Short Communication

Mass Mortality of Japanese Abalone *Haliotis discus hannai* Caused by *Vibrio harveyi* Infection

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Sixty thousand of deaths among cultured Ezo abalone *Haliotis discus hannai* occurred within a few days at an abalone farm in Japan in the middle of August, 2002. Dead animals were characterized by a hemolymphatic edema around the major circulatory system. Vibrios showing swarming motility dominated in the edema. The pathogenic vibrios were identified as *Vibrio harveyi* based on a phylogenetic analysis and a phenotypic characterization. In both immersion and injection experiments, the swarming vibrios fulfilled Koch's postulates as a pathogen for Ezo abalone. Using a GFP-tagged *V. harveyi* S20, a clump of bacterium was detected on the gills of the abalone within 48 hours after contact with the bacterium. This is the first report of *V. harveyi* infection in Ezo abalone *Haliotis discus hannai*.

Key words: *Vibrio harveyi*, mass mortality, Ezo abalone

Disease is a complex phenomenon, leading to some form of measurable damage to the host organism. Infections by micro-organisms are one aspect of disease that find ready acceptance within the general category of disease³. Because of the fluidity of water, marine animals reside with massive numbers of aquatic microorganisms. Once physiological and nutritional imbalances have occurred between these animals and microbes, mass mortality in aquatic animals may increase as a consequence of disease caused by infection.

Vibrio harveyi is widespread in marine environments. The bacterium is abundant in warm-water seasons^{26,36}, when it is likely to cause a physiological imbalance in marine animals. *V. harveyi* has been reported to be a luminescent marine bacterium¹³. Numerous *V. harveyi* strains, however, were recently reported to exhibit pathogenicity

toward sharks^{3,9,10}, groupers^{16,38}, summer flounder³¹, salmonid fish³⁹, dentex, gilthead sea bream, European sea bass²⁴, rock lobster⁶, penaeid shrimps^{2,25}, and abalone^{18,20}. *V. harveyi* is presently recognized as a fish and shellfish pathogen⁴.

The Japanese Ezo abalone *Haliotis discus hannai* is a high-quality abalone sold live as a raw foodstuff all over the world²¹. A method of intensively culturing abalone was devised by Japanese researchers in the 1950s¹². Chinese researchers subsequently adopted this technique and adapted it to conditions in China in the 1970s^{7,19}. Large quantities of cultured abalone have been produced in both Japan and China⁷. Aquaculturing of Ezo abalone was started in 1976 in southwest Hokkaido, the original habitat of the cold water-adapted species. Two major abalone farms established in Taisei and Kumaishi were capable of supplying 3 million abalone juveniles annually. Ezo abalone aquaculture farms are now established in many parts of

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Japan, due to the superior commercial value and disease resistance of the species.

Mass mortality of cultured Ezo abalone was first reported in the 1980s. This was just after the opening of a Kumaishi abalone farm. At this farm, 60,000 animals died within a few days after an outbreak, in the middle of August, without any predictive signs, such as loss of appetite. Animals ranging between 10 and 50 mm were affected. Dead animals were observed in half of the tanks on the farm. Mitigation measures employed by the farm to prevent further deaths included standard measures such as suspending the feeding of artificial diets and cleaning the tanks; these measures are known to be effective at reducing cumulative mortality in such instances. Rapid action in implementing these measures limited the mass mortality to less than 3% cumulative mortality of the abalone stock at the farm. The outbreak only occurred when the water temperature in seawater on the farm increased above 20°C. Therefore, the two-month period from August to September in most years was considered critical for the outbreak at the abalone farm. A possible causative factor has been considered to be warm-water stress of cold water-adapted abalone. In this paper, we suggest that the mass mortality of Ezo abalone was also triggered by a bacterial infection of *Vibrio harveyi*.

The mass mortality of abalone started suddenly on 20 August 2002 at a Kumaishi abalone farm. A total of 60,000 cultured abalone died within a few days. Ten dead Ezo abalone (*H. discus hannai*) were collected at the farm and transported to our laboratory on ice. Five healthy animals that had been reared in other tanks in which no deaths had occurred were also collected. After a general visual observation of the dead abalone, the hemolymph in the edema of the five dead specimens (Fig. 1) was collected aseptically using a sterilized plastic syringe (19G). No obvious lesions were observed in the healthy abalone; a homogenate of the internal organs of the healthy abalone was prepared and

then subjected to bacteriological examination. The surfaces of three healthy animals were disinfected by immersion in 70% ethanol for 1 min and the internal organs were aseptically excised. The pooled samples of the internal organs of the healthy animals were homogenized in sterile seawater using a Stomacher laboratory blender (Colworth type 80, London, UK) for 1 min. Seawater and bottom sediment were also taken from the tank in which uninfected abalones were cultured.

Serial tenfold dilutions of the hemolymph, homogenate, seawater, and bottom sediment were prepared with sterile seawater. From the 10^{-1} to 10^{-5} dilutions, 0.1 ml was inoculated on ZoBell2216E agar containing 0.5% sodium alginate²⁷⁾, then incubated at 25°C for 5 days. Viable bacterial counts were calculated from the number of colonies that developed on the agar plate. Simultaneously, for each test sample, 30 colonies were randomly picked from a plate with suitable colony counts, purified, and characterized.

All of the isolates were tested for biological and biochemical characteristics, and the representative pathogenic strains S20 and S35 are described in Table 1. In more detail, a total of 29 phenotypic characteristics were determined as a standard manual characterization established in our laboratory according to Baumann *et al.*⁵⁾, Holt *et al.*¹¹⁾, Leifson¹⁷⁾, and West *et al.*³⁷⁾. A carbon assimilation test was conducted using basal seawater medium⁵⁾. A bacterium capable of making an extended colony without an entire edge on the agar plate was recorded as positive for swarming motility. The phenotypic characterization was done at 20°C. Bacteria were tentatively classified according to the scheme for the identification of bacteria isolated from marine environments described in Sawabe *et al.*²⁷⁾.

Approximately 1400-bp gene sequences of the 16S rRNA were determined according to the method of Sawabe *et al.*²⁸⁾ using six sequence primers (24F, 530F, 1100F, 520R, 920R, and 1540R). The 16S rRNA gene sequences of *Vibrio* sp.

Fig. 1. Major symptoms of dead abalone during the mass outbreak in Japanese Ezo abalone *Haliotis discus hannai*. (A) Swelling lesion (edema) in the ventral view; (B) the edema after removing the shells. Gills of the abalone are marked by asterisks. The mouth, foot, and epipodium are marked by a double-layered-circle, closed-star, and open-star, respectively. The heart of the abalone is indicated by an open-heart.

Table 1. Phenotypic traits of the pathogenic *Vibrio harveyi*

Traits	Phenotype		
	<i>V. harveyi</i> S20 and S35	<i>V. harveyi</i> LMG7890	<i>V. harveyi</i> LMG4044 ^T
Pigmentation	—	—	—
Luminescence	—	—	+
Gram staining	—	—	—
Morphology	Rod	Rod	Rod
Motility	+	+	+
Swarming	+	—	—
Flagella	Polar	Polar	Polar
Na ⁺ requirement	H	H	H
OF test	F	F	F
Oxidase	+	+	+
Catalase	+	+	+
Hydrolysis of;			
Agar	—	—	—
DNA	+	+	+
Gelatin	+	+	+
Starch	—	—	—
Alginate	—	—	—
Nitrate reduction	+	+	+
Arginine dehydrolase	—	—	—
Lysine decarboxylase	+	+	+
Ornithine decarboxylase	+	+	+
Indole production	+	+	+
VP reaction	—	—	—
Urease	—	+	—
Growth in;			
0% NaCl	—	—	—
3% NaCl	+	+	+
6% NaCl	+	+	+
8% NaCl	—	—	—
10% NaCl	—	—	—
Growth on TCBS	+(Yellow)	+(Yellow)	+(Yellow)
Utilization of ^a ;			
Citrate	+	NT	—
Glycerol	+	NT	—
α -Ketoglutarate	—	NT	+
Cellobiose	—	NT	+

^a All strains were positive for melibiose, lactose, aconitate, erythritol, γ -aminobutyrate, L-tyrosine, D-sorbitol, DL-malate, xylose, acetate, and putrescine. All strains were negative for D-Mannose, D-galactose, D-fructose, sucrose, maltose, D-gluconate, N-acetylglucosamine, succinate, fumarate, D-mannitol, glycerol, D-glucose, trehalose, glucuronate, D-glucosamine, L-glutamate, and propionate. NT: not tested.

S20 and S35 (*E. coli* NC000913 position 163–1191) were aligned using the ClustalX program³⁵. A neighbor joining (NJ) phylogenetic analysis was done by using MEGA software, version 2.1, with the Kimura-2-parameter calculation

for generating a similarity matrix¹⁴. Figure 3 shows a subset of the final tree obtained using 100 bootstrap replications and the NJ method.

A fragment of the gene encoding glyceraldehyde-3-phosphate dehydrogenase (*gapA*) was also determined according to the methods of Lawrence *et al.*¹⁵ and Sawabe *et al.*²⁹. The sequencing was performed by Shimadzu BioTech service (Kyoto, Japan). The *gapA* gene sequences were also aligned using the ClustalX program³⁵, and the position between 48 and 920 (based on the *E. coli* AE000273 *gapA* gene) was used for a NJ analysis with 100 bootstrap replications using MEGA software, version 2.1¹⁴.

Effects of both immersion and injection of the candidate pathogenic vibrios were examined using 3- to 4-cm animals. The candidate bacterial strains were grown on ZoBell2216E agar plates at 25°C for 2 to 3 days before being suspended in sterilized aged seawater to produce a concentration of 10⁸ CFU ml⁻¹. The immersion experiments were conducted in both closed and open-flow aquaria. The immersion trial using a closed aquarium was designed to mimic the natural environment of the farm; for example, the abalone were in contact with the candidate bacteria in the summer and water quality decreased with prolonged cleaning periods (up to a week between cleanings). The bacterial suspension was added to the closed experimental aquarium (4 L plastic bag, Fujimori Kogyo, Co. Ltd., Yokohama, Japan) to obtain a final concentration of 10⁵ CFU ml⁻¹. A control aquarium without the addition of bacteria was prepared. Five abalone were reared in the experimental aquarium at 15°C and 20°C for 12 days without feeding or seawater changes. A continuous supply of air was maintained during the trials. No fatalities were recorded in the control aquarium during the immersion experiments. In the open-flow aquarium (6 L), abalone were immersed into bacterial suspensions (10⁵ CFU ml⁻¹) for 24 h at 21°C. Then, they were reared in the experimental aquarium with seawater changed 48 times per day with and without feeding at 19.5–20.7°C. An artificial diet (Nihon Nosan Kogyo, Yokohama, Japan) was used for the challenge.

For injection trials, 0.05 ml of the bacterial suspension was injected into hemolymph in the foot muscle so that the final bacterial concentration was 10³ to 10⁶ CFU in the animal body. In each injection trial, 10 animals used for the experimental challenge were reared in 2 L closed aquaria. Fifty percent of the lethal dose (LD₅₀) of the pathogen was determined by probit estimate based on the cumulative mortality results of the injection trial. *Vibrio haloticoli* isolated from the gut of abalone was used as a negative control in the injection trials and no animal deaths were observed in the

trial period.

Susceptibility to antibiotics and disinfectants was determined for the pathogenic *Vibrio*. Phylogenetically related vibrios (*V. harveyi* LMG4044^T and *V. harveyi* LMG7890) were also included in the experiments. LMG7890 was formerly named *V. carchariae*^{9,23)}. Twenty antibiotics with different antibacterial modes were tested. A subsample (0.1 ml) of fresh bacterial suspension with turbidity adjusted to approximately 0.1 at OD_{600nm} was spread onto ZoBell2216E agar. Discs containing antibiotics (Syowa Disc, Nissui Pharmacy Co. Ltd., Tokyo, Japan) were placed on the agar plate and incubated at 25°C for 5 days. The inhibitory zone was measured and the minimum inhibitory concentration (MIC) was estimated using a correlated curve supplied by the manufacturer.

Seven disinfectants were tested for their ability to inhibit growth of the pathogenic *Vibrio*. A fresh bacterial suspension (1 ml) was added to 9 ml of disinfectant solution as the final disinfectant concentration was adjusted to 10-ppm, then incubated for 30 min and 60 min at 20°C without shaking. Viable bacterial counts before and after the disinfectant treatments were measured.

GFP-tagged *V. harveyi* was prepared according to Sawabe *et al.*³⁰⁾ Healthy 1-year-old abalone (average weight 3.7 g) were placed in contact with the GFP-fluorescent cells of *V. harveyi* strain S20 in a closed 4-L aquarium (Fujimori Kogyo, Co. Ltd., Yokohama, Japan) at 20°C. The cell density was adjusted to 10⁵ CFU mL⁻¹ in the aquarium. Animals were taken from the aquarium at 6, 24, and 48 hours after contact with the bacterium, and rinsed once with artificial seawater. The animals were dissected to prepare thin-sliced samples of gill, epipodium, foot, and mouth (Fig. 1). Then, the sliced surfaces were directly observed under a fluorescence microscope equipped with a No. 10 filter set (Zeiss Axioskope, Germany).

In a ventral view of dead Ezo abalone, unique swelling (edema) (Fig. 1A), which could be attributed to the accumulation of hemolymph, was apparent. Edema was observed around the major circulatory system of the abalone examined, once the shell was removed (Fig. 1B). Hemocyte-like cells were observed in the fluid taken from the edema of the dead animals, probably due to inflammation (data not shown). No such lesions were observed in healthy abalone.

The viable bacterial count in the fluid taken from the lesions collected from the dead animals was 1.8×10⁷ CFU mL⁻¹. In the homogenate of the internal organs, including the gut, of healthy animals, the count was 1.1×10⁶ CFU mL⁻¹ (5.4×10⁷ CFU g⁻¹ of internal organ). Bacterial counts were ten times higher in the dead animals than healthy animals.

No viable bacterial counts in the hemolymph of healthy abalone were measured, indicating that the dead animals were in bacterial septicemia. Viable bacterial counts in rearing seawater and bottom sediment were 2.8×10³ CFU mL⁻¹ and 6.7×10⁴ CFU mL⁻¹, respectively. These values are in ranges commonly observed on abalone farms^{27,32,33)}. While a large number of vibrios with swarming ability were observed in the hemolymph of the dead abalone, no such bacteria were observed in either the internal organs, including the gut, of the healthy abalone or in the environmental samples (Fig. 2). The swarming vibrios constituted up to 47% of the bacterial flora isolated from the accumulated fluid from the dead abalone.

Two strains of swarming vibrios (S20 and S35) with a similar colony shape were selected for identification. The 16S rRNA gene sequences of swarming vibrios were identical in the two strains. These strains belonged to a robust clade (88% bootstrap support) with *Vibrio harveyi* strains (AY750577, X74693, and AJ312382) based on the 16S rRNA gene (Fig. 3A). Phylogenetic analysis based on *gapA* gene sequences also supports (87% bootstrap value) the pathogens being in the *V. harveyi* clade (Fig. 3B).

Major phenotypic traits among *V. harveyi* LMG4044^T, LMG7890 (previously known as *V. carchariae*), S20, and S35 were similar except for bioluminescence, urea degradation, and swarming motility (Table 1). The pathogens grew well at a temperature range of 15°C to 37°C, indicating a temperature dependency that was identical to the other strains of *V. harveyi* examined in this study. Test results for arginine dihydrolase, lysine decarboxylase, and ornithine decarboxylase, which is known as the A/L/O key¹⁾, were -/+ for the pathogens. Other *V. harveyi* strains exhibited the same results. Abalone pathogenic *V. harveyi* strains tested positive for the utilization of citrate and glycerol, and negative for the utilization of α -ketoglutarate and cellobiose, which were only minor phenotypic traits differentiating them from the *V. harveyi* type strain among 32 carbon sources tested in this study. Most of the *V. harveyi* strains tested -/+ in the A/L/O key¹⁾. Among *V. harveyi* strains, 23% had swarming motility, 47% had resistance to a 8% sodium chloride solution, 52% were positive for lysine decarboxylase, and 47% were positive for ornithine decarboxylase. In addition, they have been reported to have divergent phenotypic traits²²⁾. The pathogenic vibrios isolated in this study might be classified into Phenon 7 due to the swarming motility defined by Ortigosa *et al.*²²⁾

We tested the pathogenicity of the representative swarming *V. harveyi* strain S20 for Ezo abalone. Figure 4 shows the results of immersion and injection trials of the candidate

Fig. 2. Bacterial flora of the fluid of edema isolated from dead abalone compared with bacterial flora from healthy abalone and environmental samples. ■: *Vibrio* (swarming), □: *Vibrio*, ▨: *Alteromonas*, ▩: *Pseudomonas*, ▤: *Moraxella*, ▥: *Flavobacterium*, ▦: Gram-positive bacteria, ▧: Enterobacteriaceae, ▨: unidentified.

bacterial strain. In a immersion experiment in a closed aquarium in which the animals were inoculated with a candidate pathogen, *V. harveyi* S20, 40% mortality was observed after five days of exposure to the *Vibrio* at 20°C (Fig. 4A). Within eight days, all of the animals in the aquarium inoculated with strain S20 were dead. In contrast, no deaths occurred in a control aquarium not seeded with bacteria. Swarming vibrios were recovered from the internal organs of all of the dead abalone. A partial 16S rRNA gene sequence (500 bp of 5' termini) of the recovered swarming vibrios was identical to that of the strain used for the challenge. Conversely, no deaths occurred from the immersion trial with 10^5 CFU ml⁻¹ of the *V. harveyi* strain S20 infections, either in a closed aquarium at 15°C or in an open-flow aquarium with frequent water exchange (48 times per day) at 20°C, even with sufficient feeding (data not shown).

In the injection trial involving the inoculation of *Vibrio* at concentrations of 10^3 to 10^6 CFU per abalone body, the first incidence of mortality occurred in an aquarium inoculated with 10^6 CFU bacteria, two days after injection (Fig. 4B).

By four days after the injection, all of the animals were dead. In aquaria injected with 10^3 , 10^4 , and 10^5 CFU per abalone body, 0%, 20%, and 40% of the animals had died, respectively, by day five after the injection (Fig. 4B). Conversely, no deaths occurred in either the control uninjected with bacteria or the *V. haliotocoli*-injected control during the injection challenge. The swarming vibrios were recovered from all of the dead abalones. Viable bacterial counts in internal organs of the dead abalone were in the range of 10^6 – 10^8 CFU g⁻¹ per body. The identification of the swarming vibrios was confirmed in partial 16S rRNA gene sequences used for the challenge and in the bacteria recovered from dead abalone. In both immersion and injection trials, the swarming vibrios fulfilled Koch's postulates for being pathogens to Ezo abalone. The LD₅₀ value estimated in the injection trial was 6.4×10^5 CFU g⁻¹ per body.

Numerous *V. harveyi* strains have been reported to exhibit pathogenicity toward a wide range of marine vertebrates and invertebrates such as sharks^{3,9,10}, groupers^{16,38}, flounder³¹, salmon³⁹, sea bass²⁴, rock lobster⁶, penaeid

Fig. 3. Phylogenetic trees of the pathogenic vibrios, strains S20 and S35. (A) Unrooted tree based on 16S rRNA gene; (B) unrooted tree based on *gapA* gene sequences. Scale of bar indicates accumulated nucleotide mutations per site.

Fig. 4. Experimental challenges with swarming *Vibrio harveyi* strain S20 in Ezo abalone. (A) Immersion trial with *Vibrio* sp. S20 (●) and control (○); (B) injection trials with 10^6 CFU (●), 10^5 CFU (■), 10^4 CFU (▲), 10^3 CFU (◆: the mark overlapped with the control result) and control (○).

shrimps^{2,25}), and abalone^{18,20}). In most cases, the vibrio pathogens were reported to be *V. carchariae*. Re-classification by means of genetic characterization clearly indicated that *V. carchariae* is a junior synonym of *V. harveyi*²³). Our molecular phylogenetic data based on the 16S rRNA gene and *gapA* gene sequences also supported these findings (Fig. 3). The LD₅₀ values of these *V. harveyi* strains were reported to range from 10⁴ to 10⁶ CFU per animal body^{2,9,10,24,25,31,38}). *V. harveyi* S20 showed similar LD₅₀ values for abalone as observed in other pathogenic *V. harveyi* strains.

We used a closed aquarium without any water exchange in the experimental challenges in consideration of the worst conditions on the farm. Restrictions of water supply in such a huge abalone farm like the Kumaishi farm are a possible problem; in fact, the water exchange ratio of the farm is 16 times per day maximum. Given that the farm does not disinfect its rearing seawater, possible pathogens may freely invade culture tanks from natural environments. In addition, massive feeding of the animals may also decrease the water quality in the farm. The measured LD₅₀ of *V. harveyi* strain S20 shows that the strain has a modest virulence in abalone; however, *V. harveyi* S20 apparently infected and caused the

increased mortality rate of the abalone (Figs. 1, 2, and 4). The measured LD₅₀ of the bacterium might provide a good borderline value for predictions of an outbreak of mass mortality on the farm. Conducting a real-time monitoring of *V. harveyi* abundance using our colony-polymerase chain reaction enumeration method could be helpful for understanding the mechanism of the outbreak⁸).

The antibiotic spectra of the pathogenic vibrios were similar to those of other *V. harveyi* strains (Table 2). The abalone pathogens were resistant to benzylpenicillin, ampicillin, vancomycin, polymyxin B, colistin, josamycin, spiramycin, doxycycline, and lincomycin, and susceptible to cefotaxime, erythromycin, chloramphenicol, gentamicin, and sulfamethoxazole/trimethoprim. Antibiotic treatment of abalone is prohibited by law in Japan. The occurrence of antibiotic resistant strains of *V. harveyi* was reported³⁴). An environmentally effective strategy for preventing mass mortality of Ezo abalone by *V. harveyi* infection is required. Because no mortality was recorded in an injection trial at 15°C or in a trial with an open-flow aquarium, even with feeding at 20°C, possible prevention strategies include cooling the rearing seawater to 15°C or increasing the seawater exchange rate (Fig. 4).

Table 2. Antibiotic spectra of *Vibrio harveyi* strains

Antibiotics	MIC (μg/ml)			
	<i>V. harveyi</i> S20	<i>V. harveyi</i> S35	<i>V. harveyi</i> LMG7890	<i>V. harveyi</i> LMG4044 ^T
Benzylpenicillin	>25	>25	>25	>25
Ampicillin	>50	>50	31.2	>50
Amoxycillin	33.5	42.3	10.5	>100
Cefotaxime	0.04	0.2	0.04	0.005
Vancomycin	>10	>10	>10	>10
Polymyxin B	>200	>200	>200	>200
Colistin	>800	>800	>800	>800
Nalidixic acid	29.3	23.7	23.7	>200
Erythromycin	2.1	3.8	1.2	0.3
Josamycin	>50	>50	>50	>50
Spiramycin	>50	>50	>50	>50
Chloramphenicol	0.09	0.2	0.02	0.005
Tetracycline	7.9	12.7	3.9	0.5
Oxytetracycline	6.4	3.9	5.0	1.9
Doxycycline	>50	>50	>50	0.5
Streptomycin	8.4	4.8	11.2	26.3
Kanamycin	8.6	31.6	2.3	24.3
Gentamicin	1.9	1.9	1.1	1.1
Lincomycin	>50	>50	>50	6.7
Novobiocin	5.1	1.9	4.0	1.9
Sulfamethoxazole/Trimethoprim	0.8	0.6	0.1	0.09

Table 3. Effects of disinfectants on the pathogenic *Vibrio harveyi* strain S20

Disinfectant	Concentration	Effects (%)	
		30 min	60 min
Sodium hypochlorite	10 ppm	98.5	98.5
Benzalkonium chloride	10 ppm	99.2	99.3
Bio Talk	10 ppm	98.3	98.6
Bio Talk plus activator	10 ppm+100 ppm	>99.99	>99.99
Hydrogen peroxide	10 ppm	99.6	99.4
Povidone iodine	10 ppm	98.8	99.7

To prevent the spread of disease, we tested several bactericidal agents against the pathogenic vibrios. Among these, activated stabilized chlorine dioxide (BioTalk plus activator, Sukegawa Chemical Co. Ltd., Kobe, Japan) was most effective in terms of reducing the viable bacterial counts of the abalone pathogen, followed by hydrogen peroxide and benzalkonium chloride (Table 3). More than 99% of *V. harveyi* S20 was lost after exposure to any of these three bacteriocidal agents for 30 minutes.

Port of entry in the infection by *V. harveyi* of host marine animals has not been resolved in any animal models. We tried to observe the mechanism of initial contact by *V. harveyi* with host abalone using a GFP-tagged strain (Fig. 5). A clump of the GFP-tagged *V. harveyi* was observed on the gills of the animal after 48 hours of contact with the bacterium. No such a clump was observed anywhere else, ie. on the foot or epipodium (Fig. 1). Considering that mortality was observed 5 days after infection in the immersion experiment with *V. harveyi* (Fig. 4), the initial step of *V. harveyi* infection might occur on the surface of the gill. Then, the bacterium might increase its mass on the surface for up to 5 days since the bacterial mass has been beyond the lethal dose against the host animal. However, diseases caused by *V. harveyi* infections were gastroenteritis, vasculitis, and eye disease^{3,16,31,38}). Invasion from the foot muscle of a cultured abalone, *H. diversicolor*, was also reported²⁰). We still need further studies to understand the mode of the outbreak and the mechanism of entry by this pathogen into the host abalone using GFP-tagged strains³⁰).

In conclusion, we have identified *V. harveyi* as the causative agent of mass mortality of Ezo abalone on a Kumaishi abalone farm. In most studies of fish pathogens, the aetiology of bacterial disease in the natural environments is improperly understood³). Future research would involve experiments to understand the ecology of *V. harveyi* both inside and outside this farm in order to prevent another sudden mass mortality. Notably, a combination of *in vivo*

Fig. 5. Epifluorescence microscopic image of GFP-tagged *Vibrio harveyi* strain S20 on the gill of abalone. The image is representative of replicate experiments with GFP-tagged *V. harveyi* S20 and abalone. The artificial infection was conducted at 20°C. Scale of bar: 100 μ m.

experiments to determine the port of entry and chemotaxis to host animal of the pathogen, and *in situ* experiments to monitor the fluctuation of the pathogen in natural environments could be helpful to understand the mechanism of mass mortality caused by *V. harveyi* infection.

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