A new molecular approach to help conclude drowning as a cause of death: Simultaneous detection of eight bacterioplankton species using real-time PCR assays with TaqMan probes

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ABSTRACT

We developed a novel tool for concluding drowning as a cause of death. We designed nine primer pairs to detect representative freshwater or marine bacterioplankton (aquatic bacteria) and then used real-time PCR with TaqMan probes to rapidly and specifically detect them. We previously cultured the genus *Aeromonas*, which is a representative freshwater bacterial species, in blood samples from 94% of victims who drowned in freshwater and the genera *Vibrio* and/or *Photobacterium* that are representative marine bacteria in 88% of victims who drowned in seawater. Based on these results, we simultaneously detected eight species of bacterioplankton (*Aeromonas hydrophila*, *A. salmonicida*; *Vibrio fischeri*, *V. harveyi*, *V. parahaemolyticus*; *Photobacterium damselae*, *P. leiognathi*, *P. phosphoreum*) using three sets of triplex real- time PCR assays and TaqMan probes labelled with fluorophores (FAM, NED, Cy5). We assayed 266 specimens (109 blood, 157 tissues) from 43 victims, including 32 who had drowned in rivers, ditches, wells, sea or around estuaries. All lung samples of these 32 victims were TaqMan PCR-positive including the lung periphery into which water does not readily enter postmortem. On the other hand, findings in blood and/or closed organs (kidney or liver) were PCR-positive in 84% of the drowned victims (except for those who drowned in baths) although the conventional test detected diatoms in closed organs in only 44% of the victims. Thus, the results of the PCR assay reinforced those of diatom tests when only a few diatoms were detectable in organs due to the low density of diatoms in the water where they were found. Multiplex TaqMan PCR assays for bacterioplankton were rapid, less laborious and high-throughput as well as sensitive and specific. Therefore, these assays would be useful for routine forensic screening tests to estimate the amount and type of aspirated water.

1. Introduction

Drowning is the usual cause of death of most victims retrieved from an aqueous environment. However, concluding that death was caused by drowning can be complicated when typical signs of drowning such as overinflated lungs, frothy fluid in airways and pleural effusion are not obvious, or when the body is not fresh [1– 3]. Under such situations, diatom analysis can provide useful information for estimating the type and/or amount of aspirated water [1,3]. However, diatom tests do not always indicate antemortem water aspiration, for example, when the density of diatoms is low in the water. Moreover, diatoms can be found even in closed organs (kidney and liver) as well as in the lungs of non- drowned victims [4,5]. The presence of diatoms in closed organs (or bone marrow) usually suggests that the victim had entered water while still alive. However, many diatoms aspirated into lungs cannot enter the blood circulation because they are larger than the diameter of the alveolar capillaries [6,7]. Therefore, smaller aquatic microbes that can easily enter the blood circulation and which are detectable even in putrefied victims might serve as novel markers of water aspiration.

We previously developed a culture-based method (culture and sequencing) of detecting bacterioplankton (aquatic bacteria) in the blood of drowned victims using selective media (agar plate) containing 2–4% NaCl. We cultured freshwater (*Aeromonas* spp.) and marine (*Vibrio* spp. or *Photobacterium* spp.) bacteria from 94% to 88% of victims drowned in freshwater and seawater, respec- tively using this method [8–11]. Moreover, the aquatic bacteria could not be cultured from all non-drowned victims including two that were discovered in water. Aquatic bacteria are much smaller (0.2–2 mm) than diatoms (2 to >500 mm), and they can be cultured even from the blood of putrefied cadavers even though their numbers decrease in blood as putrefaction advances. Presently, the halophilic nature of bacteria (selective culture and oxidase tests) can be estimated within 24–36 h and estimating bacterial species (16S rRNA sequencing analysis)

requires another two days. Therefore, a rapid and less laborious method of detecting aquatic bacteria in blood or closed organs together with conventional diatom testing would provide a useful tool for routine forensic investigations.

Real-time PCR confirms rapid DNA amplification by continu- ously monitoring fluorescent signals emitted by dsDNA-specific dyes (SYBR Green I) or by using various probes (such as TaqMan, TaqMan MGB or molecular beacons), thus eliminating the need for the electrophoretic confirmation of PCR products. The specificity of real-time PCR using TaqMan probes (5'-Taq nuclease assays) is increased because specific probes and primer pairs are combined. Thus, TaqMan PCR assays are often used for the rapid, sensitive and specific detection of important human pathogens such as *Escherichia coli* O157 [12], *Legionella pneumophila* [13], *Salmonella enterica* spp. *enterica* [14], *Clostridium difficile* [15] and *Vibrio cholerae* [16]. We therefore developed a means to simultaneously detect eight species of bacterioplankton (*Aeromonas hydrophila*, *A. salmonicida*; *Vibrio fischeri*, *V. harveyi*, *V. parahaemolyticus*; *Photobacterium damselae*, *P. leiognathi*, *P. phosphoreum*) that are dominant in blood cultured from drowned bodies [9–11]. We used three sets of triplex TaqMan PCR assays to conclude or rule out drowning as the cause of death in 43 victims, including two for which the cause of death was not drowning although the victims had been discovered in water (total 109 blood and 157 tissues samples).

2. Materials and methods

2.1. Development of multiplex real-time PCR assays of bacterioplankton species

2.1.1. Target and other bacterial species

We purchased the bacterial species, *A. hydrophila* subsp. *hydrophila* (NBRC

12981), *A. salmonicida* subsp. *salmonicida* (NBRC 12659), *V. fischeri* (NBRC 101058), *V. parahaemolyticus* (NBRC 12711T), *V. harveyi* (NBRC 15632), *P. damselae* subsp. *damselae* (NBRC 15633T), *P. leiognathi* (NBRC 14169) and *P. phosphoreum* (NBRC 13896) from the National Institute of Technology and Evaluation (NITE) Biological Resource Center, Kisarazu, Japan. These species have predominantly been cultured from drowned victims [9–11]. The following bacterial species that have been cultured mainly from non-drowned victims [9–11], served as controls to confirm the absence of non-specific amplification: *E. coli* (NBRC 13891), *Citrobacter freundii* (NBRC 12681), *Enterococcus faecalis* (NBRC 12966), *Proteus vulgaris* (NBRC 3988), *Pseudomonas fluorescens* (NBRC 14808) and *Pseudomonas putida* (NBRC 14671), along with the two marine species, *Listonella anguillarum* (NBRC 12710) and *Shewanella algae* (NBRC 101017).

2.1.2. DNA preparation

Genomic DNA was prepared from cultures using a DNA purification kit (ISOPLANT; NIPPON GENE, Toyama, Japan) and then DNA concentrations were determined using NanoVue plus (GE Healthcare, Little Chalfont, Buckinghamshire, UK).

2.1.3. Design of primers and TaqMan probes

We assigned three triplex TaqMan PCR assays to detect the eight freshwater or marine bacterioplankton using triplex set *Aeromonas*, *Vibrio*, *Photobacterium* (Table 1). The *Aeromonas* triplex set was established from the aerA, gyrB and chiA genes of the freshwater bacterioplankton, *A. hydrophila* and *A. salmonicida* [17–19]. The *Vibrio* and *Photobacterium* triplex sets detected marine bacterioplankton of these two genera. These sets were established from the *kat*A, *tox*R and *vhh* genes of *V. fischeri*, *V. parahaemolyticus* and *V. harveyi*, respectively [20–22], and from the *ure*C, *sod*B and *lux*A genes of *P. damselae*, *P. leiognathi* and *P. phosphoreum* [22–24], respectively. The 5'-ends of probes were labelled with the fluorescent dyes, carboxyfluorescein (FAM), NED and cyanine-5 (Cy5) (Table

1) and the 3' ends were attached to a non-fluorescent quencher and minor groove binder (MGB), or only to a quencher (Table 1). Minor groove binder increases the melting temperature (Tm).

Information about the target genes was obtained from the DNA Data Bank of Japan (DDBJ). Primers and probes for each species were designed using the Primer Express software package v.3.0 (Applied Biosystems, Foster City, CA, USA) and GENETYX-MAC software Ver.15.0 (GENETYX, Shibuya, Tokyo, Japan). Moreover, the specificity of the new primers and probes was confirmed using the Basic Local Alignment Search Tool (BLAST, http://blast.ddbj.nig.ac.jp/top-j.html).

2.1.4. PCR amplification

Samples were assayed by TaqMan PCR using three triplex sets (*Aeromonas*, *Vibrio*, *Photobacterium*) and one positive control (TaqMan Exogenous Internal Positive Control reagent, IPC; Applied Biosystems) per sample. The 25 mL PCR mixtures also contained 1□ TaqMan Gene Expression Master Mix (Applied Biosystems), 10 pM primers, 5 pM probes and 1 mL (100 ng) of bacterial genomic DNA template (or IPC). Amplification proceeded using a 7500 Real Time PCR system (Applied Biosystems) at 50 8C for 2 min and 95 ℃ for 10 min followed by 40 cycles of 95 ℃ for 15 s and 58 ℃ for 1 min.

2.1.5. Sensitivity and specificity

The sensitivity and specificity of the PCR assay was evaluated in duplicate using 1mL of 10-fold serial dilutions (100ng/mL to 0.1pg/mL) of purified genomic DNA from each bacterial species (Section 2.1.1). The assay was repeated five times under the same conditions. Each cycle threshold (Ct) value at which threshold fluorescence was reached was determined using the software provided with the Applied Biosystems 7500. Standard curves for each of the target bacteria were generated by plotting the Ct values versus the logarithmic concentration of the dilution series of target DNA (Fig. 1) and each detection limit (DNA concentrations) was determined. The

specificity of the assay for each target bacterium was tested using 15 other bacterial species (Fig. 2 and Supplementary Table S1).

2.2. Application to routine forensic analysis

2.2.1. Differentiation of immersed and non-immersed victims

We tested blood and/or tissue samples from 43 cadavers retrieved from rivers (victims 1–13; fresh water areas), seas (victims 14–24), around estuaries (victims 25–32), bathtubs (victims 33–35) and land near an aquatic environment (victims 36–41) and non-drowned victims who discovered in water (victims 42, 43). Table 2 shows the profiles of all the victims. The causes of death were ultimately determined based on the results of diatom tests, bacterial cultures, toxicological findings, environmental aspects and evidence from police reports in addition to autopsy findings. Diatoms or bacterial cultures from all victims presented herein have already been reported [8–11].

Left cardiac, right cardiac and femoral venous blood samples were collected from each victim under aseptic conditions in situ at autopsy using a sterile needle and syringe as described [11]. Tissue samples were obtained from the inside (centre) of the lower lobe of the right lung, the periphery of the upper lobe of the left lung, the left or right kidney and the right lobe of the liver. Sampling proceeded under sterile conditions as far as possible and samples for PCR assays were stored frozen at □80℃.

2.2.2. Extraction and purification of total genomic DNA

Blood (50 mL) and homogenized tissue (300 mg) samples were digested for 1 (blood) or 2–3 (tissue) h at 70 8C in Buffer G2 containing 20 mL of proteinase K (100 mg/mL) supplied with the kit (500 mL □ n). Total genomic DNA was extracted and purified using the BioRobot EZ1 (Qiagen) and the large-volume protocol of the EZ1 DNA Investigator Kit (Qiagen, Hilden, Germany).

2.2.3. TaqMan PCR assays for forensic casework

Purified total genomic DNA (100 ng/mL) served as templates for PCR assays in duplicate as described in Section 2.1.4.

3. Results and discussion

3.1. Development of multiplex real-time PCR assays of bacterioplankton

3.1.1. Sensitivity (detection limit)

The sensitivity of the TaqMan PCR assay for each target bacterium was □1 pg/mL. The R^2 value of the linear regression analysis was >0.990 for all targets, indicating that the values were linear in the 0.1 pg to 100 ng/mL range (Fig. 1). The slopes also indicated an amplification efficiency of 85.3–94.9% (□3.734 to □3.448). The detection probability of the assay for each target DNA was 100% at □1 pg/mL.

3.1.2. Specificity

Eight target species were specifically amplified with other bacterial species and no cross reactivity was identified (Fig. 2). The amplification of target genes using a specific each primer pair was confirmed by sequencing using BigDye Terminator v1.1 Cycle Sequencing Ready Reaction Kits (Applied Biosystems) (data not shown).

We could not determine any primer pairs or probes that were specific to either *A. hydrophila* or *A. salmonicida*. However, three common primer pairs that did not cross-react with the other 14 bacterial species simultaneously detected *A. hydrophila* and *A. salmonicida* (Supplementary Table S1).

3.2. Routine forensic analysis: differentiation of immersed and non-immersed victims

3.2.1. Victims discovered in freshwater environment (n = 13)

The presence of bacterioplankton in 13 victims (1-13) who were discovered in freshwater environments such as rivers, ditches and wells was assayed

using TaqMan PCR (Tables 2 and 3). The rrepresentative freshwater bacterioplankton genus *Aeromonas* (*A. hydrophila* and/or *A. salmonicida*) was detected in all lung specimens (including periphery of upper lobe of left lung) from all of these victims (100%). However, some water might have entered the lungs after death. *Aeromonas* was detected in blood samples from 10 victims (77%). *Aeromonas* has been cultured only from victims drowned in freshwater and not from those who had drowned in seawater or in baths [8–11]. In addition, *Aeromonas* was not cultured from non-drowned victims discovered on land near water or from two non-drowned victims discovered in water [10,11]. Thus, the presence of freshwater bacterioplankton in blood suggests that a victim had aspirated freshwater. On the other hand, *Aeromonas* was detected in closed organs (kidney or liver) from six (46%) victims. Combining the results of blood and closed organs, specimens other than the lungs of 11 victims were TaqMan PCR-positive (85%) for *Aeromonas*.

Conventional testing identified diatoms in closed organs (20 g) of eight victims (62%). However, only 1–3 diatom valves were found in four of them. The presence of more diatoms in closed organs is particularly important as it raises the possibility that the victim had entered the water while still alive. Moreover, <50 diatom valves were discovered in the lungs (1 g) of victims 2, 3 and 7. Separation values for diatoms in the lung according to Hurlimann et al. range from 200 to 400 diatoms/5 g [7]. Thus, TaqMan PCR-positive results can support findings when only a few diatoms are found in closed organs or when the lungs do not harbour large numbers of diatoms. In addition, TaqMan PCR assay produced reliable results within 4 to 6 h whereas diatom tests require 3 days.

PCR assays could not usually detect bacteria in blood when <360 cfu bacteria/100 mL blood were cultured in TH agar plates containing 2–4% NaCl. Thus, the PCR assay was less sensitive than culture, but allowed more rapid, less laborious, high-throughput processing, since large numbers of agar plates per sample are required for qualitative and quantitative culture analysis.

3.2.2. Victims discovered in a marine environment (n = 11)

3.2.2.1. Victims drowned in seawater or brackish water (n = 10, 14– 23). Ten victims, who were discovered in a marine environment (harbour, beach, rocky coast or offshore) were examined using TaqMan PCR assays (Tables 2 and 4). The representative marine bacterioplankton genera, *Vibrio* (*V. fischeri*, *V. parahaemolyticus* and/or *V. harveyi*) and/or *Photobacterium* (*P. damselae*, *P. leiognathi* and/or *P. phosphoreum*) were detected in all lung samples (including the periphery of the upper lobe of the left lungs) of these victims (100%) although some water might have entered the lungs postmortem. Freshwater *Aeromonas* was also detected in victims 14, 15, 17 and 18. These findings might be attributable to live and dead freshwater microbes and/or their genomic DNA being present in water that had flowed from the river to the sea. Indeed, victims 14, 15 and 18 apparently drowned in a harbour near the mouth of a large river according to environmental aspects and police reports (Table 4).

The genera *Vibrio* and/or *Photobacterium* were detected by PCR in blood samples from 9 (90%) victims and the genus *Aeromonas* was also detected in victim 15. These results suggested that all nine of these victims aspirated seawater or brackish water. On the other hand, the genera *Vibrio* and/or *Photobacterium* were detected in closed organs (kidney or liver) from 5 (50%) victims and *Aeromonas* was undetectable.

Conventional testing detected diatoms in closed organs (20 g) of 3 (30%) victims, although only one valve was found in two of them. The presence of more diatoms in closed organs was particularly important to raise the possibility of antemortem water aspiration by these victims. Moreover, <50 valves were detected in the lungs (1 g) of victims 17, 19, 22 and 23. Thus, PCR- positive findings reinforced the probability of antemortem water aspiration particularly by victims who had drowned in seawater, when the autopsy findings and conventional diatom tests were inconclusive.

3.2.2.2. One victim (24) who might have drowned in freshwater or brackish water with low salinity despite being discovered offshore (salinity 3.08%). The PCR assay detected *Aeromonas* in the blood and closed organs of only one victim (24). The marine bacterioplankton, *Photobacterium*, was also detected in both lungs although the Ct values were very low compared with those of *Aeromonas*. This suggested that less *Photobacterium* (or target genes) than *Aeromonas* had entered the lungs. On the other hand, only *Aeromonas* was cultured in the blood. Marine and freshwater diatoms were visually evident in both lungs and the ratio of marine to freshwater diatoms was very low.

The results of the three methods indicated that this victim had aspirated freshwater or brackish water with low salinity, drowned in a freshwater river or brackish water with low salinity and then floated out to sea.

3.2.3. Victims discovered near estuaries (n = 8)

Samples from eight victims (25-32) who were discovered near the mouths of rivers (except for those who were retrieved from the site at sea), were analyzed using TaqMan PCR (Tables 2 and 5). Both or either marine (*Vibrio* or *Photobacterium*) and freshwater (*Aeromonas*) bacteria were detected in all lung samples (including the periphery of the upper lobe of the left lungs) in all victims (100%). Marine bacteria were undetectable in victim 25, who was discovered in a freshwater area (salinity < 0.05%) of the river.
Marine and/or freshwater bacteria were detected in blood samples from 5 (63%) victims and in the closed organs (kidney or liver) of 6 (75%) using PCR. These results suggest antemortem water aspiration, and the type of aspirated water was similar to that around estuaries.

Conventional diatom testing detected 1–4 diatom valves in the closed organs (20 g) of victims 31 and 32 (25%). On the other hand, <50 valves were detected even in the lungs (1 g) of victim 26. Thus, the PCR assay reinforces the findings of conventional diatom testing.
Notably, PCR detected only freshwater bacteria (*Aeromonas*) in both lungs of victim 28, although this victim was discovered in brackish water (salinity <

1.41%) at an estuary from which only marine bacteria had been cultured. Furthermore, only freshwater diatoms were evident in the lungs and only the freshwater bacterial species *Aeromonas* was cultured from the blood of this victim. Thus, the results of the three tests suggested that the victim had aspirated freshwater (or brackish water with low salinity) and that postmortem bacterial invasion of the blood or closed organs did not readily occur although some water might have entered the lungs postmortem.

3.2.4. Victims discovered in baths (n = 3)

Samples from three victims (33–35) who were discovered in baths were analyzed using PCR assays (Tables 2 and 6). No bacterioplankton were detected in any specimens although bacteria (*Bacillus cereus*, *Enterobacter hormaechei*, *Enterococcus faecalis*, *E. coli*, *Pantoea agglomerans*, *Salmonella* sp.) other than the target organisms were cultured in blood samples from all three victims.

3.2.5. Non-drowned victims discovered on land near an aquatic environment (n = 6)

Samples from six victims (36–41) who were discovered on land near an aquatic environment (sea, river, ditch or bath) were analyzed using PCR (Tables 2 and 6). No bacterioplankton were detected in any specimens from these victims, although some bacteria (*Psychrobacter faecalis*, *P.* sp.) other than the target organisms were cultured. Conventional testing revealed 1 or 2 diatom valves in the lungs (1 g) of victims 37 and 38. Routine diatom analysis sometimes uncovers a few diatoms in closed organs as well as in the lungs of non-drowned victims because diatoms are ubiquitous, being inhabitants of soil, water supplies and air [1,25]. The findings of PCR assays might help to rule out water aspiration under such circumstances.

Victim 36 was discovered wet at the side of an irrigation ditch. Traces in the ditch indicated that the victim had fallen into the ditch before death. Victim 37 was similarly wet when discovered, perhaps as a result of being splashed by waves at the shoreline. Neither PCR nor culture assays

identified bacterioplankton in any samples from these two victims. These results suggested that postmortem invasion or contamination does not readily occur.

3.2.6. Non-drowned victims immersed in water (n = 2)

Samples from victims 42 and 43 retrieved from freshwater ditches (salinity < 0.05%), were analyzed using PCR assays (Tables 2 and 6). No bacterioplankton were detected in blood and/or closed organs of both victims. Although *Aeromonas* was detected only in the right lung of victim 42, this organism was not cultured from blood samples. In addition, only 2–21 diatoms were identified in both lungs (1 g), the kidneys (20 g) and liver (20 g). A large amount of diatoms (1400 valves/mL) was detected in samples from the ditch where the victim was discovered [11], but autopsy findings of the victim did not suggest water aspiration. Thus, the results of three methods together with the autopsy findings, environmental aspects and police reports indicated that victim 42 had aspirated only a little water or none because some water might have entered the lungs postmortem.

On the other hand, *Aeromonas* was detected in both lungs of victim 43, but not cultured from blood samples. In addition, 18 and 2 diatoms were identified in the lower lobe of the right lung (1 g) and liver (20 g), respectively, but not in the periphery of the upper lobe of the left lung (1 g) and the kidney (20 g). A large amount of diatoms (32,000 valves/mL) was detected in samples from the ditch where the victim was discovered [11], but autopsy findings of the victim did not suggest water aspiration. Thus, we concluded that victim 43 had also aspirated only a little water or none.

3.3. Advantages and limitations of TaqMan PCR assay

3.3.1. Advantages

This sensitive and specific PCR assay can detect bacterioplank- ton simply, rapidly and with high throughput within 4 to 6 h. Furthermore, excluding waiting for enzymatic reactions and automated processes, the assay requires only 1 hour of actual labour. The present findings demonstrated that the

PCR assay indicated the probability of water aspiration before conventional diatom analysis generated results that would then confirm whether or not death was caused by drowning. Moreover, the type of aspirated water can be surmised from the species of detected bacterioplankton (freshwater, *Aeromonas*; seawater, *Vibrio* or *Photobacterium*).
High-throughput screening of marine or freshwater bacterioplankton is feasible for analyzing many specimens. We also designed a small (65–142 bp) target region with which to analyze samples from putrefied cadavers with cracked genomic DNA.
The PCR assay would be useful when autopsy findings do not indicate aspirated water (Table 2), or when such findings are combined with inconclusive diatom results (Tables 3–5).

3.3.2. Limitations

The ability of the PCR assay to detect bacterioplankton depends on the amount of target genomic DNA in blood or organ samples. Therefore, when for example the numbers of bacteria in water are low, the postmortem interval is short, and/or the water temperature is very low, the bacterioplankton might be undetectable in blood or organs.
Sensitivity (TaqMan PCR-positive) might be increased by preparing multiplex PCR assays that include primer pairs that are common to each genus or family, or those that target various aquatic microbes such as archaea, cyanobacteria and algae.

4. Conclusion

We designed nine new primer pairs and TaqMan probes and developed a multiplex TaqMan PCR assay to detect eight representative freshwater or marine bacterioplankton. We assayed blood and organ samples from 43 victims, including 32 who had drowned in rivers, ditches, wells, sea, or around estuaries. All lung samples from these 32 victims including the

periphery of the lungs into which water does not readily invade postmortem were PCR-positive. On the other hand, 84% of blood and/or closed organ samples were PCR-positive, whereas conventional diatom tests were positive in only 44% of closed organs. Thus, the results of the TaqMan PCR assay corroborated those of the diatom test when organs contained only a few diatoms, or when the density of diatoms was low in water at discovery sites. On the other hand, the species of bacterioplankton detected by PCR provided important information about the type of aspirated water. Thus, rapid, less laborious and high-throughput PCR screening for estimating water aspiration and type together with conventional diatom testing (morphological search) [1,3,26,27] and/or various other approaches [28–34] would increase the probability that a conclusion of death due to drowning is correct.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.forsciint.2012.04.029.

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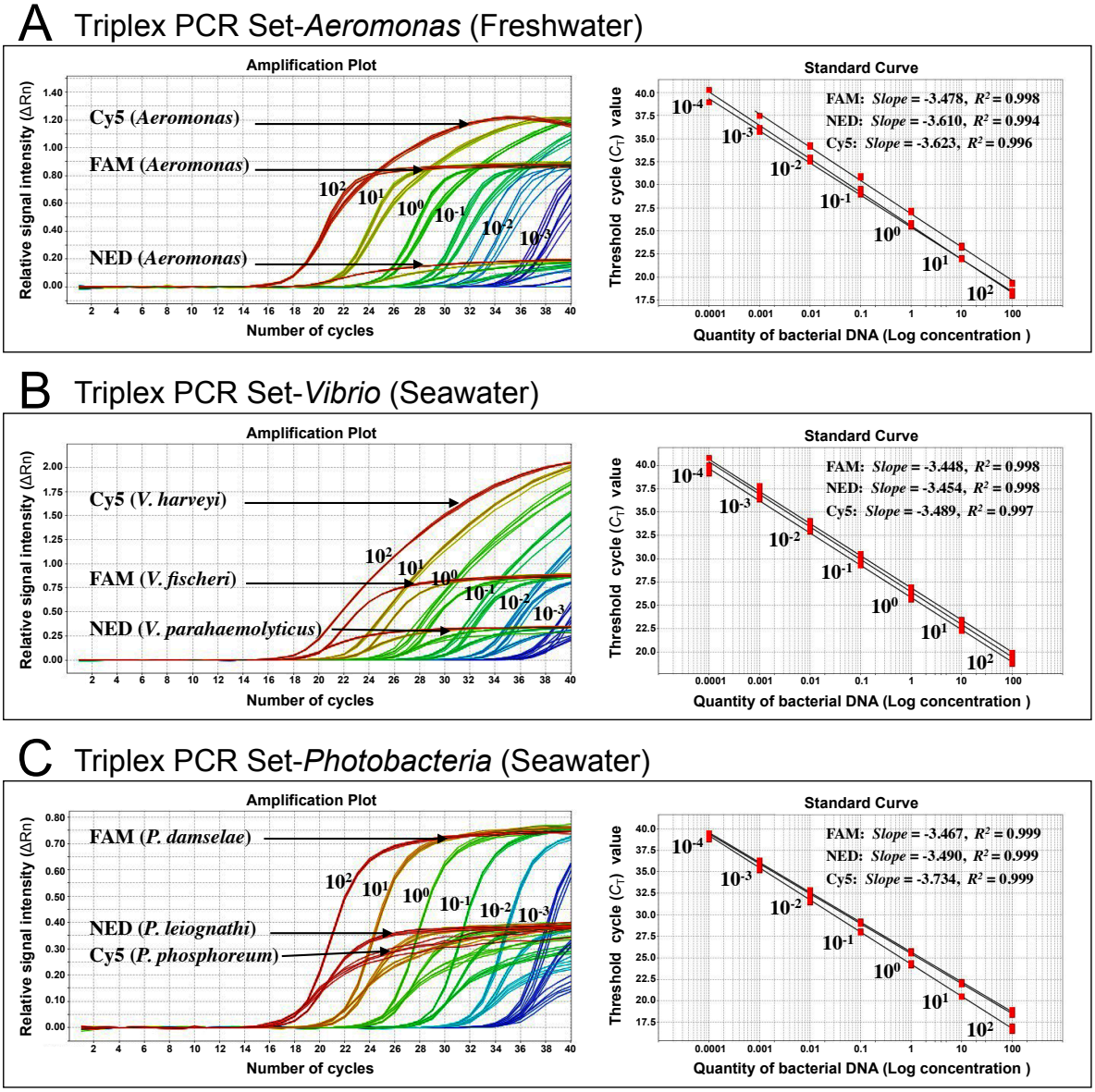

Fig. 1. Sensitivity of three sets of triplex TaqMan PCR assays for detecting eight bacterioplankton species.

(A) Aeromonas hydrophila and *A. salmonicida* (*aer*A, *gyr*B and *chi*A).

(B) Vibrio fischeri (*kat*A), *V. parahaemolyticus* (*tox*R) and *V. harveyi* (*vhh*).

(C) Photobacterium damselae (*ure*C), *P. leiognathi* (*sod*B) and *P. phosphoreum* (*lux*A).

Concentrations of 10-fold serially diluted DNA standards 1 to 7 are 10^{2}, 10, 10^{0}, 10^{-1}, 10^{-2}, $10^{-3}$and 10^{-4} ng/μL, respectively.

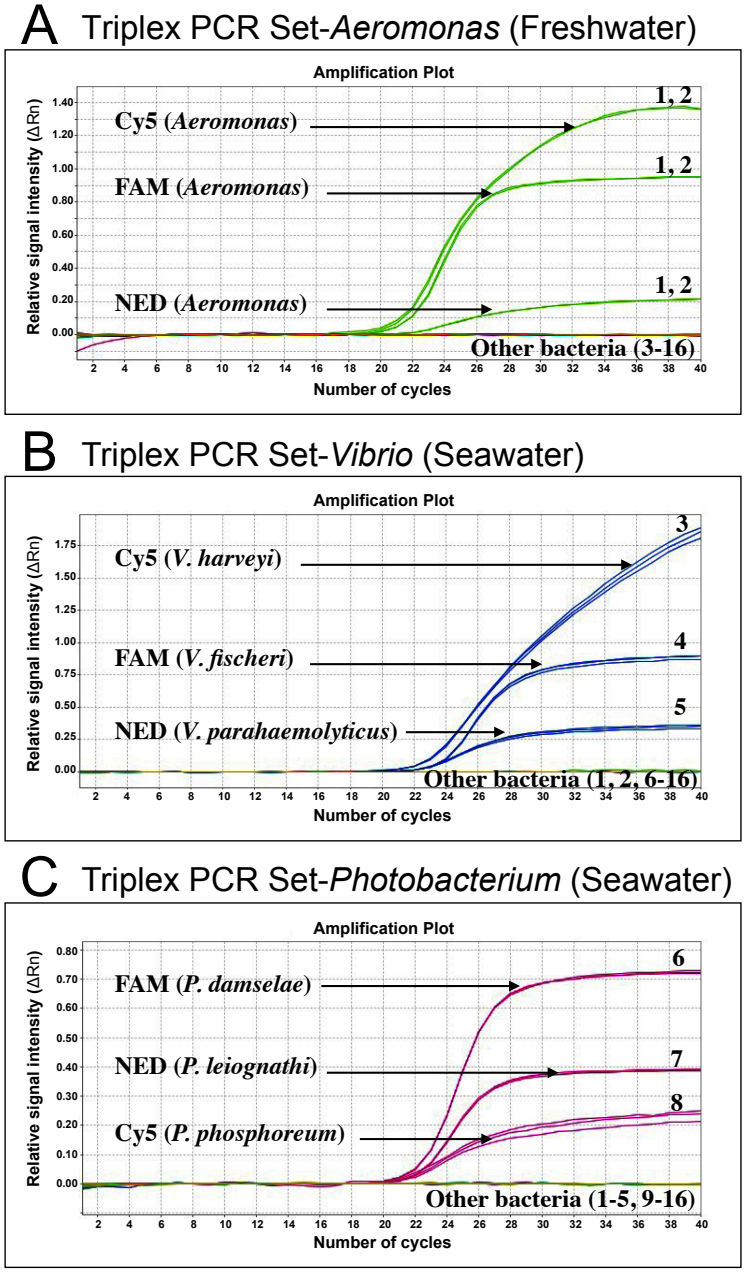

Fig. 2. Specificity of three sets of triplex TaqMan PCR detection.

Aeromonas hydrophila, 1; A. salmonicida, 2; Vibrio fischeri, 3; V. parahaemolyticus, 4; V. harveyi, 5; Photobacterium damselae, 6; P. leiognathi, 7; P. phosphoreum, 8; Listonella anguillarum, 9; Shewanella algae, 10; Escherichia coli, 11; Citrobacter freundii, 12; Enterococcus faecalis, 13; Proteus vulgaris, 14; Pseudomonas fluorescens, 15 and Pseudomonas putida, 16.

Table 1. Amplification primers and TaqMan probes to detect eight types of bacterioplankton

Target bacterial species	Target gene (Locus and accession number)	Target length (bp)	Primer and TaqMan-probe	Sequence(5'-3') and dye[a]	Mer	*T*m[b] (°C)	Reference
Triplex PCR Set-*Aeromonas* (For freshwater)							
A. hydrophila* and *A. salmonicida	***aer* A** (aerolysin: DQ186611)	**65**	Aa-Forward	AATGCCTGGTATACCCATCCG	21	59.0	This study
			Aa-Reverse	TACGGCCCGATGACGAAG	18	59.7	This study
			Aa-Probe	**FAM-** CCGAACTGGAACCACA **-BHQ-MGB**	16	70.0	This study
	***gyr* B** (gyrase subunit B: FJ608552, AY237567)	**96**	Ag-Forward	GTTCCACTTCACCACMGAGCAG	22	58.5	This study
			Ag-Reverse	GTTGTTGGTGAAGCAGTARACCC	23	58.7	This study
			Ag-Probe	**NED-** AACGAYGCCTATCAGGAA **-BHQ-MGB**	18	70.0	This study
	***chi* A** (chitinase: AF059494)	**90**	Ac-Forward	CGCACATACGAGCTCACCTCC	21	61.7	This study
			Ac-Reverse	GTGATTCATGTATTGCTGGGCG	22	61.2	This study
			Ac-Probe	**Cy5-** ACAAGATTGCCAAGGTGGACTATCGCGC -I	28	71.5	This study
Triplex PCR Set-*Vibrio* (For seawater)							
V. fischeri	***kat* A** (catalase: EU908006)	**85**	Vk-Forward	GATGCTGAAGCTGCACAAGTG	21	58.3	This study
			Vk-Reverse	GGAAATCTTGGTTATCAATGCTTTC	25	58.2	This study
			Vk-Probe	**FAM-** ATCGTGAAAGTCATCAAC **-BHQ-MGB**	18	69.0	This study
V. parahaemolyticu	***tox* R** (transmembrane regulatory protein: L11929)	**94**	Vt-Forward	CCTTGGATTCCACGCGTTATT	21	59.8	Modified [21]
			Vt-Reverse	ATCTGACGGAACTGAGATTCCG	22	59.2	This study
			Vt-Probe	**NED-** ATTTGCGTACTGCTGTTTA **-BHQ-MGB**	19	68.0	This study
V. harveyi	***vhh*** (*Vibrio harveyi* hemolysin: IFO15634)	**114**	Vv-Forward	ATCATGAATAAAACTATTACGTTACTTAGTGCA	35	60.3	Modified [22]
			Vv-Reverse	GCTTCTTACTTGAGAGGCACTGACC	25	60.7	This study
			Vv-Probe	**Cy5-** CTCACGCTGCCGAGCCAACATTGT **-BHQ**	24	70.0	This study
Triplex PCR Set-*Photobacterium* (For seawater)							
P. damselae	***ure* C** (urease large subunit: U40071)	**63**	Pu-Forward	CAGACGTCCAGCCTAATGTTGA	22	58.8	This study
			Pu-Reverse	CTTCGCCTGCCACAACTTC	19	58.0	This study
			Pu-Probe	**FAM-** ATTGTTATCGGTCCCGGTAC **-BHQ-MGB**	20	59.0	This study
P. leiognathi	***sod* B** (superoxide dismutase: AB050791)	**98**	Ps-Forward	TGAAGAACGCTGACGGCTCT	20	59.7	This study
			Ps-Reverse	AACAGTTAGTAGCGGAGTTACGCC	24	59.0	This study
			Ps-Probe	**NED-** TTGCCCAATCACAGAAGA **-BHQ-MGB**	18	70.0	This study
P. phosphoreum	***lux* A** (luciferase alpha chain: X55458)	**142**	Pl-Forward	TTAGATCAAATGTCAAAAGGCCG	23	59.1	Modified [24]
			Pl-Reverse	TTGTACCATCCATAATCATGGTGTG	25	59.4	This study
			Pl-Probe	**Cy5-** TTGGTGTTGTGCGTGGCTTGTACCAC **-BHQ**	26	69.3	This study

[a] FAM, NED and Cy5, fluorescent dyes; BHQ, quencher; MGB, minor groove binder.

[b] Melting temperature (*T*m) determined by Primer Express version 3.0 software (Applied Biosystems)

Table 2. Drowned and non-drowned victims investigated in present study (43 cadavers).

Case No.	Cause of death (final decision based on findings of autopsy, diatom test, bacteriological test, etc.)	Discovery site	Salinity of water at discovery site (‰)	In water or land at time of retrieval[a]	Duration in water[b]	Duration from recovery to autopsy [duration refrigerated at 4°C]	Putrefaction[c]	Season	Water temp. (°C)[d]	Ambient temp. (°C)[d]	Probability of drowning based on only autopsy findings	Type of sample used for TaqMan® PCR: Blood[e1]	Type of sample used for TaqMan® PCR: Tissue[f]	Reference - Case no. (Previous studies)
A. Drowned victims														
Victims retrieved from river, ditch, or well														
1	Drowning	River	< 0.05	Immersed	< 2-3 d	24 h [18 h]	±	Winter	9	9	Very high	*L, R, F*	*RL, LL, Kid, Liv*	[10] -20
2	Drowning	River	< 0.05	Immersed	< 12 h	26 h [19 h]	±	Summer	20	29	Very high	*L, R, F*	*RL, LL, Kid, Liv*	[10] -21
3	Drowning	River	< 0.05	Immersed	< 25 h	48 h [42 h]	±	Summer	20	34	Very high	*L, R, F*	*RL, LL, Kid, Liv*	[10] -22
4	Drowning	River	< 0.05	Immersed	< 4 d	24 h [15 h]	++	Spring	21	25	–	*F*[e2]	*RL, LL, Kid, Liv*	[10] -23
5	Drowning	River	< 0.05	Immersed	< 30 h	18 h [13 h]	++	Summer	26	32	–	*R, F*[e2]	*RL, LL, Kid, Liv*	[10] -24
6	Drowning	River	< 0.05	Immersed	< 10 min	45 h [40 h]	±	Autum	10	13	Very high	*L, R, F*	*RL, LL, Kid, Liv*	[11] -6
7	Drowning	River	< 0.05	Immersed	< 28 h	18 h [11 h]	±	Spring	4	14	Very high	*L, R, F*	*RL, LL, Kid, Liv*	[11] -7
8	Drowning	Ditch	< 0.05	Immersed	< 4 d	20 h [13 h]	±	Winter	9	7	–	*L, R, F*	*RL, LL, Kid, Liv*	[11] -8
9	Drowning	Ditch	< 0.05	Immersed	< 6 d	42 h [35 h]	±	Winter	8	6	–	*L, R, F*	*RL, LL, Kid, Liv*	[11] -9
10	Drowning	Ditch	< 0.05	Immersed	< 22 h	41 h [34 h]	±	Spring	13	17	Very high	*L, R, F*	*RL, LL, Kid, Liv*	[11] -10
11	Drowning	Well	< 0.05	Immersed	< 9 h	40 h [32 h]	+	Summer	21	27	Very high	*L, R, F*	*RL, LL, Kid, Liv*	[11] -11
12	Drowning	River	< 0.05	Immersed	< 25 h	70 h [60 h]	±	Summer	26	26	Very high	*L, R, F*	*RL, LL, Kid, Liv*	[11] -12
13	Drowning	River	< 0.05	Immersed	< 13 h	27 h [19 h]	±	Winter	12	16	–	*L, R, F*	*RL, LL, Kid, Liv*	[11] -13
Victims retrieved from sea														
14	Drowning	Sea	2.65	Immersed	< 3 h	11 h [5 h]	±	Spring	18	20	Very high	*L, R, F*	*RL, LL, Kid, Liv*	[10] -12
15	Drowning	Sea	2.78	Immersed	< 19 h	55 h [49 h]	±	Winter	16	17	Very high	*L, R, F*	*RL, LL, Kid, Liv*	[10] -2
16	Drowning	Sea	2.81	Immersed	< 14 h	19 h [15 h]	±	Autumn	26	28	Very high	*L, R, F*	*RL, LL, Kid, Liv*	[10] -10
17	Drowning	Sea	3.00	Immersed	< 10 h	28 h [21 h]	±	Summer	21	21	Very high	*L, R, F*	*RL, LL, Kid, Liv*	[10] -9
18	Drowning	Sea	3.00	Immersed	< 9 h	32 h [27 h]	±	Spring	19	16	Very high	*L, R, F*	*RL, LL, Kid, Liv*	[11] -3
19	Drowning	Sea	3.05	Immersed	< 12 h	59 h [51 h]	±	Spring	13	9	Very high	*L, R, F*	*RL, LL, Kid, Liv*	[11] -2
20	Drowning	Sea	3.17	Immersed	< 6 d	23 h [13 h]	++	Spring	19	24	–	*F*[e2]	*RL, LL, Kid, Liv*	[11] -4
21	Drowning	Sea	3.38	Immersed	6-14 d	101 h [91 h]	++	Winter	15	5	–	*R*[e2]	*RL, LL, Kid, Liv*	[10] -8
22	Drowning	Sea	3.48	Immersed	< 23 h	44 h [35 h]	+	Spring	21	24	Very high	*L, R, F*	*RL, LL, Kid, Liv*	[10] -11
23	Drowning	Sea	3.48	**Not immersed**[a]	< 1-2 d	19 h [11 h]	±	Summer	28	30	–	*L, R, F*	*RL, LL, Kid, Liv*	[11] -1
24	Drowning	Sea	3.08	Immersed	< 2-3 d	88 h [82 h]	++	Summer	24	30	–	*R*[e2]	*RL, LL, Kid, Liv*	[11] -5
Victims retrieved from around estuary														
25	Drowning	Estuary	< 0.05	Immersed	< 12 h	76 h [69 h]	+	Spring	21	22	Very high	*R, F*	*RL, LL, Kid, Liv*	[11] -19
26	Drowning	Estuary	0.27	Immersed	< 2 h	57 h [45 h]	+	Winter	8	2	–	*L, R, F*	*RL, LL, Kid, Liv*	[11] -18
27	Drowning	Estuary	0.80	Immersed	< 2 h	43 h [35 h]	±	Summer	27	32	–	*L, R, F*	*RL, LL, Kid, Liv*	[11] -16
28	Drowning	Estuary	1.41	Immersed	< 10 h	52 h [46 h]	+	Autumn	15	13	Very high	*L, R, F*	*RL, LL, Kid, Liv*	[11] -17
29	Drowning	Estuary	1.50	Immersed	< 3 d	52 h [47 h]	+	Spring	17	14	Very high	*L, R, F*	*RL, LL, Kid, Liv*	[9] -15
30	Drowning	Estuary	1.67	Immersed	< 10 min	42 h [34 h]	±	Winter	15	10	Very high	*L, R*	*RL, LL, Kid, Liv*	[11] -15
31	Drowning	Estuary	3.00	Immersed	< 1 d	71 h [63 h]	+	Spring	20	28	Very high	*L, R, F*	*RL, LL, Kid, Liv*	[9] -16
32	Drowning	Estuary	3.01	Immersed	< 1 h	11 h [5 h]	±	Winter	11	24	Very high	*L, R, F*	*RL, LL, Kid, Liv*	[11] -14
Victims retrieved from bathtub														
33	Suspected drowning	Bath	< 0.05	Immersion	< 2 h	15 h [10 h]	+	Summer	34	29	–	*L, R*	*RL, LL, Kid, Liv*	[10] -26
34	Suspected drowning	Bath	< 0.05	Immersion	< 2 h	44 h [33 h]	+	Winter	33	15	–	*L, R, F*	*RL, LL, Kid, Liv*	[10] -27
35	Suspected drowning	Bath	< 0.05	Immersion	< 10 min	38 h [31 h]	±	Spring	35	24	–	*R, F*	*RL, LL, Kid, Liv*	[10] -28
B. Non-drowned victims- discovered near water environment														
36	Suspected hypothermi	Near ditch	< 0.05	**Not immersed**[a]	< 8 h	27 h [19 h]	±	Winter	13	12	–	*L, R, F*	*RL, LL, Kid, Liv*	[11] -22
37	Hypothermia	Near sea	–	**Not immersed**[a]	< 11 h	29 h [22 h]	±	Winter	NI	6	–	*L, R*	*RL, LL, Kid, Liv*	[10] -31
38	Burns	Near sea	–	No immersion	< 13 h	74 h [69 h]	±	Winter	NI	16	–	*L, R*	*RL*	[10] -37
39	Hemorrhagic shock	Near river	–	No immersion	< 5 d	20 h [11 h]	+	Autumn	NI	12	–	*L, R*	–	[11] -23
40	Suspected overlaying	Near bath	–	No immersion	< 6 h	5 h [0 h]	±	Autumn	NI	21	–	*L, R*	–	[11] -24
41	Cardiac failure	Near bath	–	No immersion	< 3 d	48 h [44 h]	+	Winter	NI	15	–	*L, R*	–	[11] -25
C. Non-drowned victims- submerged in water														
42	Hemorrhagic shock	Ditch	< 0.05	Immersed	< 9 h	50 h [45 h]	±	Spring	11	12	–	*L, R*	–	[11] -20
43	Hypothermia or cardiac failure, suspicion	Ditch	< 0.05	Immersied	< 6 d	66 h [60 h]	++	Winter	9	10	–	*L, R*[e2]	*RL, LL, Kid, Liv*	[11] -21

[a1] Due to low tide. [a2] Clothes were wet (victim was thought to have fallen into ditch before death due to traces of ditch). [a3] Clothes were wet, perhaps due to wave splash.

[b] Time between discovery and reported missing.

[c] Degree of putrefaction is expressed as ± (none), + (slight: discoloration at abdomen), ++ (advanced: marbling, bloating of body, etc).

[d] Temperature at discovery. NI, not investigated.

[e1] *L*, left cardiac blood; *R*, right cardiac blood; *F*, femoral venous blood. –, Not tested. [e2] Cardiac atrium or femoral vein empty of blood due to putrefaction.

[f] *RL*, the center of the lower lobe of the right lung; *LL*, the periphery of the upper lobe of the left lung; *Kid*, kidney; *Liv*, liver. –, Not tested.

Table 3. Detection of bacterioplankton using TaqMan PCR assay in drowned victims retrieved from freshwater sites.

Case no.	Discovery site	Salinity of water at dscovery site (%)	Sample [a]	TaqMan PCR assay: Threshold Cycle (C_T) values of triplex sets: Set–Aeromonas *aer* A	*gyr* B	*chi* A	Set–Vibrio *kat* A	*tox* R	*vhh*	Set–Photobacterium *ure* C	*sod* B	*lux* A	Culture using TH agar: Number of cytochrome oxidase test–positive colonies (cfu/100ml blood) 2% NaCl	4% NaCl	Cultured bacterial species [b1]	Diatom test: Number of diatoms (valves/g *RL* or *LL*, valves/20g *Liv* or *Kid*)
1	River	< 0.05	*L*	28.38	26.87	25.43	–	–	–	–	–	–	55,000	0	*A. sp.* [b2]	NT
			R	25.34	24.83	22.65	–	–	–	–	–	–	150,000	0	*A. sp.* [b2]	NT
			F	28.00	27.79	25.61	–	–	–	–	–	–	40,000	0	*A. hydrophila, A. sp.* [b2]	NT
			RL	22.09	23.61	22.60	–	–	–	–	–	–	–	–	NT	290
			LL	23.19	24.47	24.20	–	–	–	–	–	–	–	–	NT	110
			Kid	34.28	34.93	33.32	–	–	–	–	–	–	–	–	NT	0
			Liv	–	–	–	–	–	–	–	–	–	–	–	NT	0
2	River	< 0.05	*L*	38.65	37.03	37.00	–	–	–	–	–	–	59	0	*A. hydrophila, A. sp.*	NT
			R	–	–	–	–	–	–	–	–	–	0	0	No colony	NT
			F	–	–	–	–	–	–	–	–	–	1	0	*A. hydrophila*	NT
			RL	30.94	31.86	30.50	–	–	–	–	–	–	–	–	NT	115
			LL	28.52	30.63	29.14	–	–	–	–	–	–	–	–	NT	46
			Kid	–	–	–	–	–	–	–	–	–	–	–	NT	3
			Liv	–	–	–	–	–	–	–	–	–	–	–	NT	0
3	River	< 0.05	*L*	25.14	25.65	24.49	–	–	–	–	–	–	42,000	0	*A. hydrophila*	NT
			R	25.63	26.14	25.12	–	–	–	–	–	–	12,000	0	*A. hydrophila*	NT
			F	28.22	28.91	27.96	–	–	–	–	–	–	7,600	0	*A. hydrophila*	NT
			RL	22.00	23.85	23.29	–	–	–	–	–	–	–	–	NT	130
			LL	18.59	20.38	19.48	–	–	–	–	–	–	–	–	NT	50
			Kid	–	–	–	–	–	–	–	–	–	–	–	NT	0
			Liv	36.07	–	–	–	–	–	–	–	–	–	–	NT	1
4	River	< 0.05	*F*	29.58	30.42	30.47	–	–	–	–	–	–	34,000	0	*A. hydrophila, A. veronii*	NT
			RL	23.33	22.95	23.34	–	–	–	–	–	–	–	–	NT	12,600
			LL	24.14	24.51	24.34	–	–	–	–	–	–	–	–	NT	13,000
			Kid	34.25	35.41	34.94	–	–	–	–	–	–	–	–	NT	23
			Liv	34.88	36.55	35.94	–	–	–	–	–	–	–	–	NT	68
5	River	< 0.05	*R*	29.32	30.04	28.74	–	–	–	–	–	–	23,000	0	*A. hydrophila*	NT
			F	24.56	25.17	23.77	–	–	–	–	–	–	440,000	0	*A. hydrophila*	NT
			RL	24.68	23.71	23.87	–	–	–	–	–	–	–	–	NT	9,300
			LL	25.99	24.05	25.12	–	–	–	–	–	–	–	–	NT	2,600
			Kid	33.27	34.94	33.78	–	–	–	–	–	–	–	–	NT	1
			Liv	33.92	35.90	34.86	–	–	–	–	–	–	–	–	NT	1
6	River	< 0.05	*L*	–	–	–	–	–	–	–	–	–	0	0	No colony	NT
			R	35.35	36.47	35.78	–	–	–	–	–	–	43	0	*A. hydrophila, A. veronii*	NT
			F	–	–	–	–	–	–	–	–	–	0	0	No colony	NT
			RL	25.29	25.68	24.52	–	–	–	–	–	–	–	–	NT	2,600
			LL	25.98	26.25	25.63	–	–	–	–	–	–	–	–	NT	370
			Kid	–	–	–	–	–	–	–	–	–	–	–	NT	0
			Liv	–	–	–	–	–	–	–	–	–	–	–	NT	0
7	River	< 0.05	*L*	–	–	–	–	–	–	–	–	–	3	0	*A. salmonicida*	NT
			R	–	–	–	–	–	–	–	–	–	0	0	No colony	NT
			F	–	–	–	–	–	–	–	–	–	0	0	No colony	NT
			RL	24.79	24.36	20.67	–	–	–	–	–	–	–	–	NT	300
			LL	24.28	23.29	20.90	–	–	–	–	–	–	–	–	NT	38
			Kid	34.36	33.93	31.52	–	–	–	–	–	–	–	–	NT	3
			Liv	–	–	–	–	–	–	–	–	–	–	–	NT	5
8	Ditch	< 0.05	*L*	26.39	26.73	24.48	–	–	–	–	–	–	230	0	*A. hydrophila, A. salmonicida*	NT
			R	36.59	35.01	32.76	–	–	–	–	–	–	0	0	No colony	NT
			F	–	–	–	–	–	–	–	–	–	0	0	No colony	NT
			RL	27.85	25.52	24.75	–	–	–	–	–	–	–	–	NT	310
			LL	25.02	24.07	23.20	–	–	–	–	–	–	–	–	NT	1,000
			Kid	–	–	–	–	–	–	–	–	–	–	–	NT	0
			Liv	–	–	–	–	–	–	–	–	–	–	–	NT	0
9	Ditch	< 0.05	*L*	36.93	35.22	33.28	–	–	–	–	–	–	1,400	0	*A. allosaccharophilla, A. salmonicida*	NT
			R	–	–	–	–	–	–	–	–	–	16	0	*A. allosaccharophilla, She.* sp.	NT
			F	–	–	–	–	–	–	–	–	–	0	0	No colony	NT
			RL	20.95	18.31	19.49	–	–	–	–	–	–	–	–	NT	200,000
			LL	19.16	16.77	17.75	–	–	–	–	–	–	–	–	NT	140,000
			Kid	–	–	–	–	–	–	–	–	–	–	–	NT	17
			Liv	–	–	–	–	–	–	–	–	–	–	–	NT	2
10	Ditch	< 0.05	*L*	31.11	30.37	27.29	–	–	–	–	–	–	3,300	0	*A. salmonicida*	NT
			R	–	–	–	–	–	–	–	–	–	2	0	*A. salmonicida*	NT
			F	–	–	–	–	–	–	–	–	–	0	0	No colony	NT
			RL	17.54	16.81	14.13	–	–	–	–	–	–	–	–	NT	4,000
			LL	19.81	18.81	16.44	–	–	–	–	–	–	–	–	NT	1,200
			Kid	–	–	–	–	–	–	–	–	–	–	–	NT	1
			Liv	–	–	–	–	–	–	–	–	–	–	–	NT	0
11	Well	< 0.05	*L*	–	–	–	–	–	–	–	–	–	360	0	*A. hydrophila, A. molluscorum*	NT
			R	–	–	–	–	–	–	–	–	–	0	0	No colony	NT
			F	–	–	–	–	–	–	–	–	–	0	0	No colony	NT
			RL	24.11	25.59	24.29	–	–	–	–	–	–	–	–	NT	4,500
			LL	25.73	27.24	26.18	–	–	–	–	–	–	–	–	NT	2,400
			Kid	–	–	–	–	–	–	–	–	–	–	–	NT	0
			Liv	–	–	–	–	–	–	–	–	–	–	–	NT	0
12	River	< 0.05	*L*	18.37	19.72	18.75	–	–	–	–	–	–	1,900,000	0	*A. hydrophila*	NT
			R	19.66	20.97	19.87	–	–	–	–	–	–	4,400,000	0	*A. hydrophila*	NT
			F	20.39	21.84	20.79	–	–	–	–	–	–	790,000	0	*A. hydrophila*	NT
			RL	23.97	24.35	23.53	–	–	–	–	–	–	–	–	NT	210
			LL	18.59	19.96	18.71	–	–	–	–	–	–	–	–	NT	120
			Kid	21.77	23.20	22.29	–	–	–	–	–	–	–	–	NT	30
			Liv	26.62	28.02	27.45	–	–	–	–	–	–	–	–	NT	5

13	River	**< 0.05**	***L***	–	–	–	–	–	–	–	–	–	0	0	No colony	NT		
			R	–	–	–	–	–	–	–	–	–	0	0	No colony	NT		
			F	–	–	–	–	–	–	–	–	–	0	0	No colony	NT		
			RL	**27.61**	**28.68**	**27.48**	–	–	–	–	–	–	**–**	**–**	NT	**5,400**		
			LL	**25.85**	**26.79**	**26.99**	–	–	–	–	–	–	**–**	**–**	NT	**4,900**		
			Kid	–	–	–	–	–	–	–	–	–	**–**	**–**	NT	**0**		
			Liv	–	–	–	–	–	–	–	–	–	**–**	**–**	NT	**0**		

[a] *L* , left cardiac blood; *R* , right cardiac blood; *F* , femoral venous blood; *RL* , center of lower lobe of right lung; *LL* , periphery of upper lobe of left lung; *Kid* , kidney; *Liv* , liver.

[b1] Generic name is expressed as follows: genus *Aeromonas* , *A; Shewanella, She* .

[b2] *Aeromonas* sp. closely related to *A. salmonicida* (Similarity 99.9%; Accession number, CP000644; Sequence length, 1467 bp) based on previous 16S rRNA gene analysis [10].

–, Not detected; NT, Not tested.

Table 4. Detection of bacterioplankton using TaqMan PCR assays in drowned victims retrieved from the sea.

Case no.	Discovery site	Salinity (%) of water at discovery site and situation of marine environment	Sample[a]	TaqMan PCR assay: Threshold Cycle (C_T) values of triplex sets									Culture using TH agar		Diatom test
				Set-*Aeromonas*			Set-*Vibrio*			Set-*Photobacterium*			Number of cytochrome oxidase test-positive colonies[b1] (cfu/100μl blood)		Cultured bacterial species[C]	Number of diatoms (valves/g *RL* or *LL*, valves/20g *Liv* or *Kid*)
				aer A	*gyr* B	*chi* A	*kat* A	*tox* R	*vhh*	*ure* C	*sod* B	*lux* A	2% NaCl	4% NaCl		
14	Sea	**2.65** (Harbour near river mouth; width of river mouth, 700 M)	***L***	–	–	–	–	–	–	–	–	–	0	0	No colony	NT
			R	–	–	–	–	–	–	–	–	–	0	0	No colony	NT
			F	–	–	–	–	–	–	–	–	–	0	0	No colony	NT
			RL	21.83	23.57	23.86	34.85	35.83	–	25.63	33.96	–	–	–	NT	**920**
			LL	33.08	33.51	34.87	–	–	–	38.55	–	–	–	–	NT	**120**
			Kid	–	–	–	–	–	–	–	–	–	–	–	NT	0
			Liv	–	–	–	–	–	–	–	–	–	–	–	NT	**0**
15	Sea	**2.78** (Harbour same as victim no.14)	***L***	18.25	18.89	15.23	–	–	–	–	–	–	4,100	60	*V. parahaemolyticus*, *P. damselae*	NT
			R	23.52	24.79	20.74	–	–	–	24.49	–	–	5,200	40[b2]	*P. damselae*	NT
			F	–	–	–	–	–	–	–	–	–	0	0	No colony	NT
			RL	24.35	25.94	21.10	–	–	–	2388	–	–	–	–	NT	**460**
			LL	26.47	26.51	21.04	–	–	–	24.35	–	–	–	–	NT	**87**
			Kid	–	–	–	–	–	–	–	–	–	–	–	NT	0
			Liv	–	–	–	–	–	–	–	–	–	–	–	NT	0
16	Sea	**2.81** (Ocean beach)	***L***	–	–	–	–	25.88	25.49	21.85	–	–	1,500,000	3,200,000	*V. parahaemolyticus*, *P. damselae*	NT
			R	–	–	–	–	25.95	24.04	20.96	–	–	1,900,000	350,000	*P. damselae*	NT
			F	–	–	–	–	28.50	25.98	21.38	–	–	2,900,000	28,000	*P. damselae*	NT
			RL	–	–	–	–	27.85	24.50	22.11	–	–	–	–	NT	**570**
			LL	–	–	–	–	25.15	24.89	22.40	–	–	–	–	NT	**240**
			Kid	–	–	–	–	–	34.87	34.72	–	–	–	–	NT	**1**
			Liv	–	–	–	–	–	–	–	–	–	–	–	NT	0
17	Sea	**3.00** (Harbour)	***L***	–	–	–	–	–	–	36.93	–	–	1	4[b2]	*P. damselae*	NT
			R	–	–	–	–	–	35.02	31.73	–	–	180	230	*V. harveyi*, *V.* sp., *P. damselae*	NT
			F	–	–	–	–	–	–	34.94	–	–	28	21	*V. harveyi*, *V.* sp., *P. damselae*	NT
			RL	32.46	36.43	34.92	–	33.94	27.86	24.62	–	–	–	–	–	**24**
			LL	35.10	–	–	36.06	29.97	27.68	25.24	–	–	–	–	–	**33**
			Kid	–	–	–	–	–	–	–	–	–	–	–	–	0
			Liv	–	–	–	–	–	–	–	–	–	–	–	–	**1**
18	Sea	**3.00** (Harbour near river mouth; width of river mouth, 100 M)	***L***	–	–	–	34.45	–	34.50	25.83	–	–	95	4	*V. parahaemolyticus*, *V. harveyi*, *P. damselae* etc.	NT
			R	–	–	–	–	–	–	–	–	–	4	1	*V. parahaemolyticus*, *P. damselae*	NT
			F	–	–	–	–	–	–	–	–	–	0	1	*V. parahaemolyticus*	NT
			RL	31.78	–	29.95	31.78	–	29.95	25.90	–	–	–	–	NT	**82**
			LL	32.99	32.91	30.72	34.85	34.96	32.00	26.85	35.99	–	–	–	NT	**110**
			Kid	–	–	–	–	–	–	–	–	–	–	–	NT	0
			Liv	–	–	–	–	–	–	–	–	–	–	–	NT	0
19	Sea	**3.05** (Harbour)	***L***	–	–	–	36.87	–	–	–	–	–	8,800	1,700	*V. fischeri*, *V.* sp. *Ph*. sp.	NT
			R	–	–	–	–	–	–	–	–	–	0	0	No colony	NT
			F	–	–	–	–	–	–	–	–	–	0	0	No colony	NT
			RL	–	–	–	31.19	–	–	–	–	31.91	–	–	NT	**74**
			LL	–	–	–	31.29	–	–	36.97	–	31.92	–	–	NT	**14**
			Kid	–	–	–	–	–	–	–	–	–	–	–	NT	0
			Liv	–	–	–	–	–	–	–	–	–	–	–	NT	0
20	Sea	**3.17** (Offshore)	***F***	–	–	–	31.61	–	29.48	22.73	–	–	740,000	390,000	*harveyi*, *V. aloginolyticus*, *P. damselae*	NT
			RL	–	–	–	21.80	22.18	20.69	28.84	29.11	–	–	–	NT	**360**
			LL	–	–	–	21.68	22.00	17.96	30.81	31.86	–	–	–	NT	**250**
			Kid	–	–	–	28.81	–	30.13	20.45	–	–	–	–	NT	0
			Liv	–	–	–	34.97	–	33.96	33.98	–	–	–	–	NT	0
21	Sea	**3.38** (Offshore)	***R***	–	–	–	34.56	–	–	36.94	–	–	8,700	60	*P. damselae*, *Sh. algae*	NT
			RL	–	–	–	34.96	–	–	36.12	–	–	–	–	NT	**440**
			LL	–	–	–	35.10	–	–	39.28	–	–	–	–	NT	**180**
			Kid	–	–	–	34.47	–	–	36.83	–	–	–	–	NT	0
			Liv	–	–	–	36.81	–	–	35.64	–	–	–	–	NT	0
22	Sea	**3.48** (Rocky coast)	***L***	–	–	–	26.74	–	–	24.92	30.40	–	170,000	50,000	*P. damselae*, *P. leiognathi*	NT
			R	–	–	–	26.83	–	–	23.58	30.87	–	270,000	22,000	*P. damselae*, *P. leiognathi*	NT
			F	–	–	–	28.43	–	–	21.94	29.17	–	650,000	28,000	*P. damselae*, *P. leiognathi*	NT
			RL	–	–	–	27.77	–	25.89	23.30	26.68	–	–	–	NT	**44**
			LL	–	–	–	29.80	–	27.57	24.77	30.65	–	–	–	NT	**19**
			Kid	–	–	–	30.21	–	–	35.05	–	–	–	–	NT	**3**
			Liv	–	–	–	31.32	–	–	34.43	–	–	–	–	NT	**6**
23	Sea	**3.48** (Rocky coast)	***L***	–	–	–	–	26.97	26.01	22.95	–	–	5,000,000	2,000,000	*molyticus*, *V. harveyi*, *P. damselae* etc.	NT
			R	–	–	–	–	26.74	23.25	20.56	–	–	4,300,000	2,700,000	*V. parahaemolyticus*, *P. damselae*	NT
			F	–	–	–	–	27.55	23.48	20.69	–	–	4,700,000	5,400,000	*molyticus*, *V. harveyi*, *P. damselae* etc.	NT
			RL	–	–	–	–	21.98	21.47	22.94	–	–	–	–	NT	**67**
			LL	–	–	–	–	26.82	22.82	23.70	–	–	–	–	NT	**20**
			Kid	–	–	–	–	–	35.33	33.34	–	–	–	–	NT	0
			Liv	–	–	–	–	–	–	34.10	–	–	–	–	NT	0
24	Sea	**3.08** (Offshore)	***R***	31.88	33.93	32.92	–	–	–	–	–	–	15,000	0	*A. hydrophila*	NT
			RL	19.96	21.57	19.63	–	–	–	34.24	–	–	–	–	NT	**140**
			LL	17.48	19.53	17.89	–	–	–	32.28	–	–	–	–	NT	**67**
			Kid	27.81	29.96	27.75	–	–	–	–	–	–	–	–	NT	**2**
			Liv	32.69	–	33.09	–	–	–	–	–	–	–	–	NT	0

[a] *L*, left cardiac blood; *R*, right cardiac blood; *F*, femoral venous blood; *RL*, center of lower lobe of right lung; *LL*, periphery of upper lobe of left lung; *Kid*, kidney; *Liv*, liver.

[b1] Includes bioluminescent colonies as positive colonies.

[b2] Number of blue colonies at 3% NaCl. No blue colonies at 4% NaCl were cultured, but sizes of blue colonies at 3% and 2% NaCl were similar suggesting marine bacteria according to previous findings [9-11]).

[c] Generic name is expressed as follows: genus *Aeromonas*, *A; Vibrio, V; Photobacterium, P; Shewanella, She*.

–, Not detected; NT, Not tested.

Table 5. Detection of bacterioplankton using TaqMan PCR assay in drowned victims retrieved from around estuaries.

Case no.	Discovery site	Salinity of water at dscovery site (%)	Sample[a]	TaqMan PCR assay: Threshold Cycle (C_T) values of triplex sets: Set-*Aeromonas* *aer* A	*gyr* B	*chi* A	Set-*Vibrio* *kat* A	*tox* R	*vhh*	Set-*Photobacterium* *ure* C	*sod* B	*lux* A	Culture using TH agar: Number of cytochrome oxidase test-positive colonies[b] (cfu/100µl blood) 2% NaCl	4% NaCl	Cultured bacterial species[c]	Diatom test: Number of diatoms (valves/g *RL* or *LL*, valves/20g *Liv* or *Kid*)
25	Estuary	< 0.05	***R***	23.49	24.68	22.80	–	–	–	–	–	–	**440,000**	0	*A. hydrophila*	NT
			F	23.89	25.89	23.70	–	–	–	–	–	–	**170,000**	0	*A. hydrophila*	NT
			RL	18.79	19.61	18.50	–	–	–	–	–	–	–	–	NT	**5,200**
			LL	19.91	20.32	19.47	–	–	–	–	–	–	–	–	NT	**3,100**
			Kid	23.61	25.19	23.82	–	–	–	–	–	–	–	–	NT	0
			Liv	29.99	31.40	29.43	–	–	–	–	–	–	–	–	NT	0
26	Estuary	0.27	***L***	–	–	–	–	–	–	–	–	–	0	0	No colony	NT
			R	–	–	–	–	–	–	–	–	–	0	0	No colony	NT
			F	–	–	–	–	–	–	–	–	–	0	0	No colony	NT
			RL	28.74	29.82	30.42	–	–	–	34.97	–	–	–	–	NT	**140**
			LL	29.51	29.54	31.00	–	–	–	–	–	–	–	–	NT	**25**
			Kid	–	–	–	–	–	–	–	–	–	–	–	NT	0
			Liv	–	–	–	–	–	–	–	–	–	–	–	NT	0
27	Estuary	0.80	***L***	–	–	–	–	–	–	–	–	–	0	0	No colony	NT
			R	–	–	–	–	–	–	32.76	–	–	**500**	**320**	*P. damselae*	NT
			F	–	–	–	–	–	–	–	–	–	0	0	No colony	NT
			RL	26.82	27.93	26.61	–	30.16	32.18	27.71	–	–	–	–	NT	**960**
			LL	25.70	26.81	25.64	–	30.42	–	28.72	–	–	–	–	NT	**190**
			Kid	–	–	–	–	–	–	36.24	–	–	–	–	NT	0
			Liv	–	–	–	–	–	–	–	–	–	–	–	NT	0
28	Estuary	1.41	***L***	–	–	–	–	–	–	–	–	–	14	0	*A. hydrophila*, *A. veronii*	NT
			R	–	–	–	–	–	–	–	–	–	0	0	No colony	NT
			F	–	–	–	–	–	–	–	–	–	0	0	No colony	NT
			RL	22.87	23.80	22.67	–	–	–	–	–	–	–	–	NT	**600**
			LL	24.30	25.87	24.29	–	–	–	–	–	–	–	–	NT	**140**
			Kid	–	–	–	–	–	–	–	–	–	–	–	NT	0
			Liv	–	–	–	–	–	–	–	–	–	–	–	NT	0
29	Estuary	1.50	***L***	34.33	35.12	34.61	36.74	34.90	–	–	–	–	**19,000**	**8,300**	*V. fischeri*, *V. parahaemolyticus*, *P. damselae*	NT
			R	36.28	36.86	36.90	31.73	31.24	32.82	27.27	–	–	**200,000**	**200,000**	*V. fischeri*, *V. parahaemolyticus*, *P. damselae*	NT
			F	35.40	36.81	36.26	34.82	33.64	35.27	28.75	–	–	**140,000**	**230,000**	*V. fischeri*, *V. parahaemolyticus*, *P. damselae* etc.	NT
			RL	24.35	25.22	23.89	28.94	30.94	–	25.94	–	–	–	–	NT	**550**
			LL	28.87	29.67	29.52	31.62	30.88	28.48	23.63	–	–	–	–	NT	**320**
			Kid	–	–	–	–	–	–	36.91	–	–	–	–	NT	0
			Liv	–	–	–	–	–	–	–	–	–	–	–	NT	0
30	Estuary	1.67	***L***	–	–	–	–	–	–	–	–	–	120	9	*A. hydrophila*, *V. parahaemolyticus*	NT
			R	17.76	20.26	18.43	–	–	–	–	–	–	**120**	0	*A. hydrophila*	NT
			RL	24.00	26.93	25.97	–	38.38	–	38.00	–	–	–	–	NT	**240**
			LL	25.82	27.69	27.74	–	–	–	37.26	–	–	–	–	NT	**160**
			Kid	30.58	26.72	26.56	–	–	–	–	–	–	–	–	NT	0
			Liv	35.92	32.97	32.99	–	–	–	–	–	–	–	–	NT	0
31	Estuary	3.00	***L***	–	–	–	24.11	–	–	32.35	–	–	**3,400**	**7,000**	*V. fischeri*, *V. parahaemolyticus*	NT
			R	–	–	–	32.92	–	–	–	–	–	**700**	**260**	*V. fischeri*, *V. parahaemolyticus*	NT
			F	–	–	–	–	–	–	–	–	–	16	17	*V. fischeri*, *V. parahaemolyticus*, *V. harveyi* etc.	NT
			RL	28.93	30.80	30.57	23.89	26.26	26.94	25.94	29.94	–	–	–	NT	**1,300**
			LL	30.87	31.50	31.55	27.4	28.12	29.64	27.98	35.85	28.86	–	–	NT	**550**
			Kid	–	–	–	–	–	–	36.83	–	–	–	–	NT	**1**
			Liv	–	–	–	–	–	–	–	–	–	–	–	NT	**3**
32	Estuary	3.01	***L***	–	–	–	–	–	–	–	–	–	0	0	No colony	NT
			R	–	–	–	–	–	–	–	–	–	0	0	No colony	NT
			F	–	–	–	–	–	–	–	–	–	0	0	No colony	NT
			RL	26.36	25.19	21.75	–	–	–	–	–	36.68	–	–	NT	**390**
			LL	26.71	24.88	21.31	–	–	–	–	–	–	–	–	NT	**84**
			Kid	–	31.86	–	–	–	–	–	–	–	–	–	NT	**4**
			Liv	–	–	–	–	–	–	–	–	–	–	–	NT	**1**

[a] *L*, left cardiac blood; *R*, right cardiac blood; *F*, femoral venous blood; *RL*, center of lower lobe of right lung; *LL*, periphery of upper lobe of left lung; *Kid*, kidney; *Liv*, liver.

[b] Includes bioluminescent colonies as positive colonies.

[c] Generic name is expressed as follows: genus *Aeromonas*, *A; Vibrio*, *V; Photobacterium*, *P; Shewanella*, *She*.

–, Not detected; NT, Not tested.

Table 6. Detection of bacterioplankton using TaqMan PCR assay in drowned victims recovered from bathtub or in non-drowned victims recovered near aquatic environments.

Case no.	Discovery site	Salinity of water at dscovery site (%)	Sample[a]	TaqMan PCR assay: Threshold Cycle (C_T) values of triplex sets									Culture using TH agar: Number of cytochrome oxidase test-positive colonies (cfu/100µl blood)		Culture using TH agar: Cultured bacterial species[c1]	Diatom test: Number of diatoms (valves/g *RL* or *LL*, valves/20g *Liv* or *Kid*)
				Set-*Aeromonas*			Set-*Vibrio*			Set-*Photobacterium*						
				aer A	*gyr* B	*chi* A	*kat* A	*tox* R	*vhh*	*ure* C	*sod* B	*lux* A	2% NaCl	4% NaCl		
33	Bath	< 0.05	***L***	–	–	–	–	–	–	–	–	–	**(4)**[b1]	**(1)**[b1]	(*Entb. hormaechei*, *Pan. agglomerans*, *B. cereus*)[c2]	NT
			R	–	–	–	–	–	–	–	–	–	**(13)**[b1]	**(15)**[b1]	(*Entb. hormaechei*, *Pan. agglomerans*, *E. coli* etc.)[c2]	NT
			RL	–	–	–	–	–	–	–	–	–	–	–	NT	0
			LL	–	–	–	–	–	–	–	–	–	–	–	NT	0
			Kid	–	–	–	–	–	–	–	–	–	–	–	NT	0
			Liv	–	–	–	–	–	–	–	–	–	–	–	NT	0
34	Bath	< 0.05	***L***	–	–	–	–	–	–	–	–	–	**(60)**[b1]	0	(*Sal*. sp.)[c2]	NT
			R	–	–	–	–	–	–	–	–	–	**(150)**[b1]	0	(*Sal*. sp.)[c2]	NT
			F	–	–	–	–	–	–	–	–	–	**(17)**[b1]	0	(*Sal*. sp.)[c2]	NT
			RL	–	–	–	–	–	–	–	–	–	–	–	NT	0
			LL	–	–	–	–	–	–	–	–	–	–	–	NT	0
			Kid	–	–	–	–	–	–	–	–	–	–	–	NT	0
			Liv	–	–	–	–	–	–	–	–	–	–	–	NT	**1**
35	Bath	< 0.05	***R***	–	–	–	–	–	–	–	–	–	0	0	No colony	NT
			F	–	–	–	–	–	–	–	–	–	**(26)**[b1]	**(40)**[b1]	(*Entc. faecalis*, *E. coli.*)[c2]	NT
			RL	–	–	–	–	–	–	–	–	–	–	–	NT	**1**
			LL	–	–	–	–	–	–	–	–	–	–	–	NT	**2**
			Kid	–	–	–	–	–	–	–	–	–	–	–	NT	**2**
			Liv	–	–	–	–	–	–	–	–	–	–	–	NT	**1**
36	Near ditch	< 0.05	***L***	–	–	–	–	–	–	–	–	–	0	0	No colony	NT
			R	–	–	–	–	–	–	–	–	–	0	0	No colony	NT
			F	–	–	–	–	–	–	–	–	–	0	0	No colony	NT
			RL	–	–	–	–	–	–	–	–	–	–	–	NT	0
			LL	–	–	–	–	–	–	–	–	–	–	–	NT	0
			Kid	–	–	–	–	–	–	–	–	–	–	–	NT	0
			Liv	–	–	–	–	–	–	–	–	–	–	–	NT	0
37	Near sea	–	***L***	–	–	–	–	–	–	–	–	–	0	0	No colony	NT
			R	–	–	–	–	–	–	–	–	–	–	–	No colony	NT
			RL	–	–	–	–	–	–	–	–	–	–	–	NT	**2**
			LL	–	–	–	–	–	–	–	–	–	–	–	NT	0
			Kid	–	–	–	–	–	–	–	–	–	–	–	NT	0
			Liv	–	–	–	–	–	–	–	–	–	–	–	NT	0
38	Near sea	–	***L***	–	–	–	–	–	–	–	–	–	0	0	No colony	NT
			R	–	–	–	–	–	–	–	–	–	0	0	No colony	NT
			RL	–	–	–	–	–	–	–	–	–	–	–	NT	**1**
39	Near bath	–	***L***	–	–	–	–	–	–	–	–	–	0	0	No colony	NT
			R	–	–	–	–	–	–	–	–	–	**10**	**2**	***faecalis*, *Psy*. sp.** (*Pan* spp., *Sta*. spp.)[c2]	NT
40	Near bath	–	***L***	–	–	–	–	–	–	–	–	–	0	0	No colony	NT
			R	–	–	–	–	–	–	–	–	–	0	0	No colony	NT
41	Near bath	–	***L***	–	–	–	–	–	–	–	–	–	0	0	No colony	NT
			R	–	–	–	–	–	–	–	–	–	0	0	No colony	NT
42	Ditch	< 0.05	***L***	–	–	–	–	–	–	–	–	–	0	0	No colony	NT
			R	–	–	–	–	–	–	–	–	–	0	0	No colony	NT
			RL	–	–	–	–	–	–	–	–	–	–	–	NT	**21**
			LL	–	–	–	–	–	–	–	–	–	–	–	NT	**3**
			Kid	–	–	–	–	–	–	–	–	–	–	–	NT	**3**
			Liv	–	–	–	–	–	–	–	–	–	–	–	NT	**2**
43	Ditch	< 0.05	***L***	–	–	–	–	–	–	–	–	–	0	0	No colony	NT
			R	–	–	–	–	–	–	–	–	–	**1,000**[b2]	0	***She.* sp.** (*Pro. vulgaris*, *Mor. morganii*)[c2]	NT
			RL	**35.07**	**31.35**	**33.94**	–	–	–	–	–	–	–	–	NT	**18**
			LL	–	**35.92**	**37.12**	–	–	–	–	–	–	–	–	NT	0
			Kid	–	–	–	–	–	–	–	–	–	–	–	NT	0
			Liv	–	–	–	–	–	–	–	–	–	–	–	NT	**2**

[a] *L*, left cardiac blood; *R*, right cardiac blood; *F*, femoral venous blood; *RL*, center of lower lobe of right lung; *LL*, periphery of upper lobe of left lung; *Kid*, kidney; *Liv*, liver.

[b1] Values in parentheses are show number of unstained colonies (cytochrome oxidase test-negative). Blue conlines were not cultured.

[b2] Blue colonies were not predominant and comprised <1% of total number of colnies [11].

[c1] Generic names are expressed as follows: genus *Bacillus*, B; *Enterobacter*, *Entb*; *Enterococcus*, *Entc; Morganella, Mor*; *Pantoea*, *Pan; Proteus, Pro*; *Psychrobacter*, *Psy; Salmonella, Sal*; *Shwanella*, *She; Staphylococcus, Sta*.

[c2] Parentheses indicate bacterial species identified from unstained colonies (cytochrome oxidase test-negative).

–, Not detected; NT, Not tested.

Supplementary data.

Table S1 Specificity of multiplex TaqMan PCR assay.

No.	Standard bacteria	NBRC No.	DNA concentration (ng/μl)	Threshold Cycle (C_T) values of triplex sets								
				Triplex Set-*Aeromonas*			Triplex Set-*Vibrio*			Triplex Set-*Photobacterium*		
				***aer* A**	***gyr* B**	***chi* A**	***kat* A**	***tox* R**	***vhh***	***ure* C**	***sod* B**	***lux* A**
1	*Aeromonas hydrophila*	12981	50.000	**16.69**	**19.47**	**19.62**	–	–	–	–	–	–
			12.500	**21.70**	**21.58**	**21.76**	–	–	–	–	–	–
			3.125	**23.80**	**23.61**	**23.93**	–	–	–	–	–	–
			0.780	**25.74**	**25.64**	**25.89**	–	–	–	–	–	–
			0.195	**28.17**	**28.15**	**28.32**	–	–	–	–	–	–
			0.048	**30.49**	**30.38**	**30.67**	–	–	–	–	–	–
			0.012	**32.95**	**33.03**	**33.21**	–	–	–	–	–	–
			0.003	**34.87**	**34.70**	**34.96**	–	–	–	–	–	–
2	*Aeromonas salmonicida*	12659	50.000	**18.60**	**19.36**	**19.43**	–	–	–	–	–	–
			12.500	**20.86**	**21.60**	**21.77**	–	–	–	–	–	–
			3.125	**22.45**	**23.26**	**23.37**	–	–	–	–	–	–
			0.780	**24.35**	**25.15**	**25.31**	–	–	–	–	–	–
			0.195	**26.91**	**27.82**	**27.98**	–	–	–	–	–	–
			0.048	**28.70**	**29.60**	**29.61**	–	–	–	–	–	–
			0.012	**31.05**	**32.03**	**32.13**	–	–	–	–	–	–
			0.003	**33.41**	**34.49**	**34.39**	–	–	–	–	–	–
3	*Vibrio fischeri*	101058	50.000	–	–	–	**20.38**	–	–	–	–	–
			12.500	–	–	–	**22.39**	–	–	–	–	–
			3.125	–	–	–	**24.53**	–	–	–	–	–
			0.780	–	–	–	**26.65**	–	–	–	–	–
			0.195	–	–	–	**28.72**	–	–	–	–	–
			0.048	–	–	–	**30.91**	–	–	–	–	–
			0.012	–	–	–	**32.85**	–	–	–	–	–
			0.003	–	–	–	**34.89**	–	–	–	–	–
4	*Vibrio parahaemolyticus*	12711T	50.000	–	–	–	–	**19.31**	–	–	–	–
			12.500	–	–	–	–	**21.3**	–	–	–	–
			3.125	–	–	–	–	**23.39**	–	–	–	–
			0.780	–	–	–	–	**25.58**	–	–	–	–
			0.195	–	–	–	–	**27.57**	–	–	–	–
			0.048	–	–	–	–	**29.5**	–	–	–	–
			0.012	–	–	–	–	**31.84**	–	–	–	–
			0.003	–	–	–	–	**33.83**	–	–	–	–
5	*Vibrio harveyi*	15632	50.000	–	–	–	–	–	**20.69**	–	–	–
			12.500	–	–	–	–	–	**22.59**	–	–	–
			3.125	–	–	–	–	–	**24.68**	–	–	–
			0.780	–	–	–	–	–	**26.73**	–	–	–
			0.195	–	–	–	–	–	**28.91**	–	–	–
			0.048	–	–	–	–	–	**30.95**	–	–	–
			0.012	–	–	–	–	–	**32.86**	–	–	–
			0.003	–	–	–	–	–	**35.13**	–	–	–
6	*Photobacterium damselae*	15633T	50.000	–	–	–	–	–	–	**20.38**	–	–
			12.500	–	–	–	–	–	–	**22.39**	–	–
			3.125	–	–	–	–	–	–	**24.53**	–	–
			0.780	–	–	–	–	–	–	**26.65**	–	–
			0.195	–	–	–	–	–	–	**28.72**	–	–
			0.048	–	–	–	–	–	–	**30.91**	–	–
			0.012	–	–	–	–	–	–	**32.85**	–	–
			0.003	–	–	–	–	–	–	**34.89**	–	–
7	*Photobacterium leiognathi*	14169	50.000	–	–	–	–	–	–	–	**19.25**	–
			12.500	–	–	–	–	–	–	–	**21.32**	–
			3.125	–	–	–	–	–	–	–	**23.48**	–
			0.780	–	–	–	–	–	–	–	**25.76**	–
			0.195	–	–	–	–	–	–	–	**27.87**	–
			0.048	–	–	–	–	–	–	–	**29.83**	–
			0.012	–	–	–	–	–	–	–	**31.79**	–
			0.003	–	–	–	–	–	–	–	**33.86**	–
8	*Photobacterium phosphoreum*	13896	50.000	–	–	–	–	–	–	–	–	**18.72**
			12.500	–	–	–	–	–	–	–	–	**20.91**
			3.125	–	–	–	–	–	–	–	–	**22.96**
			0.780	–	–	–	–	–	–	–	–	**25.18**
			0.195	–	–	–	–	–	–	–	–	**27.43**
			0.048	–	–	–	–	–	–	–	–	**29.68**
			0.012	–	–	–	–	–	–	–	–	**31.8**
			0.003	–	–	–	–	–	–	–	–	**33.65**
9	*Listonella anguillarum*	13896	50.000	–	–	–	–	–	–	–	–	–
10	*Shewanella algae*	101058	50.000	–	–	–	–	–	–	–	–	–
11	*Escherichia coli*	13891	50.000	–	–	–	–	–	–	–	–	–
12	*Citrobacter freundii*	12681	50.000	–	–	–	–	–	–	–	–	–
13	*Enterococcus faecalis*	12966	50.000	–	–	–	–	–	–	–	–	–
14	*Proteus vulgaris*	3988	50.000	–	–	–	–	–	–	–	–	–
15	*Pseudomonas fluorescens*	14808	50.000	–	–	–	–	–	–	–	–	–
16	*Pseudomonas putida*	14671	50.000	–	–	–	–	–	–	–	–	–

–, Not detected.