly in the stomach of these fishes, or by the filtration of the water flowing current through the gills, the bacteria will be adhere to the gills with the food particles.

In the case of pelagic type, during their rapidly swimming, the fish ingest the surrounding sea water to balance the osmotic loss of water across their gills and skin (Evans, *et al.*,

1993). Consequently, the bacteria existing in the sea water enter through their mouth opening directly to their stomach, or the reverse water current can transfer the bacteria which is previously adsorbed on the gills into the stomach.

The second question is: why does *Vibrio* sp. in particular accumulate in fish organs? During summer, when the temperature is high and in highly eutrophic waters., it is possible that the lack of upwelling and aeration in the warmer seawater allow the uneaten food and feces to accumulate within the fish holding areas. Such organic matters comprise an excellent source of nutrients for heterotrophic bacteria, (into which Vibrionaceae is included) which will undergo periods of rapid multiplication. This finding support the high percentage occurrence of *Vibrionaceae* sp. in fish organs than the other genus of microorganisms; i.e. the bacterial species depend on the water quality where fishes are living. Unfortunately, some of these common aquatic bacteria may function as fish or human pathogens (Austin, *et al.*, 1987).

Some investigators (Ishimura, *et al.*, 1988), isolated about 11 strains of *Vibrionaceae* sp. (motile *Aeromonas* sp.) from 10 samples of aquatic environments, and this ratio is considerably high. Moreover, according to the study of El Sarnagawy (1978), he isolated seven *Vibrio parahaemolyticus* strains from four salted Egyptian fish kinds. Jensen, *et al.*, (1977) found a great variety of colony types on TCBS agar plates from marine samples that showed different forms of presumptive Vibrios. This supports the present findings.

The previous results showed that, although the hemolytic activity is higher in stomach than in the gills and surface, the value of the hemolytic activities per fish species is still low (Table 2). Studies made by Lida and Yamamoto (1990) indicated that 96% of clinical isolates are KP^+ while 99% of environmental isolates are KP^-.

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