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Note

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Development of a Proficiency Test Specimen for Enumerating *Escherichia coli* in Molluscan Bivalve Shellfish

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Abstract

This study was conducted to develop a proficiency test (PT) specimen for the enumeration of *Escherichia coli*, a sanitary indicative bacterium, in molluscan bivalve shellfish. The common mussel *Mytilus edulis* was chosen as a matrix for the PT specimen. Mussels were artificially bioaccumulated for approximately 3 h with *E. coli* culture. After determining the homogeneity of samples, samples were distributed to 17 participants involved in the proficiency testing program. The enumeration of *E. coli* was performed by the most probable number method of the American Public Health Association. Statistical evaluations of the results obtained from inter- and intra-laboratory variation indicated no significant differences in the accuracy of these techniques between participants, indicating z-scores of $\leq \pm 2$ and suggesting that preparation of the PT specimen for enumerating *E coli* in shellfish was successful.

Key words: *Escherichia coli*, Proficiency test, Sanitary indicative bacterium, Shellfish

Introduction

Shellfish have been known to be an important source of food since Roman times. For over 100 years, however, the ingestion of shellfish has been recognized to cause outbreaks of bacterial and viral infections (Ingresoll, 1881). One of the first recognized outbreaks associated with the consumption of raw oysters was described in 1816 by the French physician J. P. A. Pasquier, who reported that typhoid fever was found in a group of people who had consumed sewage-contaminated oysters (Potasman et al., 2002). Human health problems associated with the consumption of bivalve shellfish are well recognized internationally. These health hazards are largely due to the filter-feeding method employed by bivalve mollusks, whereby bacterial and viral pathogens are concentrated and retained; this then causes gastroenteritis, which is often derived from sewage contamination of the waters in which the bivalves grow (Center for Environment, Fisheries and Aquaculture Science, 2010). The hazards posed by the bioaccumulation of harmful microorganisms in shellfish are compounded by the traditional consumption of certain shellfish in raw or only lightly cooked dishes (Potasman et al., 2002). Historically, fecal coliforms or *Escherichia coli*, which are used as indicators of fecal contamination in bivalve molluscan shellfish, have been adopted as surrogate indicator organisms to assess the quality of shellfish (US Food and Drug Administration, 2009; Center for Environment, Fisheries and Aquaculture Science, 2010). To enumerate sanitary indicative bacteria in shellfish samples, enrichment and subsequent selective cultivation with a series of diluted shellfish homogenates has been traditionally performed according to the National Sanitation Shellfish Program guidelines in Korea (US Food and Drug Administration, 2009).

A proficiency test (PT) is a powerful quality assurance

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tool enabling laboratories to monitor their performance and to compare their results with similar laboratories. Proficiency testing schemes are used by laboratory accreditation bodies as part of the process of assessing the ability of laboratories to competently perform tests and measurements for which accreditation is held (Duarte et al., 2007). However, no available PT specimen exists for enumerating *E. coli* in shellfish in Korea. Therefore, this study was conducted to develop a PT specimen for evaluating laboratory precision in enumerating *E. coli* in shellfish. Such a specimen is needed, since considerable health and economic consequences depend on an analyst's precision in measuring sanitary indicative bacteria.

Materials and Methods

Bacterial strain and culture

E. coli KCTC 1682 (ATCC 25922), exhibiting all the characteristics of fecal coliforms, was incubated in Nutrient Broth (Difco, Sparks, MD, USA) at 37°C. The cell population was measured by the viable cell counting method using Nutrient Agar (Difco).

Sample preparation

Samples for PT were prepared according to the standard operation procedure of Center for Environment, Fisheries and Aquaculture Science (CEFAS) with minor modification (Center for Environment, Fisheries and Aquaculture Science, 2010). The common mussel *Mytilus edulis*, collected from a commercial harvesting area at Jindong-myeon, Changwon-city, Gyeongsangnam-do, Republic of Korea, was used as a matrix to develop the PT specimen. The size, total weight, and muscle weight of samples were 7.0±0.48 cm, 14.5±2.42 g, and 8.4±2.12 g, respectively. Mud and sediment adhering to the shellfish were removed prior to opening the shellfish by rinsing/scrubbing under running tap water.

One batch, consisting of approximately 20 kg of mussels, was spread evenly across two trays. The shellfish were then immersed in water tanks that had been filled with 100 L of seawater, which was recirculated at 7 L per min for 16 h to allow the shellfish to acclimatize. After acclimation, the mussel trays were removed from the tank and the seawater was discarded. The mussel trays were reimmersed into the water tank that was refilled with 100 L of fresh seawater, and then the *E. coli* culture was inoculated. The mussels were then bioaccumulated with constant recirculation as described above. After circulation for the indicated time, the mussels were removed and the *E. coli* level that had bioaccumulated in the mussels was determined by the most probable number (MPN) method of the American Public Health Association (1970).

Sample distribution and examination

Samples comprising approximately 50 randomly selected mussels (about 720 g) were packed in accordance with the CEFAS protocol for packaging shellfish for transportation. Samples were received in an intact food-grade plastic bag and properly packed in a cool box with ice packs that reach a temperature of less than 8°C within 4 h and then maintain this temperature for at least 24 h. Packed samples were then dispatched to each laboratory participating in this PT within 24 h.

Participants analyzed the samples in duplicate immediately on receipt by the procedure for enumerating *E. coli* in bivalve molluscan shellfish based on the MPN method (American Public Health Association, 1970).

Statistical analysis

Data are expressed as means±SD, and all statistical comparisons were made using a one-way analysis of variance (ANOVA), followed by Duncan's test (Duncan, 1955). A *P*-value of <0.05 was considered to indicate statistical significance.

Proficiency evaluation of participating laboratories

The proficiency of the participating laboratories is evaluated based on their Z-score values. A Z-score was calculated for each laboratory according to the following equation:

$$Z=(x-X)/S$$

where

x is the reported result by the participant laboratory,

X is the assigned value (the median of the results reported by the reference laboratory), and

S is the standard deviation and calculated for each laboratory according to the following equation:

$$S=\sqrt{\frac{1}{n-1}\sum_{i=1}^{n}(x-X)^2}$$

The Z-score classification is as follows: $|Z|\leq 2$, acceptable; $2<|Z|\leq 3$, questionable; $3<|Z|$, unacceptable.

Results and Discussion

To develop a PT specimen for the enumeration of *E. coli* in molluscan bivalve shellfish, the common mussel *M. edulis* was chosen as the matrix for the PT specimen because the shellfish, which is a characteristic species of molluscan bivalve shellfish, grows well throughout the Korean coastal area (Je et al., 1990).

A preliminary experiment indicated that an 18 h culture of *E. coli* in Nutrient Broth at 37°C achieved 10^8 CFU per mL (data not shown). After 30 mL of the culture was inoculated into 100 L of seawater, *E. coli* was bioaccumulated into the

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mussels for 3, 6, 9, and 24 h by constant circulation.

The *E. coli* MPN results obtained from each sample indicated that approximately 10^5 viable organisms were consistently recovered from 3 h of exposure and approximately 10^6 viable organisms from 6 to 24 h of exposure (data not shown). Thus, the mussels that had artificially bioaccumulated for approximately 3 h with *E. coli* were selected as specimens for the PT, considering the *E. coli* MPN level bioaccumulated in mussels provided by the CEFAS. The agency has usually prepared and distributed mussel samples containing less than 10^6 viable organisms as PT specimens for enumerating sanitary indicative bacteria in molluscan bivalve shellfish.

To evaluate the homogeneity of the samples prepared in this study, ten samples were analyzed by the MPN method at a reference laboratory and the results were statistically analyzed (Table 1). The *E. coli* MPN levels ranged from 330,000 to 490,000 MPN/100 g. Median, geometric median (GM), and mean values were 490,000 MPN/100 g, 449,900 MPN/100 g, and 455,000 MPN/100 g, respectively. Differences between the means of each sample were estimated by one-way ANOVA. The values (mean±SE of ten replication samples) were significantly different (P<0.01). Considering the above results, we concluded that because of their stability and homogeneity (Table 1), the samples prepared in the current study could be used as PT specimens for enumerating *E. coli* in molluscan bivalve shellfish.

After determining the homogeneity of samples, samples were distributed by overnight courier at less than 8°C to 17 participants involved in the PT program. The enumeration of *E. coli* was performed by the MPN method as mentioned above (American Public Health Association, 1970). The *E. coli* MPN levels obtained from the participating laboratories ranged from 68 to 790,000 MPN/100 g. Median and GM values were 120,000 MPN/100 g and 35,653 MPN/100 g, respectively (Table 2). The statistical evaluations of the results obtained from inter- and intra-laboratory variation indicated that no significant difference existed in the accuracy of these techniques among participants, which indicated z-scores ≤±2, and they suggested that the preparation of PT specimens for the enumeration of *E. coli* in shellfish was successful (Medina-Pastor et al., 2010).

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Table 1. *Escherichia coli* level in mussel samples bio-accumulated with *E. coli* as determined by the most probable number (MPN) method at the reference laboratory

No.	Arrival date/time	Sample temperature (°C)	Date completed analysis	*E. coli* MPN/100 g*
1	Jan. 25, 2011 3:00 PM	3.0	Jan. 29, 2011	490,000
2	Jan. 25, 2011 3:00 PM	3.0	Jan. 29, 2011	330,000
3	Jan. 25, 2011 3:00 PM	3.0	Jan. 29, 2011	490,000
4	Jan. 25, 2011 3:00 PM	3.0	Jan. 29, 2011	460,000
5	Jan. 25, 2011 3:00 PM	3.0	Jan. 29, 2011	490,000
6	Jan. 25, 2011 3:00 PM	3.0	Jan. 29, 2011	490,000
7	Jan. 25, 2011 3:00 PM	3.0	Jan. 29, 2011	490,000
8	Jan. 25, 2011 3:00 PM	3.0	Jan. 29, 2011	490,000
9	Jan. 25, 2011 3:00 PM	3.0	Jan. 29, 2011	330,000
10	Jan. 25, 2011 3:00 PM	3.0	Jan. 29, 2011	490,000

Median value, 490,000 MPN/100g; Geometric median value, 449,900 MPN/100g

*Differences between the means of each sample were estimated by one-way ANOVA. Values (mean±SE of ten replication samples) are significantly different (P<0.01).

Table 2. Participants results and Z-scores

Lab ID	Arrival date/time	Sample temperature (°C)	Date completed analysis	*E. coli* MPN/100 g			Z-score
				Test 1	Test 2	Mean	S=243,663.4
1	Jan. 26, 2011 10:00 AM	2.6	Jan. 29, 2011	540,000	540,000	540,000	0.349
2	Jan. 26, 2011 10:30 AM	2.4	Jan. 29, 2011	350,000	240,000	295,000	-0.657
3	Jan. 26, 2011 7:00 PM	2.0	Jan. 29, 2011	350,000	170,000	260,000	-0.800
4	Jan. 25, 2011 4:00 PM	3.1	Jan. 29, 2011	700,000	490,000	595,000	0.575
5	Jan. 26, 2011 10:20 AM	1.8	Jan. 29, 2011	79,000	130,000	104,500	-1.438
6	Jan. 26, 2011 10:05 AM	3.0	Jan. 29, 2011	490,000	490,000	490,000	0.144
7	Jan. 25, 2011 6:00 PM	3.0	Jan. 29, 2011	120	280	200	-1.867
8	Jan. 25, 2011 5:35 PM	2.0	Jan. 29, 2011	110,000	110,000	110,000	-1.416
9	Jan. 26, 2011 10:20 AM	3.0	Jan. 29, 2011	70,000	79,000	74,500	-1.562
10	Jan. 26, 2011 10:20 AM	3.0	Jan. 29, 2011	490,000	490,000	490,000	0.144
11	Jan. 26, 2011 11:35 AM	2.1	Jan. 29, 2011	130,000	350,000	240,000	-0.882
12	Jan. 26, 2011 2:00 PM	1.8	Jan. 29, 2011	110	68	89	-1.867
13	Jan. 26, 2011 1:00 PM	2.0	Jan. 29, 2011	1,100	1,700	1,400	-1.862
14	Jan. 26, 2011 1:20 PM	2.0	Jan. 29, 2011	490,000	790,000	640,000	0.759
15	Jan. 26, 2011 11:40 PM	2.0	Jan. 29, 2011	400	390	395	-1.866
16	Jan. 26, 2011 1:10 PM	3.0	Jan. 29, 2011	790	1100	945	-1.863
17	Jan. 26, 2011 10:10 AM	2.5	Jan. 29, 2011	330,000	790,000	560,000	0.431

Median value, 120,000 MPN/100 g; Geometric median value, 35,653 MPN/100 g

MPN, most probable number.

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