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Short communication

Quantitative Method for Detecting *Vibrio* Species Using Bio-Theta DOX™ System

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Abstract

This is the first report of a quantitative method for detecting *Vibrio* species using the Bio-Theta DOX™ system. We assessed 19 strains of 9 Vibrio species and 22 strains of 22 non-*Vibrio* species and found that the positive rate in the medium-concentration (10^3 cfu/ml) and low-concentration (10 cfu/ml) samples of the 19 Vibrio samples was 100% and 89.5% (17/19), respectively. A few low-concentration samples of *V. alginolyticus* and *V. vulnificus* were negative. All *Vibrio* strains displayed good linear calibration curves for detection time vs bacterial count (r>0.94). The negative rate of the high-concentration (10^6 cfu/ml) and medium-concentration (10^3cfu/ml) samples of the 22 non-Vibrio samples was 86.4% (19/22) and 95.5% (21/22), respectively. Three positive non-*Vibrio* samples identified by the DOX system were *Pseudomonas aeruginosa* in medium- and high-concentration samples and *Enterobacter cloacae* and *Proteus mirabilis* in high-concentration samples. Shrimp are most often infected by *Vibrio* species. Microbial flora of shrimp farming pond has not been understood completely in South-east Asia, however, the bacterial counts in environmental water such as river, sea, ponds and wastewater treatment field are usually made in medium-concentration samples. In addition, the detection time for *P. aeruginosa* was notably longer than that for many of the *Vibrio* species. As the next step, although a *Vibrio* field detection study is needed, monitoring total *Vibrio* counts using the DOX system may become a useful means for early precautions against infectious diseases relating Vibrio species.

Keywords: bacteria detecting system, DOX system, early precautions, total Vibrio counts

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Introduction

Genus *Vibrio* includes Gram-negative species, most of which are oxidase-positive, rod or curved rod-shaped, and aerobic (Farmer et al., 2005). Some *Vibrio* species such as *V. cholera* and *V. parahaemolyticus* are pathogenic in humans and have been implicated in several foodborne diseases (Austin, 2009). The growth rate of *Vibrio* in rich culture and under optimal conditions is extremely high, with a mitotic time of about 8 min for *V. parahaemolyticus*. Recently, early mortality syndrome (EMS) and acute hepatopancreatic necrosis syndrome (AHPNS) in cultured shrimp have appeared to be responsible for infections seen in the shrimp aquaculture sectors of a number of Asian countries. The first occurrence of EMS/AHPNS was in southern China and on Hainan Island in 2010 (Lightner et al., 2012). Then, in 2011, EMS/AHPNS surfaced in Vietnam and Malaysia and in 2012 spread to Thailand. Causative organism of the disease was found to be *V. parahaemolyticus*, which was consistently isolated from EMS/AHPNS-infected shrimp (Litopenaeus vannamei) (Tran et al., 2013). Moreover, Kondo et al. (2014) reported that full genome sequence of *V. parahaemolyticus* was isolated from EMS/AHPNS-infected shrimp in Thailand. Total *Vibrio* count in a typical shrimp farming pond is around 10^{2-4} cfu/ml (Kaneko and Colwell, 1973; Paclibare et al., 2002; Thakur et al., 2004 ; Gopal et al., 2005), while total count of *V. parahaemolyticus*, as determined by successful immersion challenge tests of EMS/AHPNS in shrimp, was found to be 10^6 cfu/ml (Tran et al., 2013). In addition, other *Vibrio* species such as *V. harveyi*, *V. alginolyticus* and *V. penaeicida* have shown virulence against shrimps (Aguirre-Guzma´n et al., 2001). Although the epidemiology of EMS/AHPNS has not been completely clarified, shrimp farmers should regularly monitor the total *Vibrio* count in shrimp farming pond water for its increase to be able to take immediate measures such as using antibacterial products. Thus, development of a rapid method for measuring the count might be a useful means for preventing shrimp from being infected with EMS/AHPNS or other *Vibrio* species.

The DOX system (Bio-Theta, Osaka, Japan) provides a quantitative method for estimating bacterial counts based on respiration rate (Fig.1 a) (Amano et al., 1999; Katayama, 2000). An oxygen electrode measures the level of dissolved oxygen in a sample diluted with media. Over time, a high bacterial load sample will cause the level of dissolved oxygen to decrease to a given threshold value faster than a low bacterial load sample. The time required to reach the threshold level correlates with the level of bacteria in the sample. Then, by creating a standard curve for each food matrix, the level of bacterial contamination can be estimated in unknown samples. In addition, the DOX system has a special feature that enables organisms to be isolated on agar gel by using reagents in the DOX cassette (Fig. 1 b), once the sample has been detected by the DOX system. The DOX system provides rapid results and requires no special techniques for measurement. At present, Daikin Industry Ltd. staff has developed and supplied a total viable count (TVC) test kit (Amano et al., 2001), a quantitative coliforms and *Escherichia coli* detection test kit (Kawasaki et al., 2003), a *Staphylococcus aureus* detection kit, and a *Salmonella* detection kit. Furthermore, the Association of Analytical Communities (AOAC) International Research Institute has recognized the TVC test (Certificate No. 040801) and quantitative coliforms and *E. coli* test (Certificate No. 120801) in the DOX system.

Figure 1 Examination unit of the DOX system (a). The unit interfaces with a personal computer. DOX coliform cassette (b).

Conventional quantitative culture procedure takes about two days, but the DOX system use only few hours to get the results. When shrimp farmers get a quick result, they will be able to give a quick response. Here, we developed a novel quantitative method for detecting *Vibrio* species using the DOX system.

Materials and Methods

Isolates: A total of 19 strains of 9 *Vibrio* species and 22 strains of 22 non-*Vibrio* species were studied and are listed in Tables 1 and 2, respectively. Nine strains were supplied by the Research Institute for Microbial Diseases, Osaka University, Japan and 21 by the American Type Culture Collection. The other 11 strains were isolated from seawater (5 strains), a patient (1 strain), shrimp (*L. vannamei*) (2 strains), oyster (*Crassostrea gigas*) (2 strains), and clam (*Meretrix lusoria*) (1 strain).

DOX system examination of the isolates: The *Vibrio* strains were streaked on trypticase soy agar (Nissui, Tokyo, Japan) with 1.5% NaCl and the non-*Vibrio* strains were streaked on trypticase soy agar alone and incubated at 37°C for 16-20 h under aerobic conditions. The growing *Vibrio* strains were adjusted to approximately 10^5 cfu/ml (high concentration), 10^3 cfu/ml (medium concentration), and 10^1 cfu/ml (low concentration) in a custom diluted solution (a) pH 7.2

containing 34 g calcium dihydrogen phosphate, 10.2 g sodium hydroxide, and 1000 ml distilled water. Then, 1000 ml of 1.5% sodium chloride solution was mixed with 1.25 ml of (a) solution. The non-*Vibrio* strains were mixed to about 10^6 cfu/ml (high concentration) and 10^3 cfu/ml (medium concentration) in the custom diluted solution. Beforehand, concentration of the bacteria were measured using optical density (OD) value of 660 nm. Then, 1 ml custom diluted solution containing the test organisms and 1 ml DOX *Vibrio* media were inoculated in the DOX coliform cassette and shaken by hand for 30 s. The cassette was placed in the sample port of the DOX system and left for 24 h. When the DOX system revealed positive results, total measurement time were recorded. All samples were examined in triplicate.

Results and Discussion

Qualitative analysis of* Vibrio *strains: The results of the high-, medium-, and low- concentration *Vibrio* samples detected by the DOX system are shown in Table 3. The mean positive rate of the high-concentration (10^5 cfu/ml), medium-concentration (10^3 cfu/ml), and low-concentration (10 cfu/ml) *Vibrio* samples was 100%, 100%, and 89.5% (17/19), respectively. One of the 3 low-concentration (1.65 log cfu/ml) *V. alginolyticus* samples (Sample No. 8) isolated from seawater and all 3 low-concentration (1.10 log cfu/ml) *V. vulnificus* RIMD 2219009 samples (Strain No. 11) were negative in this DOX system examination.

The detection time of Sample No. 19, *V. aestuarianus* ATCC 35048, was the longest of the *Vibrio* strains (438.7, 757.3 and 1064.0 min for the high-, medium-, and low-concentration samples, respectively). In comparison, the detection time of the other *Vibrio*-positive samples was 110.0-283.0 min for the high-concentration samples, 194.7-483.3 min for the medium-concentration samples, and 278.0-660.0 min for the low-concentration samples. In particular, the detection time for the *V. parahaemolyticus* strains was 110.0-144.7 min for the high-concentration samples, 202.0-286.3 min for the medium-concentration samples, and 296.7-397.7 min for the low-concentration samples. The rate of change of *Vibrio* samples during the detection period was 0.0-17.2% for the high-concentration samples, 0.0-18.8% for the medium-concentration samples, and 0.3-12.2% for the low-concentration samples. A linear calibration curve between the detection time and bacterial count was observed for all *Vibrio* strains. The correlation coefficient (r) of the calibration curve was lowest for V. aestuarianus (0.9459), followed by *V. harveyi* (0.9738), *V. fluvialis* (0.9772), and *V. mimicus* (0.9871). The correlation coefficient for all the other *Vibrio* strains was >0.99.

Qualitative analysis of* non-Vibrio *strains: In the 22 non-*Vibrio* samples, the mean negative rate for the high-concentration (10^6 cfu/ml) samples was 86.4% (19/22) and that for the medium-concentration (10^3 cfu/ml) samples was 95.5% (21/22). The results of the 3 positive non-*Vibrio* samples detected by the DOX system are shown in Table 4. All 3 high-concentration samples and 1 of the 3 low-concentration samples of *P. aeruginosa* ATCC 27853 (Strain No. 32) were detected. One of the 3 *E. cloacae* ATCC13047 (Strain No. 25) samples, and 2 of the 3 *P. mirabilis* ATCC29909 (Strain No. 30) samples with a high concentration were also positive. In the high-concentration samples, the detection time of *E. cloacae* (Strain No. 25), *P. mirabilis* (Strain No. 30), and *P. aeruginosa* (Strain No. 32) was 818.0 min, 177.5 min, and 446.7 min, respectively. The detection time was the longest for one medium-concentration (1176.0 min) *P. aeruginosa* (Strain No. 32) sample.

Relationship between detection time and bacterial count in 9* Vibrio *species: The relationship between the detection time and bacterial count is shown in Fig. 2. Of the plural strains examined for a single species, the multiple correlation coefficient (r^2) was 0.9931 for *V. alginolyticus* (3 strains; Fig. 2 c), 0.9173 for *V. parahaemolyticus* (5 strains; Fig. 2 b), 0.7142 for *V. fluvialis* (4 strains; Fig. 2 e), and 0.5253 for *V. vulnificus* (2 strains; Fig. 3 d). *V. alginolyticus* and *V. parahaemolyticus* showed a high coefficient of determination for plural strains, while *V. vulnificus* showed the lowest coefficient of determination for plural strains.

We developed a quantitative method for detecting *Vibrio* species using the DOX system. One of the 3 low-concentration (1.65 Log cfu/ml) *V. alginolyticus* samples (Strain No. 8) and all 3 low-concentration (1.10 Log cfu/ml) *V. vulnificus* RIMD 2219009 samples (Strain No. 11) were negative, whereas the DOX system was capable of detecting the other 17 strains in the low-concentration samples. A good linear calibration curve was observed between the detection time and bacterial counts for all examined *Vibrio* strains ($r \geq 0.9459$ for all *Vibrio* strains and $r^2=0.9173$ for the 5 *V. parahaemolyticus* strains). On the other hand, in the 22 non-*Vibrio* species, 1 of the 3 high-concentration samples of *E. cloacae* and *P. mirabilis*, and all 3 high-concentration samples and 1 of 3 medium-concentration samples of *P. aeruginosa* were also positive. *P. aeruginosa* is a highly adaptable organism and has been isolated from the environment such as freshwater (Pirnay et al., 2005), waste water (Lavenir et al., 2014), ponds, rivers, and oceans (Monhandass et al., 2000; Khan et al., 2007), and soils (Ferguson et al., 2001). Lavenir et al. (2014) reported that *P. aeruginosa* was isolated from water in a lagoon in France at a concentration of 312±58 cfu/ml. The mean number of *P. aeruginosa* found in 2 ponds (Tama-ko and Inokashira-ko in Tokyo), a river (4 points of Arakawa river in Saitama and Tokyo), 2 bays (5 points of Tokyo bay in Tokyo and one point of Sagami bay in Kanagawa), and the 3 open ocean (pacific ocean near Izu islands) around Japan was 1-2 cfu/ml, 0.003-0.03 cfu/ml, 0-0.01 cfu/ml, and 0.0003-0.0083 cfu/ml, respectively (Khan et al., 2007). *E. cloacae* and *P. mirabilis* are members of *Enterobacteriaceae* and are prevalent in the intestine of both humans and a wide variety of animals. *E. cloacae* has been isolated from sewage, soil, meat, patients, and hospital environments (Patrick and Francine, 2004), while *P. mirabilis* has been isolated from patients, soil, and polluted water (Penner, 2004).

Monhandass et al. (2000) reported a typical bacterial count of *P. mirabilis*, *P. aeruginosa*, and *E. coli* in coastal Indian Ocean water of ≤10^3 cfu/ml. Although no reports on *E. cloacae* in aquatic environments such as rivers, sea, or fishponds appear to have been published, the concentration can be expected to be around ≤10^3 cfu/ml. The medium-concentration samples of *E. cloacae* and *P. mirabilis* were not detected by the DOX system in this study. One of the 3 medium-concentration *P. aeruginosa* samples was detected, but it took longer to be detected than the *Vibrio* spp. samples.

The detection time of *V. aestuarianus* was longer than that of the other *Vibrio* strains. The optimum growth temperature of *V. aestuarianus* is 20-25°C (Tison and Seidler, 1983) and our detected temperature condition was 37°C. Differences in temperature may be one of the reasons for the longer detection time in the present study.

Based on the results of the present laboratory analysis, it is likely that the DOX system can be used to detect *Vibrio* spp. contamination in environmental water samples in a relatively short time. Recently, EMS/AHPNS outbreaks have been spread by cultured shrimp in several areas of South-east Asia, and the causative organism of the disease was found to be *V. parahaemolyticus* (Kondo et al., 2014). Previous studies on the total *Vibrio* counts in shrimp farming ponds reported concentration of $10^{2\text{-}4}$ cfu/ml (Kaneko and Colwell, 1973; Paclibare et al., 2002; Thakur et al., 2004). In addition, many strains of *V. harveyi*, *V. alginolyticus* and *V. penaeicida* are virulent to shrimp (Aguirre-Guzma´n et al., 2001). Monitoring of *Vibrio* counts in shrimp farming ponds is very important for preventing *Vibrio* related disease.

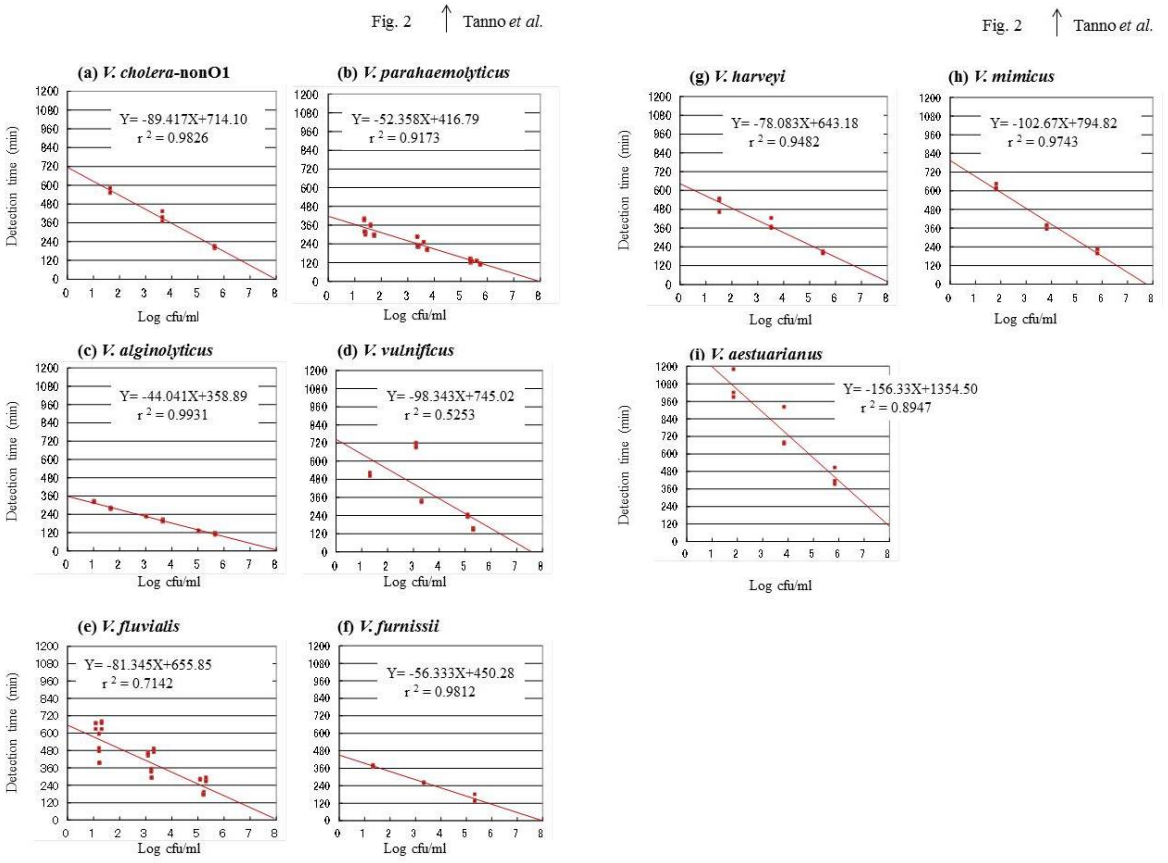

Figure 2 Relationship between detection time (min) and bacterial count (Log cfu/ml) for (a) *Vibrio cholera*, (b) *V. parahaemolyticus*, (c) *V. alginolyticus*, (d) *V. vulnificus*, (e) *V. fluvialis*, (f) *V. furnissii*, (g) *V. harveyi*, (h) *V. mimicus*, and (i) *V. aestuarianus*.

It is recommended that shrimp farmers take immediate action after detecting signs of infection such as by adding antibacterial product to the pond after shrimp begin to die. Although detailed field experiments are needed, regular measurement of the total *Vibrio* count in shrimp farming pond water is an initial step to prevent *Vibrio* related infection including EMS/AHPNS in the field. Moreover, the total *Vibrio* counts in shrimp farming pond water can be obtained easily and rapidly using the DOX *Vibrio* detection method. At present, we are working on developing a DOX detection method for *V. parahaemolyticus*.

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บทคัดย่อ

การตรวจหาปริมาณเชื้อวิบริโอโดยใช้ระบบ Bio-Theta DOX™

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รายงานนี้เป็นรายงานแรกที่ตรวจหาปริมาณของจำนวนเชื้อวิบริโอโดยใช้ระบบ Bio-Theta Daikin Oxygen (DOX ™) โดยทดลองกับเชื้อวิบริโอจำนวน 19 ตัวอย่าง (9 สายพันธุ์) และเชื้อที่ไม่ใช่วิบริโอจำนวน 22 ตัวอย่าง (22 สายพันธุ์) พบว่าอัตราผลบวกของความเข้มข้นระดับกลาง (ที่ 10^3 cfu/ml) และความเข้มข้นระดับต่ำ (ที่ 10 cfu/ml) จากเชื้อวิบริโอจำนวน 19 ตัวอย่างเป็นร้อยละ 100 และร้อยละ 89.5 (17/19) ตามลำดับ มีบางตัวอย่างของ *V. alginolyticus* และ *V. vulnificus* ที่มีความเข้มข้นระดับต่ำจึงได้ผลเป็นลบ ทุกตัวอย่างของเชื้อวิบริโอมี good linear calibration curve ระหว่างเวลาและจำนวนเชื้อ (r>0.94) ส่วนอัตราผลลบของความเข้มข้นระดับสูง (ที่ 10^6cfu/ml) ในตัวอย่างที่ไม่ใช่เชื้อวิบริโอจำนวน 22 ตัวอย่างเป็นร้อยละ 86.4 (19/22) และของความเข้มข้นระดับกลาง (10^3 cfu/ml) เป็นร้อยละ 95.5 (21/22) ตัวอย่างที่ไม่ใช่เชื้อวิบริโอจำนวน 3 ตัวอย่างที่ให้ผลบวก คือ *Pseudomonas aeruginosa* ที่มีความเข้มข้นระดับกลางและระดับสูง และ *Enterobacter cloacae* และ *Proteus mirabilis* ที่มีความเข้มข้นระดับสูง กุ้งส่วนใหญ่จะปนเปื้อนเชื้อวิบริโอ ถึงแม้ว่าเชื้อที่พบในฟาร์มเลี้ยงกุ้งในประเทศแถบทวีปเอเชียตะวันออกเฉียงใต้จะไม่ชัดเจน จำนวนเชื้อแบคทีเรียในน้ำ เช่น น้ำในแม่น้ำลำคลอง ทะเลสระ หรือ น้ำในบ่อบำบัดน้ำเสีย จะทำให้ตัวอย่างน้ำมีความเข้มข้นระดับกลาง นอกจากนี้เวลาที่ใช้ในการตรวจหา *Pseudomonas aeruginosa* นานกว่าที่ใช้ในการตรวจหาเชื้อวิบริโอ ในขั้นตอนต่อไป ควรทำการหาจำนวนเชื้อวิบริโอในฟาร์มกุ้งจริง การควบคุมจำนวนเชื้อวิบริโอในฟาร์มกุ้งยังเป็นสิ่งจำเป็น และการใช้ระบบ DOX™ ในการหาปริมาณเชื้อวิบริโอจะเป็นวิธีหนึ่งที่เป็นประโยชน์ในการป้องกันล่วงหน้าโรคติดเชื้อที่เกี่ยวข้องกับเชื้อวิบริโอในฟาร์มกุ้ง

คำสำคัญ: ระบบการหาปริมาณเชื้อแบคทีเรีย ระบบ DOX การป้องกันล่วงหน้า จำนวนเชื้อวิบริโอ

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Table 1 *Vibrio* strains examined in this study

No.	Species	Strain or source
1	*V. cholerae* non-O:1	RIMD[a)] 2203259
2	*V. parahaemolyticus*	ATCC[b)] 27969
3	*V. parahaemolyticus*	RIMD 2210001
4	*V. parahaemolyticus*	RIMD 2212197
5	*V. parahaemolyticus*	Sea water
6	*V. parahaemolyticus*	Frozen shrimp (*Litopenaeus vannamei*)
7	*V. alginolyticus*	ATCC 17749
8	*V. alginolyticus*	Sea water
9	*V. alginolyticus*	Oyster (*Crassostrea gigas*)
10	*V. vulnificus*	RIMD 2219031
11	*V. vulnificus*	RIMD 2219009
12	*V. fluvialis*	RIMD 2220001
13	*V. fluvialis*	Oyster (*Crassostrea gigas*)
14	*V. fluvialis*	Frozen shrimp (*Litopenaeus vannamei*)
15	*V. fluvialis*	Clam (*Meretrix lusoria*)
16	*V. furnissii*	RIMD 2223001
17	*V. harveyi*	RIMD 2224001
18	*V. mimicus*	RIMD 2218001
19	*V. aestuarianus*	ATCC 35048

a) RIMD: Research Institute for Microbial Diseases, Osaka University, Japan.
b) ATCC:American Type Culture Collection.

Table 2 Non-*Vibrio* strains examined in this study

No.	Species	Strain or source
20	*Bacillus cereus*	Sea water
21	*Bacillus subtilis*	ATCC[a)] 6633
22	*Candida albicans*	ATCC 10231
23	*Citrobacter freundii*	ATCC 8090
24	*Edwardsiella tarda*	ATCC 15947
25	*Enterobacter cloacae*	ATCC 13047
26	*Enterococcus faecalis*	ATCC 29212
27	*Escherichia coli*	ATCC 25922
28	*Hafnia alvei*	ATCC 13337
29	*Klebsiella pneumoniae*	ATCC 13883
30	*Proteus mirabilis*	ATCC 29906
31	*Providencia rettgeri*	ATCC 29944
32	*Pseudomonas aeruginosa*	ATCC 27853
33	*Pseudomonas oleovorans*	Sea water
34	*Salmonella* Typhimurium	ATCC 14028
35	*Serratia marcescens*	ATCC 13880
36	*Staphylococcus aureus*	ATCC 25923
37	*Staphylococcus epidermidis*	ATCC 12228
38	*Staphylococcus pasteuri*	Sea water
39	*Staphylococcus saprophyticus*	Patient
40	*Staphylococcus xylosus*	ATCC 29971
41	*Streptococcus pyogenes*	ATCC 19615

a) ATCC:American Type Culture Collection.

Table 3 Results of high-, medium-, and low- concentration *Vibrio* samples analyzed by the DOX system

No.	Species	High concentration	Medium concentration	Low concentration	Correlation coefficient (r)
1	*V. cholerae* non-O:1	5.64 a)	3.64	1.64	Y=-89.417X+714.10
		204.3 (3.7%) b)	399.7 (8.2%)	562.0 (3.1%)	0.9913
2	*V. parahaemolyticus*	5.36	3.36	1.36	Y=-63.25X+488.61
		144.7 (0.8%)	286.3 (0.4%)	397.7 (1.4%)	0.9973
3	*V. parahaemolyticus*	5.60	3.60	1.60	Y=-57.333X+453.07
		130.0 (0.0%)	250.3 (0.2%)	359.3 (0.7%)	0.9999
4	*V. parahaemolyticus*	5.73	3.73	1.73	Y=-46.667X+377.14
		110.0(3.1%)	202.0 (1.3%)	296.7 (1.7%)	0.9991
5	*V. parahaemolyticus*	5.40	3.40	1.40	Y=-44.667X+373.93
		130.7 (1.8%)	226.0 (2.5%)	309.3 (2.9%)	0.9968
6	*V. parahaemolyticus*	5.37	3.37	1.37	Y=-48.583X+385.53
		123.7 (2.8%)	223.3 (0.7%)	318.0 (0.3%)	0.9996
7	*V. alginolyticus*	5.02	3.02	1.02	Y=-47.917X+372.46
		132.0 (0.0%)	228.0 (0.0%)	323.7 (1.2%)	0.9997
8	*V. alginolyticus*	5.65	3.65	1.65	Y=-41.615X+351.18
		115.3 (3.0%)	200.3 (3.0%)	281.5 (1.8%) c)	0.9980
9	*V. alginolyticus*	5.66	3.66	1.66	Y=-41.833X+347.39
		110.7 (2.9%)	194.7 (1.1%)	278.0 (1.0%)	0.9999
10	*V. vulnificus*	5.32	3.32	1.32	Y=-89.75X+630.16
		152.0 (3.7%)	334.7 (1.5%)	511.0 (2.0%)	0.9991
11	*V. vulnificus*	5.10	3.10	1.10	Y=-234.33X+1432.20
		237.0 (3.7%)	705.7 (2.2%)	ND d)	0.9990
12	*V. fluvialis*	5.22	3.22	1.22	Y=-50.583X+456.24
		192.3 (0.8%)	292.7 (0.2%)	394.7 (0.3%)	0.9999
13	*V. fluvialis*	5.31	3.31	1.31	Y=-95.333X+789.31
		278.7 (5.0%)	483.3 (2.5%)	660.0 (4.3%)	0.9939
14	*V. fluvialis*	5.21	3.21	1.21	Y=-86.167X+624.89
		178.0 (2.0%)	344.3 (3.1%)	522.7 (12.2%)	0.9772
15	*V. fluvialis*	5.08	3.08	1.08	Y=-93.167X+751.99
		283.0 (0.9%)	455.7 (2.4%)	655.7 (3.5%)	0.9959
16	*V. furnissii*	5.33	3.33	1.33	Y=-56.333X+450.28
		151.0 (17.2%)	261.0 (1.0%)	376.3 (1.7%)	0.9906
17	*V. harveyi*	5.52	3.52	1.52	Y=-78.083X+643.18
		204.3 (3.3%)	384.3 (9.0%)	516.7 (9.1%)	0.9738
18	*V. mimicus*	5.81	3.81	1.81	Y=-102.67X+794.82
		215.0 (7.3%)	369.0 (3.5%)	625.7 (2.7%)	0.9871
19	*V. aestuarianus*	5.85	3.85	1.85	Y=-156.33X+1354.5
		438.7 (13.7%)	757.3 (18.8%)	1064.0 (9.6%)	0.9459

a) Log cfu/ml of sample
b) Mean detection period (min.) and rate of change (%) of triplicate examinations per sample
c) One of 3 samples was negative. Mean detection period and rate of change were calculated by the 2 positive samples
d) Not detected

Table 4 Results of positive non-*Vibrio* samples by the DOX system

No.	Species	High concentration			Medium concentration		
		1st	2nd	3rd	1st	2nd	3rd
		Mean (Rate of change)			Mean (Rate of change)		
25	*Enterobacter cloacae*	ND [b]	6.38 [a] ND 818.0 (0.0%)	818 [c]	ND	3.38 ND	ND
30	*Proteus mirabilis*	ND	6.64 175 177.5 (2.0%)	180	ND	3.64 ND	ND
32	*Pseudomonas aeruginosa*	439	6.56 452 446.7 (1.5%)	449	1176	3.56 ND 1176 (0.0%)	ND

a) log cfu/ml
b) Not detected
c) Detection time (minutes) of the 3rd examination at high concentration samples