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ORIGINAL RESEARCH

Abundance of *Vibrio* populations in the gut of Japanese coastal fishes

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Abstract Members of genus *Vibrio*, which are known as opportunistic pathogens of fish, often inhabit the gut of marine fishes. Polymerase chain reaction (PCR) primers specific to *Vibrio* spp. are required for rapid detection and quantification of opportunistic bacteria, which present a risk for infectious diseases. In this study, a primer set (VIB-F and VIB-R) was developed based on known probe sequences. The primer set amplified sequences from all strains of the genus *Vibrio* and related genera of the family Vibrionaceae, but not from strains of other families. In addition, clones in the library generated from marine fish gut using this primer set all corresponded to *Vibrio* spp. and other genera of Vibrionaceae, namely *Aliivibrio, Enterovibrio*, and *Photobacterium*. These results suggested that this primer set has sufficient specificity to permit estimation of the abundance of *Vibrio* spp. in the marine fish gut, facilitating the risk management of vibriosis. The quantitative PCR (qPCR) technique showed that the abundance of *Vibrio* spp. in the gut of coastal fishes was $1.1 \times 10^5 – 9.9 \times 10^{10}$ copies/g. These results revealed that the total count of bacteria in the gut of coastal fishes is relatively constant (ranging from $1.5 \times 10^9 – 2.2 \times 10^{11}$ cells/g), while the abundance of *Vibrio* spp. varies greatly. These results strongly suggest that the combination of *Vibrio*-specific primer set (VIB-F and VIB-R) and qPCR technique is a powerful tool for risk management against outbreaks of vibriosis in marine farms.

Keywords *Vibrio* . *Vibrio*-specific primer . Gut bacteria . Coastal fishes . qPCR . 16S rRNA

Introduction

Aquaculture production is increasing year-to-year on a global scale because fish are an excellent source of protein and at the same time good for people's health (FAO 2020). However, infectious diseases are one of the greatest risk factors in the aquaculture industry, sometimes causing significant economic losses. In particular, members of the family Vibrionaceae are typical pathogenic bacteria that cause considerable damage in marine aquaculture. Bacteria of the genera *Vibrio*, *Listonella*, and *Photobacterium*, which include opportunistic pathogens, often inhabit the gut of marine fishes (Buller 2004; Mohamad et al. 2019). In intensive fish farms, where fish are subjected to stressful conditions, bacterial diseases (vibriosis) caused by members of the family Vibrionaceae frequently are observed. Furthermore, recent increases in seawater temperature have had a variety of impacts on marine organisms; Green et al. (2019) reported that increased seawater temperature not only causes an increase in the growth rate and abundance of *Vibrio* spp. in coastal microbial communities, but also has a direct impact on the expression of virulence factors carried by these bacteria. That report suggested that many aquaculture farms might experience increasingly frequent outbreaks of vibriosis in the near future. Therefore, surveillance of the abundance of *Vibrio* spp.

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in the gut of fishes, in fish diets and feeds, and in environmental waters, is expected to be very important for aquaculture management. However, the conventional method of cultivating samples on solid media and identifying species and genera based on their phenotypic characteristics requires a great deal of time, effort, and skill. To effectively manage the risk of vibriosis in aquaculture, the abundance of *Vibrio* spp. must be determined rapidly and accurately. Therefore, it would be extremely advantageous if vibriosis in aquaculture farms could be managed using quantitative polymerase chain reaction (qPCR) technology employing these *Vibrio*-specific primers.

In previous work, Tanaka et al. (2012) reported that the gut bacteria in coastal fishes comprise Alphaproteobacteria, Actinobacteria, Betaproteobacteria, and Grammaproteobacteria (excluding Vibrionaceae). Analysis of the gut of many marine fishes by next-generation sequencing (NGS) yielded similar results (Yi et al. 2019). These studies demonstrated that it is very difficult to detect bacteria of the genus *Vibrio*, even by clone library analysis using 16S rRNA universal primers, given the low proportion of *Vibrio* spp. in total bacteria. However, to our knowledge, only a limited number of PCR primers specific for *Vibrio* spp. have been described (Green et al. 2019; Mansergh and Zehr 2014). In the present study, we developed *Vibrio*-specific primers based on the sequences of known probes (Giuliano et al. 1999; Kyselková et al. 2009), examined the specificity of these primers, and quantified *Vibrio* spp. in the gut of coastal fishes by qPCR using our *Vibrio*-specific primer pair.

Materials and methods

Fish

The animals analyzed here included grass puffer (*Takifugu alboplumbeus*; body weight 15–30 g per animal) and tiger puffer (*Takifugu rubripes*; 32–61 g). These animals were reared separately in indoor tanks with recirculating water systems (flow rate through the biofilter, 15 L/min) and fed *ad libitum* on a commercial feed, EP-1 (48% protein, 12% fat, 2% fiber, 17% ash; Marubeni Nisshin Feed Co., Tokyo, Japan). In addition, 14 specimens belonging to 13 coastal fish species were collected by fishing in several coastal areas of Sagami Bay (Table 1). These fish specimens were euthanized by ice cooling immediately after collection, and submitted for the following treatment.

Gut contents were obtained aseptically by dissection and squeezing extrusion. Aliquots of each gut sample were stored at -80 °C pending analysis, when these samples were subjected to amplification and clone library analysis. Separately, bacterial cells in aliquots of each gut sample were fixed with a Lugol iodine solution (Pomroy 1984) and stained with 4', 6-diamidino-2-phenylindole (DAPI); stained samples were employed to determine total counts of bacteria, using a BX50 fluorescence microscope (Olympus, Tokyo, Japan), as described by Porter and Feig (1980).

PCR primers

The 16S rDNA of the bacteria was amplified by PCR using the universal primers 20F (5'-AGAGTTTGATCCTGGCTCAG-3') and r2L 5'-CATCGTTTACGGCGTGGAC-3'; Hiraishi 1992). The 16S rDNA of the genus *Vibrio* was amplified using the primers VIB-F (5'-CTACTTGGAGGTTGTGGCCT-3') and VIB-R (5'-GCTGGCAAACAAGGATAAG-3'), which were developed based on the sequences of the GV (Giuliano et al. 1999) and Vibr1R (Kyselková et al. 2009) probes. The specificity and species coverage of the VIB-F and VIB-R primer set were tested *in silico* by conducting searches within the Ribosomal Database Project (RDP) ProbeMatch software (Cole et al. 2014). The coverage of paired primers included 64% (94 out of 147 species) of all Vibrionaceae 16S rRNA gene sequences in the RDP database, including 83% (84 out of 101) of the genus *Vibrio*, along with 100% (5 out of 5) of the genus *Aliivibrio*, 100% of (1 out of 1) of the genus *Catenococcus*, and 14% (4 out of 28) of the genus *Photobacterium*. *Photobacterium damselae* subsp. *damselae* and *P. damselae* subsp. *piscicida*, which are known as typical fish pathogens, were completely matched by the primer sets. The paired primer combinations had 1-3 mismatches with the sequences of the remaining 17 *Vibrio* spp, including 1 mismatch each to *V. cholerae, V. gallicus, V. gazogenes, V. metoecus, V. mimicus, V. quintilis, V. aerogenes, V. crosai*, and *V. pelagia*; 2 mismatches each to *V. ruber, V. cincinnatiensis, V. hippocampi, V. marisflavi, V. olivae, V. rhizosphaerae*, and *V. stylophorae*;

Table 1 Comparison between total counts of bacteria and estimated abundance of *Vibrio* spp. in the gut of wild coastal fishes

Fish (Scientific name; body weight, g)	Total counts of bacteria (cells/g)	*Vibrio* spp. (copies/g)
Bambooleaf wrasse (*Pseudolabrus sieboldi*; 37)	3.3×10^{9}	6.1×10^{6}
Cocktail wrasse (*Pteragogus aurigarius*; 62)	2.9×10^{9}	7.0×10^{7}
Croaker (*Terapon jarbua*; 13)	2.6×10^{9}	4.5×10^{8}
Dragonet No. 1 (*Repomucenus richardsonii*; 28)	4.5×10^{10}	3.7×10^{7}
Dragonet No. 2 (*R. richardsonii*; 37)	3.0×10^{10}	3.7×10^{5}
Filefish (*Thamnaconus modestus*; 240)	2.9×10^{9}	5.7×10^{6}
Japanese flounder (*Paralichthys olivaceus*; 400)	2.7×10^{9}	7.5×10^{6}
Lined silverbiddy (*Gerres equulus*; 35)	4.4×10^{9}	1.1×10^{5}
Multicolorfin rainbowfish (*Parajulis poecilepterus*; 142)	3.0×10^{9}	5.0×10^{6}
Red seabream (*Pagrus major*; 65)	4.4×10^{9}	1.3×10^{5}
Rudder fish (*Girella punctata*; 174)	2.6×10^{9}	2.2×10^{7}
Spotbelly rockfish (*Sebastes pachycephalus* subsp. *pachycephalus*; 62)	1.8×10^{9}	2.7×10^{8}
Spotnape ponyfish (*Nuchequula nuchalis*; 32)	3.5×10^{9}	9.4×10^{5}
Spotted mackerel (*Scomber australasicus*; 600)	1.5×10^{9}	1.5×10^{7}

and 3 mismatches to *V. mangrove*. These observations suggested that these *Vibrio* spp. also may be the target of amplification by PCR with these primers, although the amplification efficiency may be somewhat reduced. In any case, these *in silico* analyses suggested that the VIB-F and VIB-R primer pair should detect potentially pathogenic *Vibrio* spp., including *Photobacterium damselae*.

Construction of 16S rDNA clone libraries

The 16S rDNA clone libraries were constructed according to the method of Sugita et al. (2005b), with some modifications. Briefly, DNA was extracted from microbial cells in individual gut samples using the FastDNA SPIN Kit for Soil (MP Biomedicals, LLC, Solon, OH, USA) according to the manufacturer's instructions.

Each reaction mixture contained 1.0 µL of template DNA, 4.0 µL of 5× reaction buffer, 2 mM $MgCl_2$, 200 µM (each) deoxyribonucleoside triphosphate (dNTP), 0.06 µM (each) of 20F and r2L primers, and 1 U of Go Taq (Promega, Madison, WI, USA) in a final volume of 20 µL. DNA amplification was performed in an *iCycler* thermal cycler (Bio-Rad, Hercules, CA, USA). Cycling conditions were 95 °C for 60 s (denaturation), followed by 30 cycles of 95 °C for 20 s (denaturation), 57 °C for 20 s (annealing), 72 °C for 40 s (extension), and finally 72 °C for 120 s (extension). The amplification of DNA was confirmed by electrophoresis on a 2.0% agarose gel in Tris-acetate-EDTA (TAE) buffer of 4.0 µL of a PCR product. The amplicons then were cloned into the pGEM T-Easy vector system (Promega) and transformed into *Escherichia coli* DH5α according to the manufacturer's instructions. White colonies were screened directly for inserts by performing PCR with M13 forward and reverse primers (Invitrogen). The inserted DNA was sequenced according to the procedure of Hiraishi (1992), using 20F and r2L primers with a BigDye terminator cycle sequencing kit (Applied Biosystems, Foster City, CA, USA); products were resolved on a model 3130*xl* automated DNA sequencer (Applied Biosystems). The final sequence (approximately 690 bp) was determined from overlapping sequence data using the AutoAssembler ver. 2.1 (Applied Biosystems) computer program.

Construction of *Vibrio* clone libraries

Using the above-described method, the *Vibrio*-specific sequences (approximately 310 bp) of microbial cells in fish intestine were amplified by PCR using the VIB-F and VIB-R primer set to construct *Vibrio* clone libraries. The temperature for annealing during PCR amplification was set at 57 °C.

Sequence analysis

Chimeric sequences were identified and removed using Bellerophon (Huber et al. 2004). The clones were identified on the basis of their 16S rDNA sequences using EZBioCloud (Yoon et al. 2017). Sequences with percent similarity higher than 97 were classified to the closest type species, and those with percent similarity higher than 90 were classified into the genus of the closest type species (Chaiyapechara et al. 2012; Yarza et al. 2008). Representative sequences from this study have been deposited into the DDBJ/GenBank/EMBL databases under accession numbers LC649558 – LC649567.

Quantitative PCR (qPCR)

Abundances of *Vibrio* spp. were determined by the SYBR Green qPCR method using the *Vibrio*-specific primers VIB-F and VIB-R. Each reaction mixture contained 1.0 µL of template DNA, 10 µL of 2× QuantiFast SYBR Green PCR kit (Qiagen, Dusseldorf, Germany), and 0.4 µM of each primer in a final volume of 20 µL. Quantitative PCR was performed by three-step cycling using a PikoReal Real-Time PCR System (Thermo Fischer Scientific, Waltham, MA, USA). The cycling conditions were 95 °C for 5 min followed by 45 cycles of 95 °C for 10 s, 57 °C for 20 s, and 72 °C for 40 s. A pGEM plasmid containing a 310-bp length of 16S rDNA from *Vibrio vulnificus* RIMD 2219009 (=ATCC 27562^T) was used as the standard for the calibration curve.

Evaluation of qPCR using *Vibrio*-specific primers

The validity of the real-time PCR quantification using the *Vibrio*-specific primers was evaluated using *Listonella anguillarum* ATCC 19264. This strain was inoculated into 1/10-strength peptone-yeast extract-beef extract-glucose (1/10 PYBG) liquid medium. The 1/10 PYBG medium contains (per 1000 mL of aged seawater): Trypticase peptone (BBL, BD Diagnostics, Sparks, MD, USA), 1 g; Phytone peptone (BBL), 0.5 g; Bacto-yeast extract (BBL), 0.2 g; Lab-lemco powder (Oxoid, Basingstoke, Hamspshire, England), 0.2 g; and glucose, 0.1g; the resulting solution was adjusted to pH 7.5. The inoculated medium was incubated at 25 ºC for 48 hr under aerobic conditions. After incubation, the culture was washed twice with filter-sterilized artificial seawater at 12,000 × g for 20 min (4 ºC), and the resulting cell pellet was resuspended in filter-sterilized seawater. Aliquots of the cell suspension were stained with DAPI to measure the total count of bacteria. The washed cell suspension was subjected to 10-fold serial dilution in filter-sterilized seawater, and the abundance (copies/mL) was measured by real-time PCR using *Vibrio*-specific primers. According to Ribosomal RNA Data Base (rrnDB) version 5.7 (https://rrndb.umms.med.umich.edu/), the mean copy number of 16S rRNA gene in *Listonella anguillarum* is 8.9 (standard deviation, 1.2), ranging from 7 to 10 copies/cell. This number, 8.9 (copies/cell), was used to estimate the cell density (cells/mL) of each diluted cell suspension.

Results and discussion

Specificity of *Vibrio*-specific primers

The specificity of the primer set (VIB-F and VIB-R) was examined using 74 bacterial strains belonging to seven families; these strains are stock cultures in our laboratory. The 16S rDNA universal primers 20F and r2L amplified DNA from all strains, whereas the *Vibrio*-specific primers VIB-F and VIB-R amplified DNA only from Vibrionaceae, which consisted of 12 *Vibrio* species and two *Photobacterium* species (Table 2). These results suggested that this primer set has sufficient specificity to at least estimate the abundance of *Vibrio* spp. in the marine fish gut, facilitating the risk management of vibriosis.

Next, we constructed clone libraries from the gut of two grass puffer (Nos. 1 and 2) specimens using 16S rDNA universal primers. The intent of this experiment was to understand the predominant microbiota in the gut of marine fishes; the species composition of the resulting clones is shown in Table 3. The two libraries contained nine species of bacteria from eight families. Among them, *Aliarcobacter* sp. (FN650333; 91.4–93.1% identity) of the Arcobacteraceae and *Pseudomonas*

Table 2 PCR amplification of 16S rDNA from bacterial strains isolated from the gut of goldfish, grass puffer and tiger pufer using the 16S rDNA-universal and *Vibrio*-specific primers

Family	Closest species (accession no.; identity)	No. of isolates in PCR test using:			
		Universal primers		*Vibrio*-specific primers	
		Positive	Negative	Positive	Negative
Aeromonadaceae	*Aeromonas allosaccharophila* (S39232; 100%)	1	0	0	1
	Aeromonas hydrophila (CP000462; 100%)	3	0	0	3
	Aeromonas ichthiosmia (X71120; 100%)	10	0	0	10
	Aeromonas veronii (X60414; 99.9–100%)	10	0	0	10
Bacillaceae	*Bacillus cereus* (AE016877; 99.0%)	1	0	0	1
	Bacillus fortis (AY443038; 99.2%)	1	0	0	1
Corynebacteriaceae	*Corynebacterium glutamicum* (BA000036; 98.8%)	1	0	0	1
Enterobacteriaceae	*Citrobacter freundii* (AJ233408; 99.6%)	2	0	0	2
	Escherichia coli (EU014689; 100%)	1	0	0	1
	Escherichia vulneris (AF530476; 97.5%)	1	0	0	1
	Yersinia nurmii (FJ717338; 98.4%)	1	0	0	1
Halomonadaceae	*Halomonas variabilis* (AJ306893; 99.7%)	1	0	0	1
Micrococcaceae	*Micrococcus luteus* (CP001628; 99.4%)	1	0	0	1
Vibrionaceae	*Photobacterium damselae* subsp. *piscicida* (X78105; 99.3–99.9%)	5	0	5	0
	Photobacterium swingsii (GQ386822; 99.3–99.7%)	2	0	2	0
	Vibrio alfacsensis (JF316656; 99.6%)	1	0	1	0
	Vibrio atlanticus (EF599163; 99.3–99.5%)	3	0	3	0
	Vibrio atypicus (FJ009624; 98.8%)	2	0	2	0
	Vibrio comminis (GU078672; 98.9%)	1	0	1	0
	Vibrio gigantis (EF094888; 100%)	1	0	1	0
	Vibrio harveyi (X74706; 99.4–99.5%)	2	0	2	0
	Vibrio inhibens (FN687911; 99.3–99.9%)	7	0	7	0
	Vibrio jasicida (AB562589; 99.3–99.7%)	3	0	3	0
	Vibrio sagamiensis (AB428909; 96.9–97.0%)	2	0	2	0
	Vibrio scophthalmi (AFWE01000105; 98.5–100%)	8	0	8	0
	Vibrio variabilis (GU929924; 97.4–97.5%)	2	0	2	0
	Vibrio vulnificus (X76333; 100%)	1	0	1	0
Total		74	0	40	34

Table 3 Distribution of bacterial species in clone libraries constructed from the gut of grass puffer using 16S rDNA universal primers

Family	Closest species (accession no.; identity, %)	No. 1	No. 2	Total
Arcobacteraceae	*Aliarcobacter sp*. (FN650333; 91.4–93.1%)	56	91	147
Brevinemataceae	*Brevinema sp*. (DQ340184; 94.5%)	1	0	1
Desulfovibrionaceae	*Mailhella sp*. (GQ867364; 91.8–92.5%)	4	0	4
Mycoplasmataceae	*Mycoplasma muris* (M23939; 92.2–92.7%)	3	0	3
Peptostreptococcaceae	*Romboutsia timonensis* (LT629862; 98.1%)	0	1	1
Pseudomonadaceae	*Pseudomonas mosselii* (AF072688; 99.6–100%)	20	9	29
Rikenellaceae	*Mucinivorans sp*. (HM630238; 93.4–93.5%)	2	0	2
Vibrionaceae	*Photobacterium damselae* subsp. *piscicida* (X78105; 99.6%)	0	1	1
	Vibrio hyugaensis (LC004912; 100%)	0	1	1

mosselii (AF072688; 99.6–100%) of the Pseudomonadaceae were the most frequently detected bacteria, accounting for 65.1–88.3% and 8.7-23.3%, respectively. In contrast, *Vibrio* spp. included one clone each of *Photobacterium damselae* subsp. *piscicida* (X78105; 99.6%) and *Vibrio hyugaensis* (LC004912; 100%) in the library of animal No. 2. These results indicated that the Vibrionaceae were not the predominant bacteria in the gut of grass puffer, consistent with the data of Tanaka et al.

Table 4 Distribution of bacterial species in clone libraries constructed from the gut of different coastal fishes using the *Vibrio*-specific primers

Closest species (accession no.; identity, %)	Number of clones in libraries of:						
	Grass puffer No. 1	Grass puffer No. 2	Tiger puffer	Japanese flounder	Rudder fish	Spotted mackerel	Total
Aliivibrio finisterrensis (EU541604; 98.4%)	0	0	0	0	0	1	1
Aliivibrio fischeri (BBEE01000115; 100%)	0	0	0	0	0	1	1
Enterovibrio coralii (LNTY01000037; 97.8–100%)	4	12	0	8	0	0	24
Photobacterium damselae subsp. *damselae* (ADBS01000001; 98.2–100%)	0	2	0	1	0	0	3
Photobacterium damselae subsp. *piscicida* (X78105; 99.1–100%)	6	3	0	1	0	0	10
Photobacterium frigidiphilum (AY538749; 98.7–100%)	0	0	0	0	1	9	10
Vibrio aestuarianus (X74689; 100%)	2	0	0	0	0	0	2
Vibrio alfacsensis (JF316656; 99.5–100%)	1	1	0	5	0	0	7
Vibrio aquaticus (MG754467; 99.1–100%)	1	0	0	0	2	0	3
Vibrio cidicii (LOMK01000001; 98.2–100%)	1	0	2	10	0	2	15
Vibrio cincinnatiensis (FUXB01000057; 98.2–99.6%)	1	2	0	0	0	0	3
Vibrio crosai (JQ434120; 99.1%)	1	0	0	0	0	0	1
Vibrio diabolicus (X99762; 99.1%)	2	0	0	0	0	0	2
Vibrio fluvialis (BCZR01000036; 99.5–100%)	0	18	0	0	0	0	18
Vibrio halioticoli (BAUJ01000001; 97.8–100%)	17	0	0	8	0	0	25
Vibrio harveyi (BCUF01000119; 99.1–100%)	10	6	0	4	6	1	27
Vibrio hispanicus (AY254039; 98.1–100%)	1	0	0	8	0	1	10
Vibrio pomeroyi (AJ491290; 99.5–100%)	3	0	0	0	0	0	3
Vibrio rarus (DQ914239; 100%)	0	0	0	0	1	0	1
Vibrio scophthalmi (AFWE01000105; 98.7–100%)	0	2	1	0	20	0	23
Vibrio shilonii (ABCH01000080; 98.6–100%)	0	0	0	0	15	0	15
Vibrio tapetis subsp. *tapetis* (Y08430; 97.8–99.1%)	0	0	6	0	0	0	6
Vibrio sp. (Y08430; 96.7%)	0	0	1	0	0	0	1
Vibrio tasmaniensis (AJ514912; 98.1–100%)	4	5	90	5	1	33	138
Vibrio toranzoniae (EU541606; 98.2–100%)	0	3	0	0	12	0	15
Total	54	54	100	50	58	48	364

(2012).

Next, *Vibrio* libraries were constructed from the gut contents of grass puffers No. 1 and 2, tiger puffer, Japanese flounder, rudder fish, and spotted mackerel using the *Vibrio*-specific primer pair. Table 4 shows the species composition of clones in the six resulting libraries. The data showed that all 364 clones corresponded to bacteria of the family Vibrionaceae, which consists of 25 species within the genera *Vibrio*, *Aliivibrio*, *Enterovibrio*, and *Photobacterium*. *P. damselae* subsp. *piscicida* was detected only in the library constructed from grass puffer No. 2, while *V. hyugaensis* was not detected in any of the six libraries. The absence of *V. hyugaensis* may be due to the fact that the target sequence of the *Vibrio*-specific primers was too short (approximately 270 bp) to be identified accurately. In any case, based on the specificity of the primer set described above, it was determined that the bacteria amplified with this primer set were *Vibrio* spp. and included some species of the genera *Aliivibrio*, *Enterovibrio*, and *Photobacterium*.

Abundance of *Vibrio* in guts of coastal fishes

In this study, the total counts of bacteria and the abundance of *Vibrio* in the guts of 14 specimens of 13 fish species were determined (Table 1). The total counts of bacteria ranged from 1.5×10^9 cells/g (in the spotted mackerel) to 4.5×10^{10} cells/g (in the dragonet No. 1). qPCR analysis, using *V. vulnificus* RIMD 2219009 as the standard DNA, showed that the reaction was highly linear (r^2=0.997; Fig. 1). The detection limit of *Vibrio* spp. was 7.7×10^2 copies/g. *Vibrio*-specific sequences were detected in all 14 fish specimens, with abundances ranging from 1.1×10^5 copies/g (in the lined silverbiddy) to 4.5×10^8 copies/g (in the croaker). Table 5 shows the total count of bacteria and the abundance of *Vibrio* in the guts of grass puffer

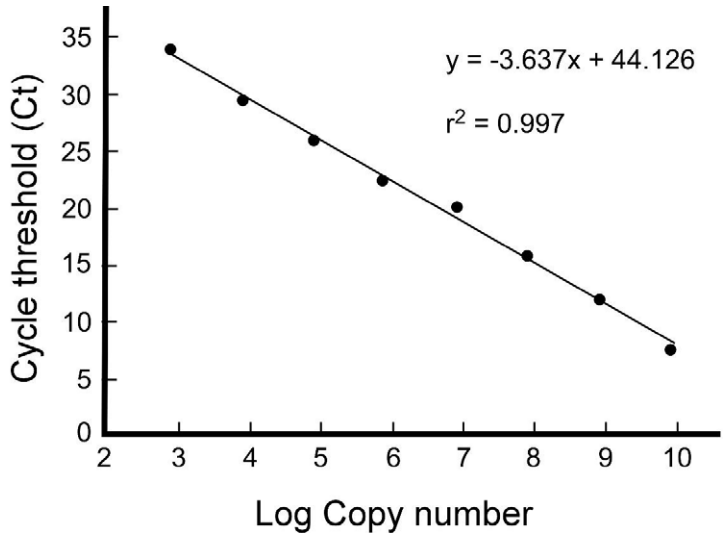

Fig. 1 Quantitative PCR (qPCR) analysis of a 10-fold serial dilution of standard DNA. Cycle threshold values (Ct) are plotted against the calculated copy number. The equation and r^2 values of the regression line are indicated.

Table 5 Comparison between total counts of bacteria and estimated abundance of *Vibrio* spp. in the gut of grass puffer and tiger puffer raised in indoor tanks

Specimen	Total counts of bacteria (cells/g)	*Vibrio* spp. (copies/g)
Grass puffer No. 1	7.8×10^{9}	2.5×10^{7}
Grass puffer No. 2	5.6×10^{9}	1.4×10^{8}
Tiger puffer No. 1	2.1×10^{9}	5.7×10^{8}
Tiger puffer No. 2	1.3×10^{10}	5.2×10^{8}
Tiger puffer No. 3	1.9×10^{10}	4.5×10^{7}
Tiger puffer No. 4	2.5×10^{10}	5.2×10^{6}
Tiger puffer No. 5	2.4×10^{10}	4.7×10^{8}
Tiger puffer No. 6	2.3×10^{10}	4.0×10^{9}
Tiger puffer No. 7	1.6×10^{10}	5.4×10^{9}
Tiger puffer No. 8	1.6×10^{10}	2.8×10^{8}
Tiger puffer No. 9	2.2×10^{11}	9.9×10^{10}
Tiger puffer No. 10	1.9×10^{10}	3.0×10^{9}
Tiger puffer No. 11	2.0×10^{11}	3.0×10^{10}
Tiger puffer No. 12	1.8×10^{10}	3.9×10^{9}
Tiger puffer No. 13	1.9×10^{10}	3.2×10^{9}
Tiger puffer No. 14	2.3×10^{9}	1.3×10^{9}

and tiger puffer raised in indoor tanks. The total count of bacteria ranged from 2.1×10^{9} cells/g (in the tiger puffer No. 1) to 2.2×10^{11} cells/g (in the tiger puffer No. 9), and the abundance of *Vibrio* spp. was 5.2×10^{6} copies/g (in the tiger puffer No. 4) to 9.9×10^{10} copies/g (in the tiger puffer No. 9). These results indicated that the total count of bacteria (10^{9}–10^{11} cells/g) in guts of the coastal fish is relatively constant, as previously reported by Sugita et al. (2005a), while the estimated abundance of *Vibrio* spp. (10^{5}–10^{10} copies/g) varies greatly.

Fig. 2 shows the relationship between the total count and estimated cell density in the diluted culture of *L. anguillarum* ATCC 19264. The qPCR using *Vibrio*-specific primers was highly linear ($r^2 = 0.940$), demonstrating that this quantification system accurately measures the abundance of *Vibrio* spp.

In this study, a PCR primer set, VIB-F and VIB-R, for *Vibrio* spp. was developed based on the sequences of DNA probes reported by Giuliano et al. (1999) and Kyselkova et al. (2009). Examination of the predominant gut microbiota of coastal fishes using this primer set detected bacterial species consisting primarily of *Vibrio* spp., with *Photobacterium, Aliivibrio*, and *Enterovibrio* spp. of the Vibrionaceae serving

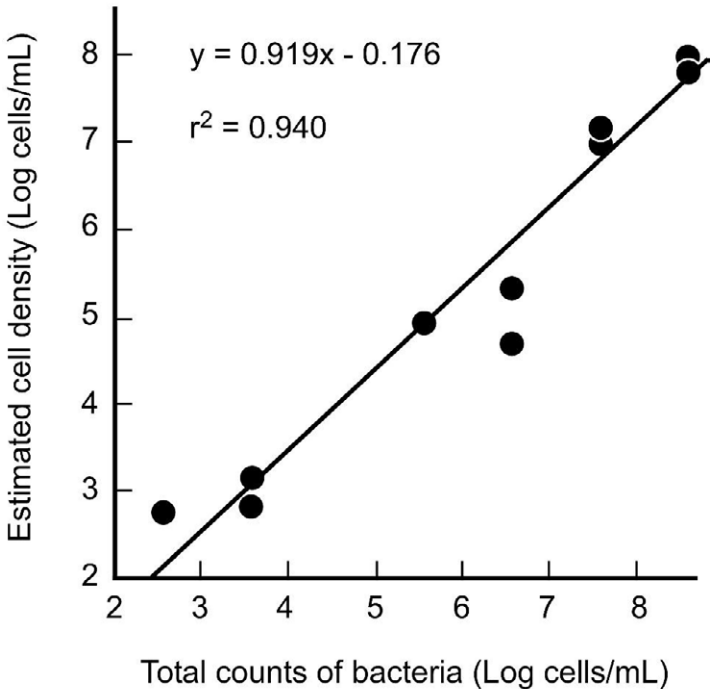

Fig. 2 Estimated cell densities in the diluted culture of *Listonella anguillarum* ATCC 19264 are plotted against the total counts of bacteria. The equation and r^2 values of the regression line are indicated.

as minor components (Table 4). Furthermore, when the reactivity of the primer set was examined using the genomic DNA of *Vibrio* spp. and other strains, it was found that the primer set amplified sequences from strains of *Vibrio* spp., and not from strains of other families (e.g., *Aeromonas* spp.) that dominate in the intestine of freshwater fishes (Cahill 1990; Sugita et al. 1995). These results showed that this primer set is highly specific and suitable for detecting *Vibrio* spp. in fish gut. Furthermore, when the abundance of *Vibrio* spp. in the gut of 15 fish species was examined by qPCR technique using this primer set, the abundance of *Vibrio* spp. was estimated to range between $1.1 \times 10^5 – 9.9 \times 10^{10}$ copies/g (Tables 1 and 5). In any case, it is expected that the qPCR technique using the *Vibrio*-specific primers can be applied not only to the gut of marine fishes but also to risk management of vibriosis in marine fish farms.

Previous work using non-selective agar media showed that *Vibrio* spp. constitute the dominant bacteria in the fish gut (Cahill 1990; Chen et al. 2013; Shiina et al. 2006). However, molecular methods revealed that *Vibrio* spp. are not necessarily dominant in the gut of marine fish (Tanaka et al. 2012), which may reflect the fact that the gut microbiota comprises unknown species that do not grow under general laboratory cultivation conditions (e.g., certain media compositions, levels of salinity, O_2 tension, and temperatures). In addition, marine bacteria can consist of bacterial cells in a viable but non-culturable state (Colwell and Grimes 2000). The results of these previous studies also were confirmed in the present study by qPCR using *Vibrio*-specific primers. NGS is currently being used to analyze gut microbiota, but this technique has the drawback that the needed sequencer and consumables are expensive, and the analysis can require a long time frame. On the other hand, qPCR is relatively inexpensive to perform and analyze, and requires less time than NGS, meaning that qPCR may be suitable for fish farms once primers specific for pathogenic bacteria are defined. The present results strongly suggest that the combination of a *Vibrio*-specific primer pair (VIB-F and VIB-R) and the qPCR technique is a powerful tool for risk management against outbreaks of vibriosis in marine aquaculture.

Conclusion

Vibrio-specific primers based on the sequences of known probes showed high specificity in polymerase chain reaction (PCR) and high linearity against standard DNA in quantitative PCR (qPCR). Therefore, the

application of a *Vibrio*-specific primer set to qPCR is recommended for risk management against outbreaks of vibriosis in aquaculture. These primers then were used with qPCR to quantify *Vibrio* spp. in the gut of coastal fishes. The results showed that the total count of bacteria in the gut is relatively constant (1.5×10^9 – 4.5×10^{10} cells/g), although the abundance of *Vibrio* spp. (1.1×10^5 – 9.9×10^{10} copies/g) varies greatly. In the future, we plan to analyze why the population of *Vibrio* spp. fluctuates greatly and to improve our understanding of vibriosis in marine fish.

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Authors' contributions CHC performed molecular genetic analysis; KS, RM, YY, and DY collected material and identified clonal species. SI discussed the results and commented on the manuscript. HS designed the experiment and wrote the manuscript. All authors reviewed the manuscript.

Compliance with ethical standards All applicable international, national, and/or institutional guidelines for the care and use of animals were followed by the authors.

Conflicts of interest The authors declare that they have no conflict of interest.

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