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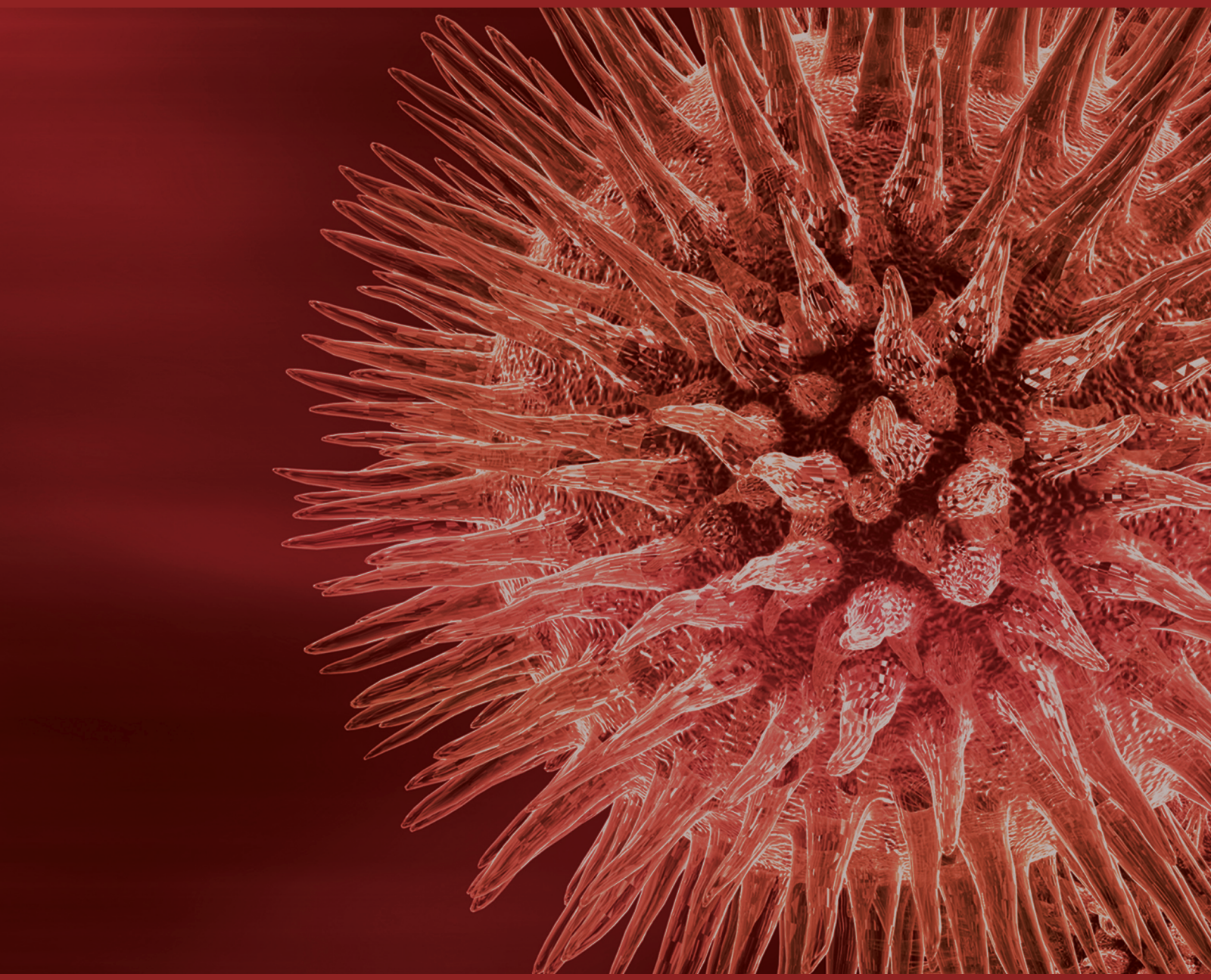
Guest Editors: Hiroshi Asakura, Holger Brueggemann, Sou-ichi Makino, and Yoshiko Sugita-Konishi



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Editorial

Molecular Approaches for the Classification of Microbial Pathogens of Public Health Significance

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A number of pathogenic microorganisms are widely disseminated, surrounding us and sometimes infecting our body, causing a wide range of health problems, ranging from mild to life-threatening diseases. It is now widely accepted to use a variety of molecular approaches to classify or type the causative microbial pathogens in both host and hostile environments, which improve our global understanding on the epidemiology, pathogenesis, ecology, and evolution of the microorganisms, contributing to the prevention of infection, better infection control, and environmental sanitation.

This special issue focuses on molecular approaches for microbial classification and typing, which would be contributable to our better understanding on the microbial features, risks, and potent strategies for its controlling from the viewpoint of public health.

Microbial infections in humans could occur in a number of ways, including food-borne, human-to-human, or environmental transmissions. To reduce the food-borne infections, it is well recognized that the control of microbial safety in foodstuffs is a first-line of preventive strategy. Such an assessment of 126 natural cheese products manufactured in Hokkaido, Japan, was reported by F. K. Esho et al. The authors examined prevalence of some pathogens (*Listeria monocytogenes*, pathogenic *Escherichia coli*, and *Salmonella* spp.) as well as enumeration of indicator bacteria, revealing no detection of those pathogens despite the detection of coliforms in 25 of 126 tested samples (19.8%). Considering the use of pasteurized milk, it could be evidenced that the microbiological quality and hygienic status of the natural cheese tested in this study were in the most fine and satisfactory status.

Microbial quality of meat processing at slaughter is now one of the most public health concerns because of the frequent contamination with a numbers of pathogenic bacteria in meat products attributing to human infections [1]. In this relation, H. Asakura et al. reported the prevalence of Shiga toxin-producing *E. coli* (STEC) O157 in bovine feces and bovine offal at preslaughter and their characterization. At preslaughter, the STEC O157 was detected in 31 of 301 cattle feces (10.3%). Throughout slaughtering, this pathogen was detected from bovine offal and carcasses, and some of which exhibited identical macrogenotypes, suggesting their cross-contamination at preslaughter.

Development of the protocol for the detection of food-borne pathogens was reported by Hayashi et al. The authors demonstrated a quick screening methodology by the development of cocktail PCR dipstick DNA chromatography (CPDC) assay, which enabled finalizing the simultaneous detection of multiple enteric pathogens including *Salmonella* spp., *Shigella* spp. enteroinvasive *E. coli* (EIEC), and enterohemorrhagic *E. coli* (EHEC) from food samples, within 45 min after 4–6 h of enrichment in a recently developed FPE broth [2]. Such method offers rapid report to food suppliers and

helps the quick shipment of safety-confirmed food products to markets.

Vibrio cholerae is an aquatic bacteria that causes cholera, a major public health problem especially in developing countries [3]. For the rapid and sensitive detection of this pathogen, E. Yamasaki et al. report the development of immunochromatographic test strip targeting cholera toxin (CT-IC). The authors evaluated the high sensitivity (detection limit of 10 ng/mL) and no cross-reactions of this developed tool, confirming its feasible use for the rapid detection and surveillance of toxigenic *V. cholerae* that is a public health threat. The rapid detections in early stage of epidemic would also allow quick triggering of control measures.

Some pathogens could withstand under hostile environments. *Legionella pneumophila* that causes Legionnaires' disease and Pontiac fever [4] is one of the representatives to achieve adaptation to aquatic environments. The article of M. Tachibana et al. reports the prevalence and virulence characteristics of this pathogen in environmental water and foot spa in Yamaguchi, Japan. Finally, *L. pneumophila* was isolated from 5 of 22 samples, which exhibited virulence characteristics to humans. The authors thus concluded the potent risks for the transmission of this pathogen from the spa via generated aerosols.

Staphylococcus aureus, a gram positive coccal bacterium, is either commensal that colonizes healthy nasal mucosa or pathogen of humans [5]. During the last five decades, *S. aureus* clones that resist methicillin (methicillin-resistant *S. aureus*, MRSA) disseminated and caused a medical and public health problem worldwide [6]. N. Indrawattana et al. performed genotypic and phenotypic classification of 92 *S. aureus* isolates from periodic monitoring in Thailand. The authors confirmed the link between the possession of virulence genes and resistance to methicillin as well as the fact that about 73% of the isolates formed biofilms on abiotic surface. The results of this study provide insight information on molecular and phenotypic markers of *S. aureus* clinical isolates in Thailand which should be useful for future active surveillance that aimed to control a spread of existing antimicrobial resistant bacteria as well as early recognition of a newly emerged variant.

In summary, this special issue covers a range of diverse topics related to the microbial classification of public health significance. We hope the papers published will serve to further highlight the microbial safety in foods and environments, as well as in stimulating further researches into the virulence features of microbes and development of diagnostic tools, thereby contributing to the improved patient treatment and microbial safety in sources of infection.

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Hiroshi Asakura
Holger Brueggemann
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Yoshiko Sugita-Konishi

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Research Article

Molecular Approach for Tracing Dissemination Routes of Shiga Toxin-Producing *Escherichia coli* O157 in Bovine Offal at Slaughter

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Bovine offal is currently recognized as one of the sources of human Shiga toxin-producing *Escherichia coli* (STEC) infection in Japan. Here, the prevalence and genetic characterization of STEC O157 in bovine feces, offal, and carcasses at slaughtering were examined between July and October in 2006. STEC O157 was detected in 31 of 301 cattle feces (10.3%) delivered from 120 farms. Simultaneously, 60 bovine-originated offal (tongue, liver, and omasum) and carcasses were randomly selected and the detection of O157 STEC was examined as well. STEC O157 was isolated from 4 tongues (6.7%), 1 liver (1.7%), 3 omasa (5.0%), and 2 carcasses (3.3%), respectively. All the O157 isolates were positive for *eae* and *hlyA* genes, and 37 of 41 isolates (90.2%) exhibited *stx2c* genotype. PFGE analysis revealed the identical macrogenotypes of 4-tongue- and 1-liver-originated isolates and among 2 fecal isolates from animals slaughtered consecutively. Considering their continuous detection according to the slaughtering order, we concluded that these distributions of O157 in bovine offal and feces might be due to cross-contamination at (pre)slaughter. Our data thus reposes implication of better sanitary control in diapedesis from both upper and lower sites to prevent spread of this pathogen to bovine offal at slaughtering.

1. Introduction

Shiga toxin (Stx)-producing *Escherichia coli* (STEC) is one of the major foodborne pathogens that causes diarrheal illness in humans worldwide. Among a number of serotypes categorized in the STEC, O157 is the most major serotype associated with human infection [1]. Infection with STEC O157 can be achieved at least in part through the intake of contaminated foods, in which dairy products and meats contaminated with animal feces or intestinal contents during/after slaughtering are considered as the most common sources [2].

In Japan, bovine offal which include liver, heart, tongue and intestines are customarily eaten, a part of which are consumed raw and the contamination with pathogenic microorganisms in these meat products is therefore considered a high risk for human health. Indeed, as epidemiological records for foodborne O157 infections in Japan, 6 of 52 cases (11.5%) were associated with the bovine offal in years 2010-2011 [3]. More recently, we examined the prevalence of STEC in retail bovine offal products in Japan, revealing that 38 of 229 samples (16.6%) were positive for *stx* gene and four O157 and one O26 STEC were finally isolated from small intestine and omasum products [4]. However, the routes of contamination in these products remain unclear, especially the issue of how and whether cross-contamination might occur during slaughtering processes.

Given the background, here we examined the prevalence and genetic characterization of STEC O157 in bovine feces and offal at slaughterhouse in Japan.

2. Materials and Methods

2.1. Sampling, Isolation, and Identification of STEC O157. Fecal samples were collected with cotton swab from a total of

301 bovine from 120 farms between July and September, 2006, at a slaughterhouse in Japan. Simultaneously, 60 of these animals were randomly selected and 100 cm^2 surface areas of their offal (livers, tongues, and omasum) and carcasses were swabbed, thereby being subjected to the detection procedure of STEC O157 as well. The swab samples were incubated in 10 mL of novobiocin-supplemented mEC broth (Eiken Kagaku, Tokyo, Japan) at 42°C for 24 h. The cultures were then subjected to screening of O157 using Path-Stik *E. coli* O157 (Celsis, Cambridge, UK) and mini-VIDAS (bioMérieux-Vitek, France). The O157-positive culture samples were then plated on CT-SMAC (Eiken Kagaku, Tokyo, Japan), CHROMagar O157 (CHROMagar, Paris, France), and Rainbow agar O157 (Biolog, Hayward, CA, USA). After incubation at 37°C for 24 h, suspected colonies were biochemically and/or genetically identified to be STEC O157 with API-20 kit (bioMérieux), O157 PCR screening set (Takara Bio, Shiga, Japan), and NH immunochromatography (Nippon-Ham, Tokyo, Japan) accordingly. The above culturing flow for each sample was started immediately within the day of slaughter.

2.2. Genetic Characterization and Toxin Production of STEC O157 Isolates. To characterize virulence gene possession of these isolates, total DNA was extracted from bacterial isolates with DNeasy kit (Qiagen, Hilden, Germany) according to the manufacturer's instruction. The *eaeA* and *hlyA* (*ehxA*) genes were detected by PCR using primers as described previously [5, 6]. The *stx* genes were subtyped by PCR as described [4, 7]. Shiga toxin (Stx) production was assayed by VTEC-RPLA (Denka Seiken, Tokyo, Japan).

2.3. Pulsed-Field Gel Electrophoresis (PFGE). Representative O157 isolates were subjected to PFGE with *Xba*I endonuclease (New England BioLabs, Ipswich, MA, USA) using the CHEF Mapper system (Bio-Rad Laboratories, Hercules, CA, USA) as described previously [8]. The gel images were obtained using ethidium bromide stain. The electrophoretic patterns from PFGE were compared based on band position using FingerPrinting II software (Bio-Rad Laboratories, Hercules, CA, USA) and derived using the Dice coefficient with a maximum position tolerance of 1%. The strains were clustered using the unweighted pair group (UPGMA) method with arithmetic averages according to the manufacturer's instructions.

3. Results and Discussion

3.1. Dynamics for the Prevalence of STEC O157 at Slaughter. The majority of primary STEC infections are considered to be food- or water-borne, in which bovine and its products are one of the main sources of infection [9]. Epidemiological studies have mount evidence for the high prevalence of STEC especially O157 serotype in cattle intestines [10–12]. Throughout the screening tests herein, STEC O157 was detected from 31 of 301 samples from bovines feces that were slaughtered between July and September in 2006 in Japan (Table 1), of which 6 isolates (isolates # 7–12) were originated from animal slaughtered at the same day, even though they were delivered from different farms (Table 1). 60 offal (tongues, livers, and omasum) and carcass samples were simultaneously subjected to the O157 detection tests, resulting in that the STEC O157 was isolated from 4 tongues (6.7%), 1 liver (1.7%), 3 omasa (5.0%), and 2 carcasses (Table 1). Among them, 4 tongues and 1 liver isolates (isolates number 1–5) were originated from samples consecutively slaughtered (Table 1). Thus, these data showed the prevalence of STEC O157 in bovine feces and offal at slaughter. The consecutive detection of O157 from fecal and offal samples (i.e., isolates #1–3, 5) suggested that these isolates might be originated from identical sources.

The prevalence date herein at 10.5% is likely to be similar to that in a previous nationwide study in Japan [13]. Comparatively, little is known about the prevalence of STEC O157 in bovine offal at slaughter worldwide. Such biased information is likely to depend on the intake custom, as the individuals who eat them raw are limited and very few in westerns. Our data herein thus provided the implication of these foods for microbial risks to human infection.

3.2. Genetic Characterization of STEC O157 Isolates. Genetic characterization assays showed that all isolates were positive for *eaeA* and *hlyA* (Table 1). The most frequent *stx* subtype was *stx2c* (37 of 41, 90.2%), followed by *stx1*+*stx2c* (isolates number 6 and #21, 4.9%), and *stx1* (isolate #12, 2.4%), *stx1*+*stx2* (isolate #41, 2.4%), respectively (Table 1). The high yields of *stx2c* among the bovine isolates herein are in agreement with the previous reports [13]. We confirmed the Stx production in all isolates by VTEC-RPLA. Thus, these data indicated the potent virulence of these isolates.

3.3. Macrogenotypes of Representative O157 Isolates. Having nonconsecutive isolation of O157 STEC from fecal samples originated from identical farm (i.e., isolates #8, 9, 27, and 28 from farm F) and almost consecutive detection from tongue/liver (i.e., isolates #1–4 and #5) and omasum/fecal samples (isolates #21-22 and 17–20), their genetic associations were examined by pulsed-field gel electrophoresis (PFGE). This approach then revealed the identical macrogenotypes among four tongue isolates and one liver isolate that were slaughtered almost consecutively (Figure 1). Likewise, two omasum isolates (isolates #21-22) also exhibited identical PFGE pattern with fecal isolates #17–20 that were obtained at the same date (24/Aug/2006) (Figure 1). These suggested that cross-contamination at slaughter might be a possible factor for this dissemination.

Moreover, four fecal isolates #8-9 and #27-28 exhibited identical PFGE patterns with close phylogenic lineages (Figure 1). Because these isolates were originated from animals fed at the same farm (farm F, Table 1), it could be considered that this O157 might be widely disseminated at that farm continuously. Indeed, a previous study demonstrated that a part of bovine animals shed high doses of O157 longitudinally (so-called "super shedders") [14]. A recently trialed vaccine against type III secreted proteins [15] might be effective for the reduction of such continuous spread of this pathogen at farms. In this relation, two beef carcass isolates #40 and 41 showed different PFGE genotypes although they were

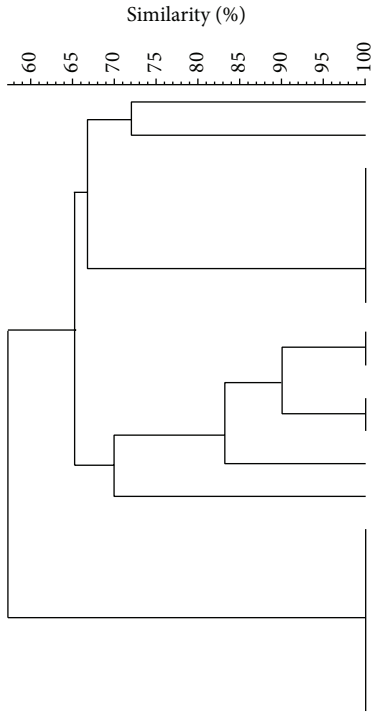
Table 1: Summary of STEC O157 isolates obtained in this study.

Isolate number	Date of slaughter	Animal number*1	Place*2	Farm*2	Source	Virulence gene		
						stx	*eaeA*	*hlyA*
1	6/Jul/2006	206	i	A	Tongue	2c	+	+
2	6/Jul/2006	207	ii	B	Tongue	2c	+	+
3	6/Jul/2006	208	ii	B	Tongue	2c	+	+
4	6/Jul/2006	210	iii	C	Tongue	2c	+	+
5	6/Jul/2006	207	ii	B	Liver	2c	+	+
6	20/Jul/2006	842	iv	D	Omasum	1 + 2c	+	+
7	27/Jul/2006	1202	v	E	Feces	2c	+	+
8	27/Jul/2006	1207	v	F	Feces	2c	+	+
9	27/Jul/2006	1208	v	F	Feces	2c	+	+
10	27/Jul/2006	1242	iii	G	Feces	2c	+	+
11	27/Jul/2006	1257	i	H	Feces	2c	+	+
12	27/Jul/2006	1263	i	I	Feces	1		
13	28/Jul/2006	1315	vi	J	Feces	2c	+	+
14	28/Jul/2006	1316	vi	J	Feces	2c	+	+
15	28/Jul/2006	1318	vi	J	Feces	2c	+	+
16	23/Aug/2006	834	vii	K	Feces	2c	+	+
17	24/Aug/2006	852	iii	L	Feces	2c	+	+
18	24/Aug/2006	868	vii	M	Feces	2c	+	+
19	24/Aug/2006	869	vii	M	Feces	2c	+	+
20	24/Aug/2006	883	vii	N	Feces	1 + 2c	+	+
21	24/Aug/2006	851	iii	O	Omasum	2c	+	+
22	24/Aug/2006	853	iii	L	Omasum	2c	+	+
23	25/Aug/2006	936	viii	P	Feces	2c	+	+
24	25/Aug/2006	937	viii	P	Feces	2c	+	+
25	30/Aug/2006	1048	v	E	Feces	2c	+	+
26	30/Aug/2006	1050	v	Q	Feces	2c	+	+
27	30/Aug/2006	1057	v	F	Feces	2c	+	+
28	30/Aug/2006	1058	v	F	Feces	2c	+	+
29	30/Aug/2006	1068	vii	R	Feces	2c	+	+
30	9/Sep/2006	383	i	I	Feces	1		
31	9/Sep/2006	388	i	S	Feces	2c	+	+
32	9/Sep/2006	389	i	S	Feces	2c	+	+
33	9/Sep/2006	392	i	T	Feces	2c	+	+
34	9/Sep/2006	399	vii	U	Feces	2c	+	+
35	9/Sep/2006	400	vii	U	Feces	2c	+	+
36	9/Sep/2006	401	vii	U	Feces	2c	+	+
37	9/Sep/2006	424	ix	V	Feces	2c	+	+
38	14/Sep/2006	601	viii	P	Feces	2c	+	+
39	30/Sep/2006	1152	ix	W	Feces	2c	+	+
40	17/Aug/2006	635	vii	N	Carcass	2c	+	+
41	24/Aug/2006	890	vii	N	Carcass	1 + 2	+	+

*1Animals were numbered monthly according to the slaughtering order. *2The places (prefectures) and farms, where the animals were fed, were shown in Arabic numerals or alphabetic orders.

slaughtered almost consecutively (Figure 1). It is likely that the super shedders can have a disproportionate effect on the animal's hide and subsequent carcass contamination while low-shedding animals are also linked to the contamination on beef carcass [16]. Minimizing or eliminating the super shedding animals would thus contribute, at least in part, to the reduction of contamination with O157 STEC on beef carcasses.

As a molecular classification tool, we used PFGE to genetically discriminate representative STEC O157 isolates.



Isolate number	Animal	Farm	Source
6	842	D	Omasum
31	388	S	Feces
1	206	A	Tongue
2	207	B	Tongue
3	208	B	Tongue
4	210	C	Tongue
5	207	B	Liver
8	1207	F	Feces
9	1208	F	Feces
27	1057	F	Feces
28	1058	F	Feces
40	635	N	Carcass
41	890	N	Carcass
17	852	L	Feces
18	868	M	Feces
19	869	M	Feces
20	883	N	Feces
21	851	O	Omasum
22	853	L	Omasum

Figure 1: Pulsed-field gel electrophoresis (PFGE) patterns of representative STEC O157 isolates from bovine samples at slaughter. UPGMA dendrogram was constructed with the use of FingerPrinting II software.

The PFGE types are known to be altered during passage in bovine intestine [17], and therefore the identical PFGE patterns of O157 isolates from offal and feces of bovines at slaughter suggest that this dissemination of O157 might be due to animal-to-animal contact at preslaughter (including farm environment) and/or cross-contamination at slaughter, respectively. In this relation, Arthur et al. (2011) reported that the O157 cells could survive on cattle hides for up to 9 days after infection [18], suggesting that animal-to-animal contact at preslaughter might be also one of the important factors for the bacterial dissemination. In addition, the detection of STEC O157 at the surface of tongues and omasum further provided an idea that this pathogen could be disseminated at slaughter by diapedesis of oral and gastric contents to the surroundings. In this support, Bergholz and Whittam reported the superior ability of STEC O157 in acid resistance to the other serotypes of STEC [19].

4. Conclusion

Our data showed the prevalence of STEC O157 in bovine feces and offal at slaughter in Japan. The genetic characterization reposes the importance for the proper salinity control at bovine (pre)slaughtering processes to prevent spread of STEC O157 to beef carcasses and offal.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Microbial Assessment and Prevalence of Foodborne Pathogens in Natural Cheeses in Japan

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The production and consumption of domestic natural cheese in Japan is increasing year by year. More than ninety percent of domestic natural cheese is produced in Hokkaido region of Japan, while information on its quality and safety related to foodborne pathogens is limited. To assess the microbiological safety of domestic natural cheese, a total of 126 natural cheese samples produced in Hokkaido were collected from December, 2012, to July, 2013. In addition to standard plate count (SPC) and coliform counts, the prevalence study of three pathogens (*Listeria monocytogenes*, pathogenic *Escherichia coli*, and *Salmonella* spp.) was performed on each sample. Real-time PCR and matrix-assisted laser desorption-ionization time-of-flight mass spectrometer methods were employed for identification of presumptive pathogens. Coliform was detected in 25 samples (19.8%) with a minimum of 25 cfu/g and a maximum of more than 3.0×10^6 cfu/g. *Salmonella* spp. and *L. monocytogenes* were not isolated from any of the samples. Only one sample (0.80%) showed positive PCR amplification for *ipaH* gene suggesting possible contamination of enteroinvasive *E. coli* or *Shigella* in this product. Overall results indicate that natural cheeses produced in Hokkaido region were satisfactory microbiological quality according to existing international standards.

1. Introduction

Cheese consumption became popular in Japanese culinary system following the exposure of public to the western food cultures, which lead to substantial increase in domestic production and imports [1, 2]. Although the Japanese cheese market relies on imports by nearly 80%, domestic production of natural cheese is growing year by year. A recent estimate shows that over 50,000 metric tons of natural cheese is produced in Japan per year [2] out of which the majority comes from Hokkaido region. Production of natural cheese in this region accounts for more than 90% of the overall domestic production. The number of farm dairies producing natural cheese is doubled in a decade.

Consumption of cheese has been associated with foodborne outbreaks in reports from different parts of the world raising the safety concern of the product [3]. *Listeria monocytogenes* is a ubiquitous foodborne pathogen and human listeriosis outbreaks are often associated with ready-to-eat food products including cheese. Ingestion of foods contaminated with this pathogen results in a severe disease with higher fatality rate where certain risk groups including pregnant, newborn, elderly people, and immunocompromised patients are affected [4]. Several outbreaks and sporadic cases of disease associated with the consumption of pasteurized milk, cheeses made from unpasteurized milk, and other dairy products in USA and Europe in the past decades [3, 5–10]. Previous domestic studies from 1992 to 1994 reported that *L. monocytogenes* contamination was found in raw milk [11]. However, a foodborne listeriosis outbreak was occurred in 2001 due to contaminated natural cheese [12]. This is the first and only reported foodborne listeriosis in Japan so far.

In addition to *L. monocytogenes*, other enteric pathogens such as Shiga toxin-producing *Escherichia coli* (STEC) and *Salmonella* are also the causes of concern on public health in relation to the consumption of cheese worldwide [3, 5–10]. STEC is another important foodborne pathogens responsible for outbreaks which may result in hemorrhagic colitis (HC)

and lethal hemolytic uremic syndrome (HUS) [13]. Although most outbreaks of HC and HUS have been attributed to serotype O157:H7, infections are also caused by other serotypes, such as O26:H11, O103:H2, O111:H8, and O145:H28 [8, 10]. *Salmonella* is another important organism which represents well-recognized foodborne bacterial pathogens. It causes a number of illnesses and deaths worldwide.

Natural cheeses, regardless of their varying characteristics, can support the growth of microorganisms including foodborne pathogens. Given the continuous increase of natural cheese consumption and following earlier reported outbreak of *L. monocytogenes* in Hokkaido associated with cheese [12], there is concern for microbiological safety of domestically produced natural cheeses. The purpose of this study was to assess a hygienic quality and safety of natural cheeses produced in Hokkaido, Japan. We performed standard plate counts (SPC) and coliform counts on each sample to obtain general hygienic information. Since a survey targeting other important pathogens in domestic natural cheese has not been performed yet, the prevalence of three significant pathogens such as *Salmonella* spp., pathogenic *E. coli,* and *L. monocytogenes* was also examined in this study.

2. Materials and Methods

2.1. Sample Collection. A total of 126 domestic natural cheeses made from raw cows' milk were collected from local retail stores and farmers' markets in Hokkaido, Japan, from December 2012 to July 2013. Some samples were obtained from online source. Sampling was centered in the Eastern Hokkaido because many natural cheese producers are located in this area, and most of cheese samples used in this study were produced on farm-dairies. The samples included soft type ($n = 66$) those were mostly ripened, semihard ($n = 33$), and hard type ($n = 27$) of cheeses. Samples were stored at refrigeration temperature (4 to 8°C) during delivery to the laboratory. Cheese type, producing companies, expiry dates, and packaging were recorded and the samples were stored in refrigerator until examined within the shelf life.

2.2. Bacterial Count. The homogenates of samples were prepared by aseptically removing 25 g of cheese into sterile strainer/filter-stomaching bags (Filterbag type P, GSI Creos Corporation, Tokyo, Japan). A 225 mL of buffered peptone water (BPW) was also added into the stomaching bags containing the samples and homogeneously mixed in pulsed stomacher (AES Laboratoire, Combourg, France) twice for 30 sec. The sample homogenates were serially diluted with BPW. Each serial dilution of sample homogenates was pour-plated on SPC agar (OXOID, Basingstoke, UK) and Deoxycholate agar (MERCK and Eiken Chemical, Tokyo, Japan) for SPC and coliform, respectively, followed by incubation of the plates at 37°C for 48 h.

2.3. Detection of Pathogens. Isolation of *L. monocytogenes* was conducted as described in International Organization for Standardization ISO 11290-1 [14]. In brief, 25 g of samples were preenriched in 225 mL of half Frazer broth (OXOID) and incubated for 24 h at 30°C. From preenriched sample, 0.1 mL of culture was enriched in 10 mL of Fraser broth (OXOID) and incubated at 35°C for 48 h. Then, a loopful of the cultures were streaked on PALCAM agar (OXOID) and incubated at 37°C for 48 h. Typical or suspect colonies were picked up, streaked on BHI agar (Becton Dickinson, NJ, USA), and incubated at 37°C for 24 h for further identification.

Isolation of *Salmonella* spp. was carried out following the procedures indicated in US Food and Drug Administration (FDA) Bacteriological Analytical Manual Online [15]. Briefly, 25 g of samples was preenriched in 225 mL buffered peptone water (MERCK) and incubated at 35°C for 18 h. Next, 0.1 mL of each sample homogenate was enriched into 10 mL of Rappaport-Vassiliadis (RV) broth (OXOID) and incubated at 42°C for 18 h. Then, a loopful of RV culture was streaked on deoxycholate hydrogen sulfide lactose agar (DHL; Eiken Chemical) and CHROMagar *Salmonella* and incubated at 37°C for 24 h. Suspicious colonies were collected and streaked on BHI agar for further analysis by Matrix-assisted laser desorption-ionization time-of-flight mass spectrometer (MALDI-TOF MS).

Screening for pathogenic *E. coli* (STEC, enterotoxigenic *E. coli*, enteropathogenic *E. coli*, enteroaggregative *E. coli,* and enteroinvasive *E. coli*) was performed by real-time PCR based on their associated genetic markers. Briefly, 25 g of samples was preenriched in 225 mL of mEC broth with novobiocin (MERCK) and incubated at 37°C for 24 h. DNA was extracted from 2 mL of preenriched broth by using PrepMan Ultra Sample Preparation kit (Applied Biosystems, Foster City, CA, USA). Real-time PCR was performed using QuickPrimer kit (*stx 1*, *stx 2*, *ipaH*, *LT*, *EASTI*, *STI*) (Takara Bio, Shiga, Japan) according to the manufacturer's instruction. QuickPrimer Control DNA sets were used as positive control while DDW was used as negative control of the DNA amplification.

2.4. Identification of Bacteria by MALDI-TOF MS. MALDI-TOF MS analysis was used to identify presumptive isolates. Bacterial cells of single colony grown on BHI agar plates were transferred to a 96 well stainless steel target plate (Bruker Daltonik, Germany) using a disposable loop. The sample on the plate was then overlaid with 1 μL of α-Cyano-4-hydroxycinnamic acid and allowed to dry at room temperature. The plate was then subjected to MALDI-TOF Mass Spectrometer machine (autoflex-04S, Bruker Daltonik) and profile spectra were analyzed using MALDI Biotyper 2.0 software (Bruker Daltonik) according to the reference database.

3. Result

Amongst the total samples inspected (126 samples), 66, 33, and 27 of them were soft, semihard, and hard type of cheeses, respectively. All natural cheese samples in this study was already precut and prepacked individually at the time of purchase. Soft cheeses tested (66 samples) include brie, camembert, cream cheese, gorgonzola, mozzarella, and wash-type soft cheeses. Semi-hard (33 samples) and hard type

Table 1: Coliform-positive rates in the different types of cheese and packaging.

Cheese types	Package types (% of coliform positive)							Total
	AF/other[a]	Paper A[b]	Paper B[c]	Plastic A[d]	Plastic B[e]	Plastic C[f]	Can	
Soft	1/5 (20.0)	5/5 (100.0)	7/22 (31.8)	3/16 (18.8)	2/8 (25.0)	0/9 (0.0)	0/1 (0.0)	**18/66 (27.3)**
Semi-hard	0	0	0	3/32 (9.4)	0/1 (0)	0	0	**3/33 (9.1)**
Hard	0	0	0	4/27 (14.8)	0	0	0	**4/27 (14.8)**
Total	**1/5 (20.0)**	**5/5 (100.0)**	**7/22 (31.8)**	**10/75 (13.3)**	**2/9 (22.2)**	**0/9 (0.0)**	**0/1 (0.0)**	**25/126 (19.8)**

[a]Primary packaging: aluminum foil wrap, secondary: none, plastic bag or wooden box.
[b]Paper wrapping only.
[c]Primary packaging: paper, secondary: carbon box, wooden box or plastic bag.
[d]Plastic vacuum seal.
[e]Plastic film wrapping.
[f]Plastic container.

cheese (27 samples) samples include cheddar, caciocavallo, emmental, gouda gruyere, and raclette.

SPC of natural cheese tested in this study ranged from below detection limit (<10 cfu/g) to 6.4×10^6 CFU/g (Figure 1(a)). Five (4.0%) out of 126 samples were negative (<10 cfu/g) for viable aerobic bacteria. SPC counts are generally used for monitoring microbial quality and spoilage levels. However, fermented products like cheese generally show high number of SPC because "good" microorganisms present in food to ferment properly. High level of SPC in natural cheese samples seems to be natural, since some lactic bacteria and mold are known to grow on SPC agar.

No coliforms were detected in 80.2% of samples tested in this study, and 25 of cheese samples (19.8%) were found to be positive for coliform bacteria (Table 1). Coliform counts ranged from below detection limit (<10 cfu/g) to over detection limit (>3.0×10^6 CFU/g) (Figure 1(b)).

Soft type cheese showed the highest positivity of coliform compared to other cheese types. Out of the total 66 soft cheese samples, 18 samples (27.3%) were positive for coliform with minimum and maximum values of 2.8×10^1 and more than 3.0×10^6 CFU/g, respectively. On the other hand, from the 33 semi-hard types of cheese samples, 9.1% of them were positive for coliform with minimum and maximum values of 8.0×10^2 and 8.0×10^5 CFU/g, respectively. Among 27 samples of hard type cheese, four samples (14.8%) were positive for coliform count with minimum and maximum values of 2.5×10^1 and 2.7×10^4 CFU/g, respectively.

We further classified the type of packaging into 7 types such as AF/other (primary packaging: aluminum foil wrapping, secondary: none, plastic bag or wooden box), Paper A (paper wrapping only), Paper B (primary: paper, secondary: carbon box, wooden box or plastic bag), Plastic A (plastic vacuum seal), Plastic B (plastic film wrapping), Plastic C (plastic container), and Can (canned). Table 1 shows the relatedness between coliform contamination and cheese and packaging types. As shown in this Table, various packagings were applied for soft cheeses, whereas most of semi-hard and hard type cheeses were plastic vacuum sealed. Soft type cheeses wrapped with paper (Paper A in Table 1) showed the highest coliform-positive rate such as 100.0%. Although the number of samples were as low as 5, this is a notable difference compared to the other packagings. The prevalence of coliform

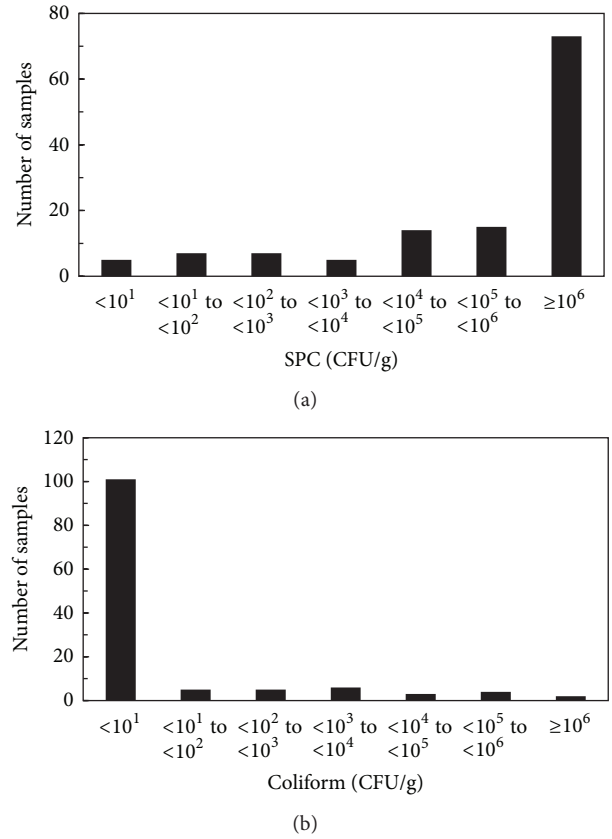

Figure 1: Hygienic quality of natural cheese samples. Distribution of SPC (a) and coliform counts (b) in natural cheese samples.

in soft cheeses that were paper-wrapped but also employed secondary outer packaging such as carbon box, wooden box, or plastic bags (Paper B in Table 1) showed lower contamination. Coliform-positive rate of plastic packaging including film wrapping (25.0%), vacuum seal (18.8%), and container (0.0%) were much lower compared to paper wrapping.

For detection of pathogens, we used standard analytical methods of ISO and FDA as well as Japanese method of Ministry of Health, Labor and Welfare (MHLW). For identification of presumptive isolates, we used MALDI-TOF MS analysis and real-time PCR analysis. The result showed that

among the 126 samples tested for *L. monocytogenes* none of them was positive for this pathogen. Likewise, no *Salmonella* spp. was detected from 126 natural cheese samples. However, under real-time PCR inspection, none of the 126 samples inspected was positive for pathogenic *E. coli*, except one sample (0.79%) that showed positive result for *ipaH* gene, indicating possible presence of EIEC or *Shigella* spp. [16]. However, we failed to isolate the pathogen from the cheese sample by culture-based detection method using specific media for *Shigella* spp.

4. Discussion

Despite the growing popularity of domestic natural cheeses among consumers, information about microbiological safety of these products is limited. The incidence of high coliform count in food is considered as indicator for reduced hygienic condition in production process. The results of this study shows that about 80.2% of the 126 natural cheese samples examined were negative for coliforms. Although microbiological criteria for *L. monocytogenes* in natural cheese (negative per 25 g) are described by the Ministry of Health, Labour and Welfare, other criteria, for example, coliform count, *E. coli,* and the other pathogenic bacteria, are not specified. However, it is a prerequisite to use pasteurized milk for natural cheese production in Japan. In case of using unpasteurized raw milk, producers must process products under equivalent temperature condition (63°C for 30 min) to eliminate the risk of pathogenic organisms (Ministry of Health, Labour and Welfare, 2003). Thus, coliform bacteria are not expected to exist due to the heating process in the production of natural cheese. Although milk pasteurization is regarded as an effective method to eliminate bacteria including foodborne pathogens, it is suggested that postpasteurization contamination and poor hygienic practices such as inappropriate pasteurization of raw milk and equipment also one of the causative factor in cheese-related foodborne illness [17].

The present study revealed significant correlation between packaging type and rate of coliform contamination in soft type cheese as a results of their packaging in paper with or without of secondary packaging (31.8% and 100.0% resp.), plastic packaging by film wrapping, vacuum or container (25.0%, 18/8% and 0.0% resp.), aluminum foil (20.0%) and canned (0.0%). The microbiological quality of natural cheese is influenced by equipment and environmental hygiene during production, packaging and handling and storage conditions as well as by the quality of raw milk. It was not determined in this study whether the highest coliform prevalence in paper-wrapped cheese is due to pre-packaging or post-packaging. However, our results clearly indicate that appropriate packaging such as plastic packaging and/or combination of primary and secondary packaging will reduce the risk of contamination. To choose an appropriate packaging that is suitable for the products is a simple and practical measure to reduce the risk of unnecessary contamination and ensure the food safety. We are planning to discuss about our data with the relevant cheese manufacturers whose products were observed in the high levels or continuous contamination of coliform during the survey for making a good hygiene practice plan and appropriate HACCP plan, by collecting and analyzing the data of coliform contamination levels in pre-and post production process including raw milk, pasteurization, factory environment such as utensils and equipment, storage and ripened shelf, and packaging materials, and so forth.

Most of the natural cheese samples tested in this study complied with the microbiological criteria by Hokkaido regional accreditation body, which is the food standard (criteria) uniquely given by Hokkaido Government. Since Hokkaido prefecture is the nation's first and largest area in the production of a wide array of agricultural, seafood and fresh dairy products including natural cheese (Spotlight on Hokkaido, NatureJobs article in 2011), ensuring food safety of the food products those made in Hokkaido region is the one of the important issues. Hokkaido regional accreditation requires natural cheese to be negative for coliform and *L. monocytogenes* in 25 g by the standard microbiological test (http://www.dairyfoodsconsulting.com/pdf/EU_cheese_safety_report.pdf). Similarly, the natural cheese samples tested in this study were within the limit of US ordinance for pasteurized grade "A" milk which states coliform should be lower than 10 cfu/mL [18]. Applying criteria in the European standard that requires coliform in cheese produced from heat-treated milk to be less than or equal to 10^5 cfu/g [16], 95.2% of samples inspected in this study were within this limit.

The prevalence of foodborne pathogens including *L. monocytogenes*, pathogenic *E. coli* (STEC, ETEC, EPEC, EAEC, and EIEC), and *Salmonella* spp. in natural cheese was investigated in this study. *L. monocytogenes*, STEC, and *Salmonella* are reportedly associated with foodborne outbreaks related to the consumption of cheese [3, 5–10]. Apart from the single one, all the other samples inspected had no prevalence of the foodborne pathogens such as *L. monocytogenes*, *Salmonella* spp., and pathogenic *E. coli*. The results of the present study also demonstrate that natural cheeses produced in Hokkaido are negative for these relatively frequent foodborne pathogens of the recent times. The *ipaH* gene detected from one sample (0.79%) in this survey indicates the possibility of contamination by EIEC or *Shigella*, because this gene is a multicopy gene which is exclusively found in those pathogens [19]. The source of contamination is uncertain, but fecally contaminated water and unsanitary handling by food handlers are the probable causes of contamination. Previous report of *S. sonnei* outbreak in Spain was associated with regionally manufactured fresh pasteurized milk cheese that caused large outbreak affecting over 200 people in the region [20]. Epidemiological investigation suggests that infected employee at the cheese factory might have been the source of contamination.

Several prevalence studies conducted across the globe on these pathogens in natural cheeses show varying results from place to place. No pathogens such as *E. coli* O157:H7, *L. monocytogenes*, *Salmonella*, or *Campylobacter* was detected in US study [21]. In UK study, *Salmonella* was not detected; however, 2% of samples were at unsatisfactory level of

Staphylococcus aureus, *E. coli,* and *L. monocytogenes*. Study in Italy showed that no *L. monocytogenes* was detected; however, high levels of *S. aureus* and *E. coli* were detected in particular samples [22]. In Spanish study, 2.4% of samples were positive for STEC [23]. In Peru, 7.8% of samples were positive for *E. coli* O157:H7 [23, 24]. From our results and the fact that outbreaks of food borne pathogens related to consumption of cheese are not common in Japan, natural cheese produced in Hokkaido seems to be in relatively better hygienic status as compared to those produced in other countries mentioned above.

The outbreaks of food borne pathogens related to consumption of dairy products are not common in Japan so far. Several reports predict that the market for domestically produced natural cheese will continue to grow from year to year [1, 2]. The average cheese consumption of the Japanese is about 2 kg a year per person, which is 10 times lower than those in European countries. This may account for lower incidence of foodborne outbreak associated with cheese. However, increase in consumption and production amounts may influence the risk of foodborne illness in future. Conducting similar survey in small and large scale on periodic basis would be important to ensure food safety of domestic natural cheese and to prevent possible incidence of foodborne pathogen outbreaks and related public health hazards.

In conclusion, the current result indicates that the microbiological quality and hygienic status of the natural cheese tested in this study was in the mostly fine and satisfactory status. This may be attributed to the required use of pasteurized milk for production of dairy products and implementation of good manufacturing and hygienic practices across all production stages from farm to table. The results from this study also suggest that current regulation and ministerial guidance would be considered appropriate for safe natural cheese production.

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Research Article

A New Protocol to Detect Multiple Foodborne Pathogens with PCR Dipstick DNA Chromatography after a Six-Hour Enrichment Culture in a Broad-Range Food Pathogen Enrichment Broth

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A quick foodborne pathogen screening method after six-hour enrichment culture with a broad-range food pathogen enrichment broth is described. Pathogenic factors of *Salmonella enterica*, *Shigella* spp., enteroinvasive *Escherichia coli*, and enterohemorrhagic *E. coli* are amplified with a cocktail primer and rapid polymerase chain reaction (PCR), which finishes amplification in 30 min. The PCR amplicon was differentiated with a dipstick DNA chromatography assay in 5–10 min. Starting from a four- to six-hour enrichment culture, this assay was finished within 45 min. Detection sensitivity of this protocol was less than 2.5 CFU/25 g for *S. enterica* and 3.3 CFU/25 g for enterohemorrhagic *E. coli* in spiked ground meat experiments.

1. Introduction

Infectious gastroenteritis is a leading cause of morbidity and mortality worldwide, particularly in developing countries [1]. Risk factors for infectious gastroenteritis include exposure to various contaminated food products [2]. Several methods to detect pathogens directly in food samples have been reported [3, 4]; however, most food analysis requires a 25 g food sample. Ideally, pathogen detection in food should be at the single-cell level [5].

Several methods based on polymerase chain reaction (PCR) have been developed to detect a single-cell pathogen from enrichment culture [6–8]. Cocktail PCR, carried out in a single PCR tube for simultaneous detection of more than one bacterial target, has been investigated as a more cost-effective and time-saving method [9, 10]. However, it is difficult for small food laboratories to use ethidium bromide-based agarose gel. On the other hand, real-time PCR assays employing various types of fluorescence systems allow multiple detection during PCR [11–13]. This is an excellent method, but it requires an expensive real-time thermal cycler and reagents. Thus, small laboratories cannot afford this real-time method.

Another aspect of food analysis is the analysis time. Fresh food products must arrive to the market quickly, but current culture-based protocols require several days to confirm that the products are pathogen-free. Confirmation that fresh food is safe before shipping is, therefore, desired, but difficult in practice.

To solve these problems, we developed a quick cocktail PCR and dipstick DNA chromatography to differentiate PCR amplicons for *Salmonella enterica*, *Shigella* spp., enteroinvasive *Escherichia coli* (EIEC), and enterohemorrhagic *E. coli* (EHEC) from a single enrichment culture broth. In our previous report, we described a food pathogen enrichment (FPE) broth that supports the growth of *Campylobacter* without adding lysed blood and carbon dioxide [14]. The method detected a few *Campylobacter* cells in 25 g of chicken within 24 hours and was better than the conventional Bolton-based enrichment culture.

In this report, we describe a new protocol to detect *S. enterica*, *Shigella* spp., EIEC, and EHEC from the FPE broth. DNA preparation from the FPE broth was simplified and the cocktail PCR was designed to finish within 30 min. The PCR amplicon was visually differentiated using dipstick DNA chromatography within 5–10 min.

2. Materials and Methods

2.1. Bacterial Strains. The bacterial strains of *S. enterica*, *Shigella* spp., EIEC, and EHEC and other strains are listed in Table 1. All strains were supplied from the Gifu Type Culture Collection of the Microbial Genetic Resource Stock Center, Gifu University Graduate School of Medicine (Gifu, Japan), supported by the Ministry of Education, Culture, Sports, Science, and Technology of Japan. All strains were cultured on heart infusion agars (BD, Tokyo, Japan) at 37°C under an aerobic atmosphere overnight. A fresh culture was used for each experiment.

2.2. Determination of Optimal Enrichment Culture with FPE Broth. FPE broth is designed to support the growth of *Campylobacter* species without blood and carbon dioxide [14]. This broth was used in the present study to simplify the total food analysis protocol because the FPE broth supported most food borne pathogens in our preliminary experiment. The growth of the foodborne pathogen in the FPE broth was compared with that in conventional selective enrichment broth (Figure 1). In the spiked ground meat experiment, diluted fresh bacterial solution and 25 g of beef were mixed in 225 mL of FPE broth and incubated at 37°C.

2.3. DNA Extraction. DNA was extracted from 1 mL of culture broth using a physical disruption method (MORA-EXTRACT, AMR, Gifu, Japan) according to the manufacturer's instructions, with a final DNA elution volume of 200–400 μL.

DNA extraction from FPE broth was performed using a simplified protocol. One milliliter of 2 to 18 hours enrichment culture was collected in a 2 mL Eppendorf tube and centrifuged at 12,000 g. The supernatant was completely removed and 1 mL of T10E1 buffer was added and centrifuged under the same conditions. After the complete removal of the supernatant, 200 μL of T10E1 buffer was added to the tube and mixed. The solution was transferred to a tube containing beads and physically disrupted for 1 min with a Disrupter Genie (Scientific Industry Inc., Bohemia, NY, USA). The tube was boiled at 100°C for 3 min. Five microliters of the solution were used for the cocktail PCR assay.

2.4. Cocktail PCR. The cocktail PCR conditions used in the present study are described below. The primers used are described in Table 2. PCR amplification was performed in 10 μL of reaction mixture containing 5 μL of 2× premix Ex *Taq* (Takara Bio, Shiga, Japan), 2.5 μL of primer mixture, 0.5 μL of distilled water, and 2 μL of DNA template. PCR was carried out using the QuickBath thermal cycler (ThermoGen Ltd., Nagano, Japan) under the following conditions: 95°C for 3 min, 40 cycles of 95°C for 10 s, and 65°C for 10 s. The PCR cycles finished within 30 min.

2.5. Dipstick DNA Chromatography. The 5 terminus of the cocktail PCR amplicon was labeled with biotin, and the 3 terminus was labeled with four different tags. Streptavidin-coated blue latex, kindly provided from Fujikura (Saitama, Japan), bound the biotinylated 5 terminal amplicon and the tagged 3 terminus was bound on the antitag lines printed on the DNA strip (Figure 2).

After PCR, 30 μL of streptavidin-coated blue latex solution was added to the PCR tube, and then the DNA strip was inserted into the PCR tube. The hybridized PCR amplicon was visualized in 5 to 10 min as a blue line, which represented the bound streptavidin-coated blue latex and biotin-labeled 5 terminus of the PCR amplicon.

2.6. A Protocol to Detect Multiple Foodborne Pathogens after 6 Hours Enrichment Culture of Ground Meat. For detection of foodborne pathogens with our protocol, 25 g of beef and 225 mL of FPE broth were homogenized in Stomacher bags (Eiken Chemical Co., Tokyo, Japan). The entire homogenate was transferred to a culture bottle and incubated with shaking at 37°C. After incubation, 1 mL of the supernatant was collected, and DNA was extracted using the physical disruption method described above. Subsequently, 5 μL of the extracted DNA was analyzed by cocktail PCR primers (Table 2) using premix Ex *Taq* (Takara Bio, Shiga, Japan) and the QuickBath thermal cycler. After 30 min, the PCR amplicon was analyzed by dipstick DNA chromatography. Thirty microliters of streptavidin-coated blue latex solution were added to each tube. Subsequently, the DNA strip was inserted into each tube. After 5–10 min at room temperature, the amplicon bound on the appropriate line on the dipstick surface (Figure 2) was visualized by the blue latex of the biotin-labeled 5 terminus of the PCR amplicon.

2.7. Sensitivity and Specificity of the Cocktail PCR Dipstick DNA Chromatography (CPDC) Assay. To measure the sensitivity of the CPDC assay, purified chromosomal DNA of *E. coli* O157 GTC14510 and *S. enterica* serovar Enteritidis GTC03838 were prepared at six different concentrations (2 ng, 200 pg, 20 pg, 2 pg, 200 fg, and 20 fg) and assayed (Table 3). Another sensitivity assay starting from quantitatively diluted culture supernatants was also performed (Table 4). The specificity of the CPDC assay was determined using the 176 strains listed in Table 5.

Table 1: List of bacterial strains used in this study.

Bacteria	Serotype	Toxin	Strains	Number of strain
EHEC (Enterohemorrhagic *E. coli*)	O26: H-	Shiga toxin 1	GTC14538, GTC14548, GTC14605, GTC14606	4
	O26: H11	Shiga toxin 1	GTC14516, GTC14540, GTC14549, GTC14557, GTC14558	5
		Shiga toxin 1 and 2	GTC14515, GTC14539, GTC14567	3
	O111: H-	Shiga toxin 1	GTC14517, GTC14528, GTC14541	3
		Shiga toxin 1 and 2	GTC14508, GTC14582	2
	O115: H10	Shiga toxin 1	GTC14518	1
	O119: H2	Shiga toxin 1	GTC14529	1
	O121: H19	Shiga toxin 2	GTC14530, GTC14577, GTC14601, GTC14602	4
	O128: H-	Shiga toxin 1 and 2	GTC14603	1
	O157: H7	Shiga toxin 2	GTC14513, GTC14514,GTC14524, GTC14525, GTC14537 GTC14546, GTC14547, GTC14550, GTC14553, GTC14560	10
		Shiga toxin 1 and 2	GTC14510, GTC14511, GTC14512, GTC14521, GTC14535 GTC14536, GTC14544, GTC14545, GTC14551, GTC14552,	10
	O157: H-	Shiga toxin 1 and 2	GTC14507, GTC14520, GTC14530, GTC14543, GTC14555 GTC14556, GTC14566, GTC14571, GTC14587, GTC14588	10
EIEC (Enteroinvasive *E. coli)*	O28: H-		GTC14240, GTC14243, GTC14251, GTC14259, GTC14260	5
	O124: H-		GTC14241, GTC14242, GTC14245, GTC14262	4
	O136: H-		GTC13248, GTC14254	2
	O144: H-		GTC14249, GTC14252, GTC14256	3
	O164:H-		GTC14244, GTC14246, GTC14247,	3
Salmonella enterica				
subsp.*enterica*	serovar Typhimurium		GTC02557, GTC02561, GTC02562, GTC02563, GTC02564 GTC02571, GTC02572, GTC-2573, GTC02574, GTC02575	10
subsp.*enterica*	serovar Enteritidis		GTC03838, GTC00131, GTC02377, GTC02382, GTC02387 GTC02389, GTC02390	7
subsp.*enterica*	serovar Dublin		GTC13214, GTC13215, GTC13216, GTC13217,GTC13218 GTC13219, GTC13220, GTC13221. GTC02558, GTC02560	10
subsp.*enterica*	serovar Typhi		GTC3P001, GTC3P074,GTC3P076, GTC3P081, GTC3P085 GTC3P087, GTC3P091, GTC3P095, GTC3P100, GTC3P106	10
subsp.*enterica*	serovar ParaTyphi A		GTC3P002, GTC3P082, GTC3P083	3
Salmonella bongori			GTC03793T	1
Shigella boydii			GTC00779T, GTC01912, GTC01913, GTC01914, GTC01915	8
Shigella dysenteriae			GTC01915, GTC01916, GTC01917, GTC01057T, GTC00786, GTC01929, GTC01930, GTC14808 GTC14809, GTC14810, GTC14811, GTC14812, GTC14813, GTC14814, GTC14815, GTC14816, GTC14817, GTC14818, GTC14819, GTC14820,	17
Shigella flexneri			GTC 0780T, GTC01918, GTC01920, GTC02007, GTC02008 GTC02009, GTC02010, GTC02011, GTC02012, GTC02013, GTC02015, GTC02016, GTC02014,	13
Shigella sonnei			GTC00781T, GTC01909, GTC01910, GTC01911, GTC01931 GTC01932, GTC01933,	7
Escherichia coli			GTC00503 T	1

Table 1: Continued.

Bacteria	Serotype	Toxin	Strains	Number of strain
Escherichia albertii			GTC 16441T	1
Escherichia fergusonii			GTC 01720T	1
Escherichia vulneris			GTC 10613T	1
Escherichia hermannii			GTC 10612T	1
Escherichia blattae			GTC 01342T	1
Citrobacter freundii			GTC 14890T	1
Citrobacter diversus			GTC 00114T	1
Citrobacter rodentium			GTC 14911T	1
Citrobacter youngae			GTC 14914T	1
Klebsiella pneumoniae			GTC 14868T	1
Enterobacter cloacae			GTC 00109T	1
Enterobacter aerogenes			GTC 14962T	1
Cronobacter sakazakii			GTC 14952T	1
Serratia marcescens			GTC 14672	1
Yersinia enterocolitica			GTC 00127T	1
Pseudomonas aeruginosa			GTC 00002T	1
Vibrio parahaemolyiticus			GTC 02055	1
Staphylococcus aureus			GTC 00286T	1

GTC is the Gifu Type Culture Collection supported by the National Bioresource Project (NBRP: http://www.nbrp.jp/) of the Ministry of Education, Culture, Sports, Science,and Technology.
"T" after the strain number means type strain.

Table 2: Cocktail primer list.

Pathogen	Targeted gene	Primer name	Sequence (5′-3′)
EHEC	*stx1*	Forward *stx1*	Biotin-ACAGGATTTGTTAACAGGAC
		Reverse *stx1*	Tag1-TCTGTATTTGCCGAAAACGT
	stx2	Forward *stx*2	Biotin-GATACAGAGAGAATTTCGTC
		Reverse *stx*2	Tag1-GCCAGTTATCTGACATTCTG
Shigella spp. and EIEC	*ipaH*	Forward *ipaHF*	Biotin-CTCGCAGAGAAACTTCAGCTCT
		Reverse *ipaHR*	Tag2-TTCTCTTCACGGCTTCTGACCAT
Salmonella spp.	*invA*	Forward *invA*	Biotin-TGACAGAATCCTCAGTTTTTCA
		Reverse *invA*	Tag3-AGATAAGACGGCTGGTACTGAT
Internal control		Forward IPC	Biotin-ACTCTTCCTAGCAGGATCCCTCTAAG
		Reverse IPC	Tag4-GCAATTCTAATACGACTCACTATAGG

Table 3: Detection sensitivity of CPDC assay.

DNA concentration	EHEC serovar O111, GTC14517 (*stx*1)	EHEC serovar O157, GTC14513 (*stx*2)	*Shigella dysenteriae* serovar 2, GTC01929 (*ipaH*)	*Salmonella enterica* serovar Enteritidis GTC03838 (*invA*)
2 ng/assay	+	+	+	+
200 pg/assay	+	+	+	+
20 pg/assay	+	+	+	+
2 pg/assay	+	+	+	+
200 fg/assay	+	+	+	+
20 fg/assay	−	−	−	−

Serially diluted purified DNA of each strain was used to count detection sensitivity.

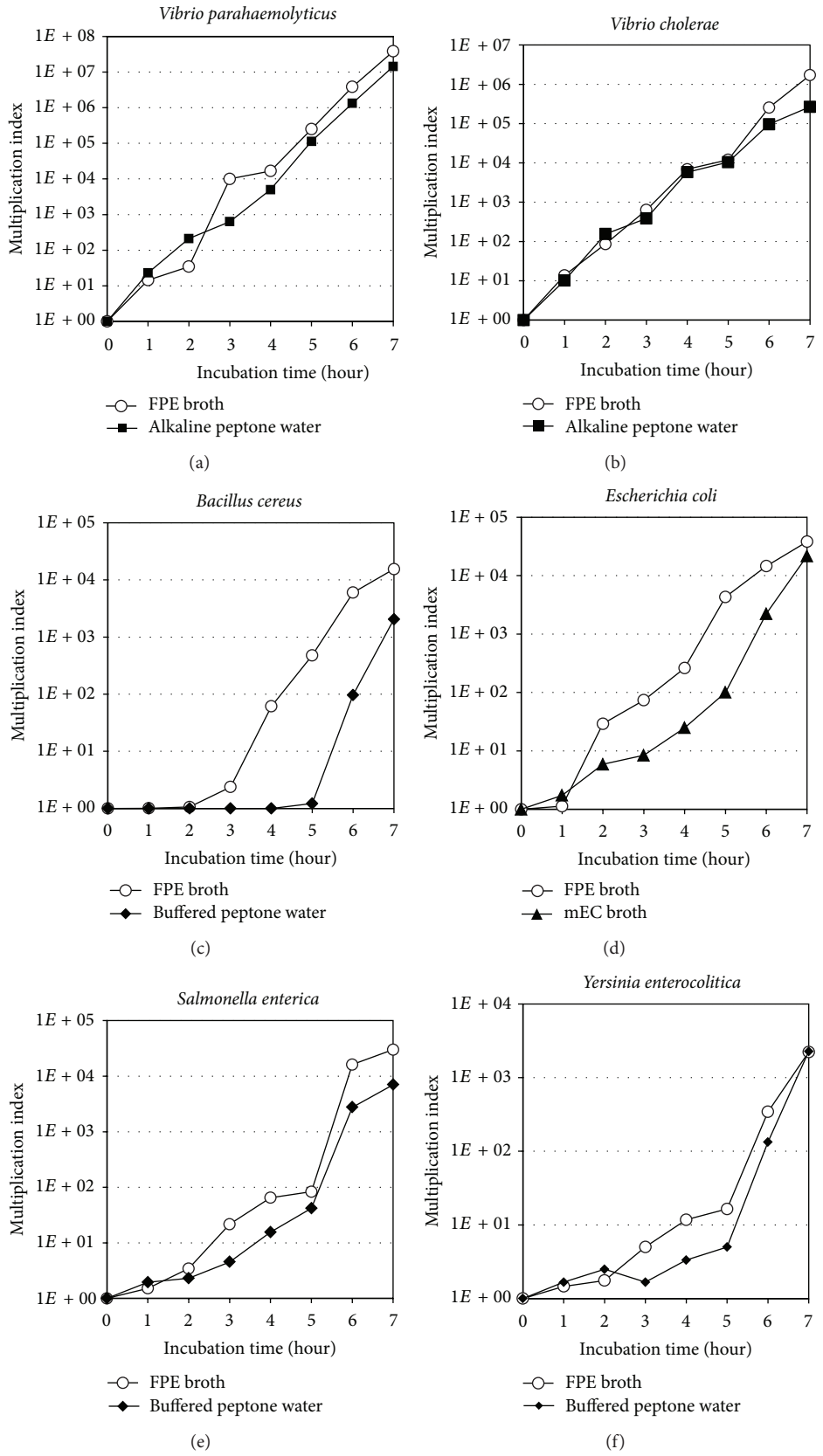

Figure 1: Growth of reference strains in FPE broth and established reference media. The initial number of bacteria was defined as one and the multiplication number is indicated on the y-axis. The x-axis represents incubation time.

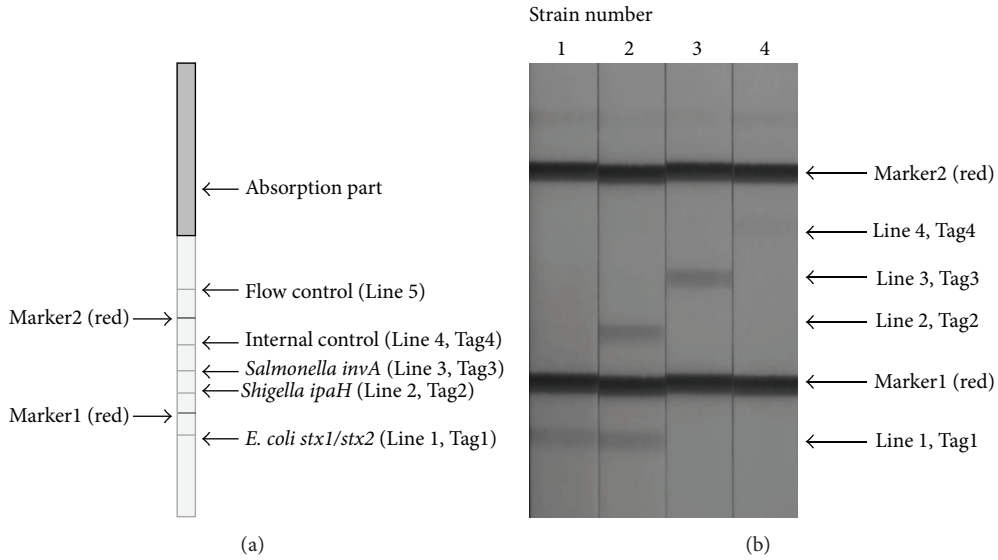

Figure 2: DNA strip for chromatography (a) and reaction results (b) for three pathogens and a negative control. (a) Line 1 (Tag1): EHEC, Line 2 (Tag2): *Shigella* spp. and EIEC, Line 3 (Tag3): *Salmonella* spp., Line 4 (Tag4): internal control, Line 5: flow control. (b) Strains: no. 1 *Eschesrichia coli* O157 (shiga toxin 1 and 2) GTC14510; no. 2 *Shigella dysenteriae* O1 (shiga toxin 1 and IpaH) GTC01057; no. 3 *Salmonella enterica* serovar Enteritidis GTC03838; and no. 4 internal positive control. All of amplicons are reacted with each tag.

Table 4: Detection sensitivity of CPDC assay and immunochromatography.

Bacterial concentration (CFU/mL)	EHEC O157 GTC14510		Bacterial concentration (CFU/mL)	*Salmonella enterica* serovar Enteritidis GTC03838	
	Immunochromat.	CPDC assay		Immunochromat.	CPDC assay
3.3×10^9	+	+	2.5×10^9	+	+
3.3×10^8	+	+	2.5×10^8	+	+
3.3×10^7	+	+	2.5×10^7	+	+
3.3×10^6	+	+	2.5×10^6	+	+
3.3×10^5	+	+	2.5×10^5	+	+
3.3×10^4	−	+	2.5×10^4	−	+
3.3×10^3	−	+	2.5×10^3	−	+
3.3×10^2	−	−	2.5×10^2	−	−
3.3×10	−	−	2.5×10	−	−
3.3	−	−	2.5	−	−

2.8. Evaluation of CPDC Assay with Spiked Ground Meat. The CPDC assay after enrichment culture in FPE broth was compared with a commercial immunochromatography system (Wako Pure Chem. Industries, Ltd., Osaka, Japan). Ground beef collected from local supermarkets was immediately transported to our laboratory in an insulated cooler box at 4°C. However, the isolation frequency of the target *Shigella* spp., *Salmonella enterica*, and *E. coli* O157 : H7 was less than 0.1% by culture-based conventional methods in our preliminary experiments. We decided, therefore, to evaluate the CPDC assay with spiked ground meat experiments. Ground meat was collected from a supermarket and confirmed target to be pathogen free by conventional methods. One milliliter of a mixed culture containing *Shigella dysenteriae*, *S. enterica* subspecies *enterica* serovar Enteritidis, and *E. coli* O157:H7 at three different concentrations were mixed with 25 g of ground meat, and 225 mL of FPE broth was then added. The total volume was incubated at 37°C. Immediately, and 4 hours, 6 hours, 8 hours, and 18 hours, 1 mL of the enrichment was used for extraction and then used in the CPDC assay. Another aliquot was used for the commercial immunochromatography kit for *S. enterica* serovar Enteritidis and *E. coli* O157.

3. Results and Discussion

Conventional culture has been the "gold standard" method for the detection of enteric bacterial pathogens. The advantages of this method include identification, facilitation of outbreak management, and generation of an antimicrobial susceptibility profile [15]. However, this conventional method has many disadvantages. Many different enrichment broths and solid media are used to screen for all possible foodborne

Table 5: Specificity of CPDC assay.

Bacteria	Serotype (Virulence factor)	Reacted Dipstick line	CPDC assay (positive/strains)
EHEC	O26: H- (*stx*1)	Line 1	4/4
	O26: H11(*stx*1)	Line 1	5/5
	O26: H11(*stx*1/2)	Line 1	3/3
	O111: H- (*stx*1)	Line 1	3/3
	O111: H- (*stx*1/2)	Line 1	2/2
	O115: H10(*stx*1)	Line 1	1/1
	O119: H2(*stx*1)	Line 1	1/1
	O121: H19(*stx*2)	Line 1	4/4
	O128: H- (*stx*1/2)	Line 1	1/1
	O157: H7(*stx*2)	Line 1	10/10
	O157: H7(*stx*1/2)	Line 1	10/10
	O157: H-(*stx*1/2)	Line 1	10/10
EIEC	O28: H- (*IpaH*)	Line 2	5/5
	O124: H- (*IpaH*)	Line 2	4/4
	O136: H- (*IpaH*)	Line 2	10/10
	O144: H- (*IpaH*)	Line 2	3/3
	O164:H- (*IpaH*)	Line 2	3/3
Shigella boydii	(*IpaH*)	Line 2	8/8
Shigella dysenteriae	(*IpaH*)	Line 2	16/16
Shigella flexneri	(*IpaH*)	Line 2	13/13
Shigella sonnei	(*IpaH*)	Line 2	7/7
Salmonella enterica			
subsp.*enterica*	serovar Typhimurium (*InvA*)	Line 3	10/10
subsp.*enterica*	serovar Typhi (*InvA*)	Line 3	10/10
subsp.*enterica*	serovar Enteritidis (*InvA*)	Line 3	7/7
subsp.*enterica*	serovar Dublin (*InvA*)	Line 3	10/10
subsp.*enterica*	serovar paratyphi A (*InvA*)	Line 3	3/3
*Salmonella bongori*GTC 03793 T	(*InvA*)	Line 3	1/1
Escherichia coli GTC00503 T		N*	0/1
*Escherichia albertii*GTC 16441 T		N	0/1
*Escherichia fergusonii*GTC 01720 T		N	0/1
Escherichia vulneris GTC 10613 T		N	0/1
*Escherichia hermannii*GTC 10612 T		N	0/1
*Escherichia blattae*GTC 01342 T		N	0/1
*Citrobacter freundii*GTC 14890 T		N	0/1
*Citrobacter diversus*GTC 00114 T		N	0/1
Citrobacter rodentium GTC 14911 T		N	0/1
*Citrobacter youngae*GTC 14914		N	0/1
Klebsiella pneumonia GTC 14868 T		N	0/1
*Enterobacter cloacae*GTC 00109 T		N	0/1
*Enterobacter aerogenes*GTC 14962 T		N	0/1
*Cronobacter sakazakii*GTC 14952 T		N	0/1
*Serratia marcescens*GTC 14672		N	0/1
*Yersinia enterocolitica*GTC 00127 T		N	0/1
Vibrio parahaemolyticus GTC02055		N	0/1
*Pseudomonas aeruginosa*GTC 00002 T		N	0/1
*Staphylococcus aureus*GTC 00286 T		N	0/1

N*: no positive line.
Reacted line no. 1 is *stx*1 and 2 for EHEC, line 2 is *ipaH* for *Shigella* spp. and EIEC, and line 3 is for *invA* for *Salmonella*.

Table 6: Result of CPDC assay in spiked ground meat samples.

Inoculated level (CFU/25 g)	CPDC assay Enrichment time (h)					Immunochromatography Enrichment time (h)				
	0	4	6	8	18	0	4	6	8	18
	Enterohemorrhagic *E. coli* O157 GTC14510									
13.3	−	−	+	+	+	−	−	−	−	+
6.7	−	−	+	+	+	−	−	−	−	+
3.3	−	−	+	+	+	−	−	−	−	+
Control	−	−	−	−	−	−	−	−	−	−
	Salmonella enterica serovar Enteritidis GTC03838									
10.0	−	+	+	+	+	−	−	−	−	+
5.0	−	+	+	+	+	−	−	−	−	+
2.5	−	+	+	+	+	−	−	−	−	+
Control	−	−	−	−	−	−	−	−	−	−

pathogens, and time-consuming protocols are prepared to generate a result. FPE broth is designed to support *Campylobacter* without adding lysed blood and carbon dioxide. *Campylobacter*, however, is a slow-growing organism and needs 24 hours to reach 10^4 CFU/mL. Therefore, addition of selective antibiotics to the FPE broth was essential to suppress contaminating other bacteria for 24 hours enrichment culture. FPE broth could also support the growth of *Listeria* without adding blood, but the growth of *Listeria* is also slow, needing 24 hours to reach 10^4 CFU/mL (unpublished data).

The growth of pathogens in conventional enrichment culture and FPE broths were measured (Figure 1). Approximately 1–10 bacteria were spiked in 225 mL of enrichment broth. *S. enterica* and *E. coli* reached 10^4 CFU/mL after 6-hours incubation in FPE broth, buffered peptone water broth, and mEC broth, as shown in Figure 1. *V. parahaemolyticus* reached 10^4 CFU/mL after 4-hours incubation in both FPE and alkaline peptone broths. Based on these data, we selected 6 hours incubation for the CPDC assay.

In the present study, cocktail PCR was capable of simultaneously determining the presence of *Salmonella* spp., *Shigella* spp., EIEC, and EHEC by targeting *invA*, *ipaH*, *stx1*, and *stx2* genes (Figure 2).

To evaluate the detection limit of the CPDC assay for each pathogen, 2 ng to 20 fg of DNA per reaction was prepared. The sensitivity and specificity of this assay are shown in Tables 3–5. The detection limit was 200 fg for each pathogen per CPDC assay (Table 3). The presence of the products with expected sizes was also confirmed by agarose gel electrophoresis, and nonspecific products were not observed (data not shown). The specificity of this CPDC assay was evaluated using 157 target strains (45 strains of *Shigella* spp., 54 strains of EHEC, 17 strains of EIEC, and 41 strains of *Salmonella* spp.) and 19 nontarget strains shown in Table 5. The detection limit of *Salmonella* and *Escherichia* from FPE culture supernatant (Table 4) was 10^3 CFU/mL, while the commercial immunochromatography kit required 10^5 CFU/mL. No false positive lines appeared on the dipstick DNA chromatography for any of the nontarget strains.

Immunochromatography is a simple technology to detect antigen in culture supernatant. However, immunochromatography targeting EHEC serotypes is not useful for testing food, because many kinds of *E. coli* serotypes produce shiga toxins. Thus, it is practically difficult to cover all of the EHEC serotypes by immunochromatography. Our method targeted both shiga toxin 1 and shiga toxin 2 toxins and detected non-O157 shiga toxin-producing serotypes (O26, O111, and O121). Three serotype (O45, O103, and O145) strains were not used because they are not available from our collection.

Shiga toxin 1 and shiga toxin 2 PCR products were designed to be bound on line 1 of the dipstick (Figure 2). The two genes were not equally amplified. The signal of the shiga toxin 2 amplicon was always bigger than the signal of the shiga toxin 1 amplicon.

The invasion plasmid antigen H (*ipaH*) gene is often used to diagnosis dysentery [16], because *ipaH* is carried by all four *Shigella* species as well as EIEC. In our cocktail primer, therefore, we selected the *ipaH*-specific primer to detect both *Shigella* and EIEC. The *ipaH* amplicon was designed to react on the second line of the dipstick chromatography strip, as shown in Figure 2. The CPDC assay was found to be effective at detecting *Shigella* species and EIEC from 4 to 6 hours FPE broth.

The CPDC assay required 4 to 6 hours FPE broth for the detection of *S. enterica* serovar Enteritidis. The detection limit of chromatography for *Salmonella* was, however, 10^5 CFU/mL in culture supernatant. To reach this cell number in FPE broth, incubation for more than 6 hours was necessary (Table 6). There is another disadvantage to using immunochromatography. The commercially available immunochromatography kits for *Salmonella* serovars are limited. The products only detect serovar Enteritidis. Thus, it is difficult to screen many different *Salmonella* serotypes simultaneously, such as serovar Typhimurium, serovar Choleraesuis, serovar Dublin, serovar Typhi, and others.

The CPDC assay for *Salmonella* was evaluated by spiked ground meat experiments with three different inoculation levels from 2.5 to 10 CFU/25 g (Table 6). The CPDC assay

detected target pathogens on the third line of a dipstick DNA strip from 4 to 6 hours culture with FPE broth. On the other hand, the commercial immunochromatography kit only detected antigens from 18 hours enrichment culture because the method requires 10^5 CFU/mL of organism (Tables 4 and 6).

Multiplex PCR to detect many *Salmonella* serovars has been reported [17, 18]; however, we selected the *invA* gene for our assay because all *Salmonella* serovars carry this gene [19].

Internal amplification control (IAC) is designed to bind to line 4 of the dipstick chromatography strip to check for the presence of PCR inhibitor and false negatives [20]. A general guideline proposed for PCR testing of foodborne pathogens also requires the presence of IAC in the reaction mixture [21].

Systematic review of clinical implications, public health considerations, and the cost effectiveness of rapid diagnostic tests for detection and identification of bacterial pathogens in feces and food suggests that adoption of rapid test methods, especially for PCR, in combination with a routine culture is unlikely to be cost-effective [7, 22]. However, as the cost of rapid technologies decreases, total replacement with rapid technologies may be feasible.

The clinical impact of the decreased turnaround time means that bacterial diarrhea is more promptly ruled out using the CPDC assay compared to using conventional culture in small laboratories. This reduces the expenditure of infection control resources and, in particular, in cases of sporadic diarrhea, helps to reduce the requirement for scarce isolation rooms. In addition, the earlier availability of results is helpful in community-based management of outbreaks.

For detection of *Salmonella* spp., *Shigella* spp., EIEC, and EHEC, the overall time to confirm a positive result by conventional culture plus immunochromatography is at least two working days. Generating a negative report requires 48 hours. In contrast, a report can be generated for the CPDC assay within one working day. One advantage of an early laboratory report is early judgment of contamination, which can prevent food poisoning and additional outbreaks. The method also contributes to the quick shipment of fresh food products to the markets.

4. Conclusion

Cocktail PCR targeting multiple foodborne pathogens and simple dipstick DNA chromatography to differentiate the PCR product was designed. The method was applied to detect pathogens in ground meat after 6 hours enrichment broth, which supports the growth of broad range foodborne pathogens. This single tube PCR and enrichment method help to simplify food analysis protocol. As a result, the method offers rapid report to food suppliers and helps the quick shipment of safety-confirmed food products to markets.

Acknowledgments

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Research Article

Development of an Immunochromatographic Test Strip for Detection of Cholera Toxin

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Because cholera toxin (CT) is responsible for most of the symptoms induced by *Vibrio cholerae* infection, detection of CT is critical for diagnosis of the disease. In this study, we constructed an immunochromatographic test strip for detection of CT (CT-IC) with polyclonal antibodies developed against purified recombinant whole CT protein. The detection limit of the CT-IC was 10 ng/mL of purified recombinant CT, and it could detect the CT in culture supernatant of all 15 toxigenic *V. cholerae* isolates examined, whereas no false-positive signal was detected in all 5 nontoxigenic *V. cholerae* isolates examined. The specificity of the CT-IC was examined with recombinant heat-labile toxin (LT), which shares high homology with CT, and it was revealed that the minimum detection limit for LT was 100 times higher than that for CT. In addition, *lt* gene-positive enterotoxigenic *Escherichia coli* (ETEC) was examined by CT-IC. The false-positive signals were observed in 3 out of 12 ETEC isolates, but these signals were considerably faint. The CT-IC did not develop false-positive signals with all 7 *V. parahaemolyticus* isolates. These results showed the high specificity of CT-IC and the feasible use of it for the detection and surveillance of toxigenic *V. cholerae*.

1. Introduction

Cholera remains a major public health problem especially in developing countries. The seventh pandemic of cholera which began in 1961 is still ongoing. In recent years, cholera cases have steadily increased. In 2011, the cholera cases reported to WHO were from 58 countries and accounted for 589,854 cases including 7,816 deaths [1]. The most recent epidemic is striking in Sierra Leone where over 20,000 cases including 280 deaths had been reported before October 2012. Furthermore, the actual number of cholera cases is assumed to be much higher than those reported. This discrepancy is attributed to the lack of dissemination of effective surveillance system. Because underreporting or underestimation impedes implementation of sufficient control measures, further improvement in surveillance system, which contributes largely to determine the true number of incidences, is still required.

Currently, several diagnostic procedures including the "gold standard" of culture test and rapid diagnostic tests are available for *V. cholerae* detection [2]. In culture test, alkaline peptone water (APW) and TCBS are commonly used as enrichment and selective media, respectively. As many have noted, the cultivation test is time-consuming, but it has the advantage of being able to isolate the causative bacterium which can be used for further characterization. On the other hand, utility of various rapid diagnostic tests such as polymerase chain reaction (PCR), quantitative PCR (qPCR), loop-mediated isothermal amplification (LAMP), enzyme-linked immunosorbent assay (ELISA), reverse passive latex agglutination test (RPLA), and immunochromatographic test (IC) has been demonstrated. These rapid methods facilitate timely, in some cases, on-site responses. And, the rapid detections in early stage of epidemic allow quick triggering of control measures. In the case of diagnosis of cholera, after or

Table 1: Profiles of *V. cholerae* strains used in this study.

Year of isolation	Isolated from*	Serotype	*ct* gene
1993	Traveler's diarrhea case (India)	O1 El Tol Ogawa	+
1993	Traveler's diarrhea case (Philippines)	O1 El Tol Ogawa	+
1995	Traveler's diarrhea case (China)	O1 El Tol Ogawa	+
1996	Traveler's diarrhea case (Thailand)	O1 El Tol Ogawa	+
1996	Traveler's diarrhea case (Thailand)	O1 El Tol Ogawa	+
1996	Traveler's diarrhea case (Thailand)	O1 El Tol Ogawa	+
2006	Cholera patient lacking detailed information	O1 El Tol Ogawa	+
1997	Domestic case	O1 El Tol Inaba	+
2000	Traveler's diarrhea case (Thailand)	O1 El Tol Inaba	+
2001	Traveler's diarrhea case (China)	O1 El Tol Inaba	+
2004	Traveler's diarrhea case (Thailand)	O1 El Tol Inaba	+
2007	Traveler's diarrhea case (India)	O1 El Tol Inaba	+
2007	Traveler's diarrhea case (India)	O1 El Tol Inaba	+
1993	Traveler's diarrhea case (India)	O139	+
2004	Domestic case	O141	+
1998	Traveler's diarrhea case (Thailand)	O1 El Tol Ogawa	−
1999	Traveler's diarrhea case (Egypt, Greece)	O1 El Tol Ogawa	−
1997	Traveler's diarrhea case (Iran)	O1 El Tol Inaba	−
2001	Domestic case	O1 El Tol Inaba	−
2003	Cholera patient lacking detailed information	O139	−

*The places where the traveler's diarrhea patients were staying are indicated in brackets.

along with the detection of bacterium, verification of cholera toxin (CT) production is required because only the *V. cholerae* which can produce CT is responsible for cholera symptoms such as acute "rice water" diarrhea. Some detection methods for toxigenic *V. cholerae* have been described previously. The approaches to assay for CT can be divided in terms of features to be detected: (1) bioassay including rabbit ileal loop test, rabbit skin test, and cultured CHO cell assay, (2) immunoassay including ELISA and RPLA, and (3) DNA-based assay including PCR, qPCR, DNA hybridization, and LAMP [3, 4]. Combined use of more than one detection method would be required to increase the accuracy of a diagnosis. At that time, combination of different target analytes; for example, immunoassay which detects the existence of toxin and DNA-based assay which detects the existence of toxin-coding DNA must be chosen.

While DNA-based assays may be more sensitive than immunoassays, the latter has an important advantage in the detection of extracellular bacterial toxin. Recently, some new methodology of immunoassay with extremely high sensitivity has been reported [5, 6]. However, IC is still one of the most commonly utilized immunoassays because it is rapid and very easy to conduct. In the present study, we constructed IC for CT detection (CT-IC). To increase sensitivity, we used the polyclonal antibodies established against whole toxin which contains both A (active) and B (binding) subunits. We demonstrated that the constructed CT-IC could detect CT in *V. cholerae* culture in which more than 10 ng/mL of CT was expressed.

2. Materials and Methods

2.1. Bacterial Strains. Fifteen *ct* gene-positive *V. cholerae* isolates (7 O1 El Tor Ogawa, 6 O1 El Tor Inaba, and 1 each of O139 and O141) and 5 *ct* gene-negative *V. cholerae* strains (2 each of O1 El Tor Ogawa and O1 El Tor Inaba and 1 of O139) were kindly provided by Saitama Institute of Public Health, Saitama, Japan. These strains were isolated in Japan from 1993 to 2007. Each strain was individually isolated from diarrhea patients including 15 traveler's diarrhea patients who traveled to India, Philippines, China, Thailand, Egypt, Greece, or Iran, and 3 patients who are infected domestically, and 2 patients lacking detailed information (Table 1). Twelve enterotoxigenic *E. coli* isolates were laboratory stock strains which were isolated from stools of diarrhea patients in India. Seven *V. parahaemolyticus* strains were the strains isolated from food samples or patients in Hokkaido, Japan.

2.2. Preparation of Purified CT and LT. The laboratory stocks of anti-CT antiserum and anti-LT antiserum were used for preparation of anti-CT IgG conjugated column and anti-LT IgG conjugated column. These antisera stocks were prepared according to a protocol previously described [7] by using purified recombinant CT and LT which were purified as described in a previous report [8] as an antigen. For antiserum conjugated column preparation, the laboratory stocks of antisera were coupled to HiTrap NHS-activated HP (5 mL, Amersham Biosciences) according to the manufacture's instructions.

For CT and LT purification, previously constructed *E. coli* MC1016 (pKTJ5-15x) strain and *E. coli* HB101 (pKTN1003b) strain in which recombinant CT and LT were overexpressed respectively [8], were inoculated into LB broth supplemented with ampicillin (50 μg/mL). After overnight cultivation at 37°C, the bacterial cells were collected by centrifugation, resuspended in PBS, and then disrupted by sonication. The obtained cell sonicates were centrifuged to obtain soluble fractions. Proteins in the soluble fractions were precipitated with 80% $AmSO_4$, resuspended in PBS, treated with RNase and DNase, and then applied to anti-CT antisera conjugated column and anti-LT antisera conjugated column. After the columns were washed with PBS, recombinant CT and LT proteins were eluted with 0.1 M glycine-HCl, pH3.0. After dialysis, purified samples were condensed by using of Centricon YM-10 (Merck Millipore). Purities of the samples were analyzed by SDS-PAGE and CBB staining. Concentrations of the purified samples were determined by Bio-Rad Protein Assay Dye Reagent Concentrate (Bio-Rad) by using bovine serum albumin as a standard.

2.3. Preparation of IgG Specific for CT. Anti-CT polyclonal antibodies were raised in a rabbit against purified recombinant CT according to a protocol previously described [7]. In brief, purified CT (100 μg protein in 1 mL of PBS) was mixed with the same volume of complete Freund's adjuvant and injected intradermally into 7-week-old Slc:JW/CSK rabbit. Three weeks after the first injection, the rabbit was boosted with the same amount of antigen mixed with complete Freund's adjuvant. One week after the second injection, 50 μg of the antigen without adjuvant was injected, and from then on, the same amount of the antigen without adjuvant was injected once a week. Antibody titers were determined by an Ouchterlony double immunodiffusion test with purified recombinant CT. Rabbits were bled 9 weeks after the first injection. Whole blood collected from immunized rabbit was left at room temperature for 4 hours, incubated at 37°C for 2 hours, and kept standing under refrigeration overnight. Clear serum obtained after centrifugation of the blood clot at 3,000 rpm for 10 min was used for the purification of anti-CT specific IgG. After the proteins were precipitated by adding of Na_2SO_4 (1.27 M) to the serum, the precipitate was resuspended in phosphate buffer (0.02 M NaH_2PO_4, 0.02 M Na_2HPO_4, pH 7.0) and dialyzed thoroughly against the same buffer. The preparation was loaded onto 1 mL HiTrap NHS-activated HP coupled with purified recombinant CT. After incubation of the column at 4°C for 2 hours, the column was washed with 10 bed volumes of phosphate buffer. And then, the bound CT-specific IgG was eluted with 0.1 M glycine-HCl, pH 3.0. Elute was monitored by measuring the absorbance at 280 nm. After elute was neutralized by adding of Tris base (0.1 M), the fractions containing CT-specific IgG were dialyzed thoroughly against PBS. Protein concentration for purified IgG specific for CT was determined by using of previously measured molar-absorbance coefficient.

2.4. Immunochromatographic Test. The test strip was prepared according to a protocol previously described [9]. For immunochromatographic test, 100 μL of the samples was loaded onto the test strip placed on horizontal table, and after migration of the sample through the membrane for 15 min at room temperature, the appearance of red lines at the test (T) zone and the control (C) zone was analyzed. The results with the appearance of red lines at both the T and C zones were interpreted as positive detection of CT.

2.5. Quantitative Analysis of CT Production in Bacterial Culture Supernatant by Bead-ELISA. Preparation of quantitative bead enzyme-linked immunosorbent assay (Bead-ELISA) for CT and quantitative analysis of concentration of CT in culture supernatant were done as described previously [10]. To construct the Bead-ELISA, anti-CT-specific IgG prepared in this study was used. The concentrations of CT in culture supernatant were calculated with four-parameter logistic curve fit for points on the standard curve for purified recombinant CT.

The bacterial culture supernatants were obtained after the organisms were cultured under AKI-SW condition [11]. Briefly, the organisms were cultured initially in stationary test tubes (height, 150 mm; diameter, 15 mm) for 4 h at 37°C, and then all the culture was transferred into 100 mL Erlenmeyer flasks. Subsequent cultivation was done at 37°C for 20 h with shaking (130 rpm). The amount of medium was maintained constantly at 10 mL. AKI medium (1.5% Bacto Peptone (Becton, Dickinson and Company), 0.4% yeast extract (Becton, Dickinson and Company) and 0.5% NaCI, and 0.3% $NaHCO_3$) was used for all bacterial strains. The bacterial culture supernatant was obtained after centrifugation at 900 ×g for 5 min.

3. Results

3.1. Detection Limit and Specificity of the Immunochromatographic Test Strip. To develop the high sensitive immunochromatographic test (IC), polyclonal antibodies were developed by using purified recombinant whole CT protein as an antigen. And, to increase the specificity, CT-specific IgG was isolated from antiserum by using purified recombinant whole CT conjugated column, and the obtained CT-specific IgG was used for construction of IC. We examined the lower detection limit of established CT-IC with 10-fold serial dilutions of purified CT (Figure 1(a)), and it was reveled that the CT-IC can detect as low as 10 ng/mL of purified CT within 15 min.

Heat-labile toxin (LT) produced in enterotoxigenic *E. coli* (ETEC) was used for specificity verification because it shares around 80% amino acid homology with CT; therefore, it is known that LT is antigenically similar to CT. Examination with serial dilution of purified LT revealed that detection limit for LT was about 100 times higher than that for CT (Figure 1(b)), indicating that established test strip has high specificity.

3.2. Detection of CT from V. cholerae Culture. The optimal condition for CT production had been persistently investigated because the amount of CT produced in *V. cholerae*

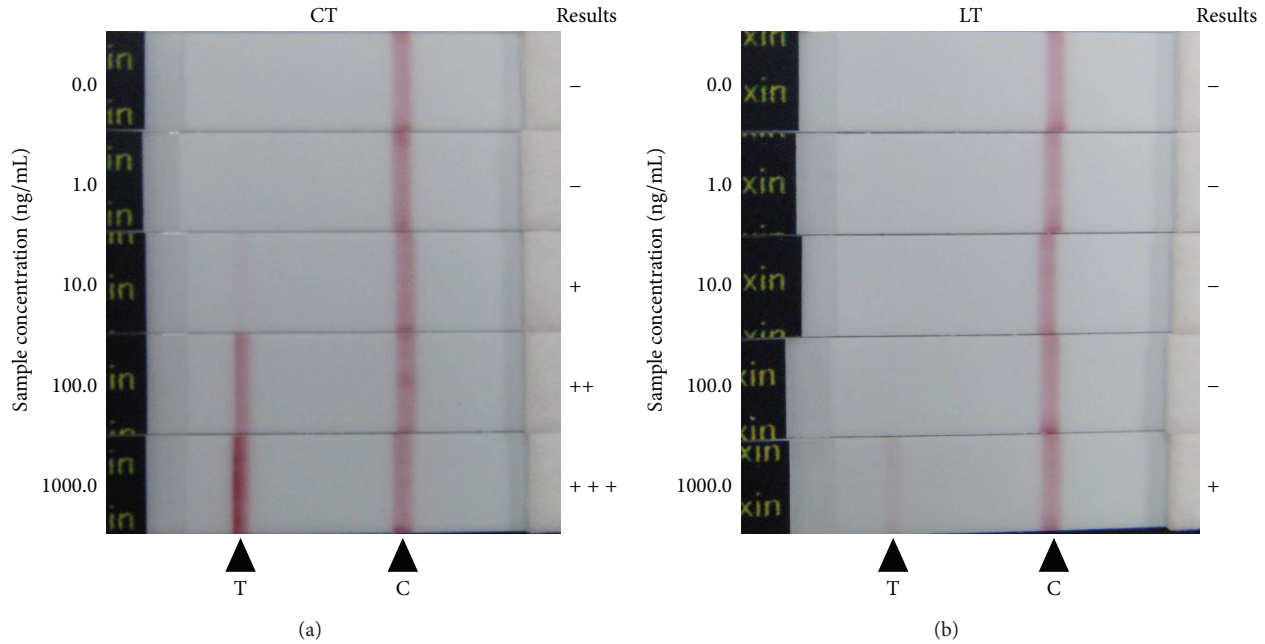

Figure 1: Reactivity of CT-IC with purified recombinant CT and LT. 0.1 mL of serial diluted purified recombinant CT (a) or LT (b) was applied to the test strips. After 15 minutes, development of red color at position for test (T) or control (C) lines was monitored. Concentrations of samples applied are indicated on right side of each strip. The "+++", "++", "+", or "–" symbols are placed on the left side of the strips developing "strong," "medium," "faint," or "no" bands at test lines, respectively.

El Tor vary according to the medium used and culture conditions (i.e., temperature and aeration status) [11–14]. In this study, we used AKI medium with biphasic culture condition, that is, 4 h cultivation in a stationary test tube followed by 16 h cultivation in a shaking flask at 37°C (AKI-SW condition), because, under this condition, it was reported that the most of the *V. cholerae* El Tor strains could produce more than 10 ng/mL of CT proteins [13]. The quantitative analysis revealed that, in the case of *V. cholerae* strains we used in this study, 14 out of 15 *ct* gene-positive strains produced substantially high amount of CT (Figure 2(a)). And, even in the strain with low level of CT expression, concentration of CT in cultured supernatant was higher than the detection limit (10 ng/mL) of CT-IC. CT in culture supernatant of all 15 of *ct* gene-positive stains could be detected by CT-IC, whereas no false-positive signal observed in all 5 *ct* gene-negative *V. cholerae* strains (Figure 2(b)).

As indicated above, CT-IC can discriminate CT from LT and did not react with nontoxigenic *V. cholerae* strains. The specificity of CT-IC was further evaluated by examining the culture of bacteria other than *V. cholerae*. In this study, we examined the reactivity of CT-IC against cultures of ETEC and *V. parahaemolyticus*. As mentioned above, ETEC strains have the ability to express LT protein which shares high homology with CT. *V. parahaemolyticus* is the most frequently isolated species among genus *Vibrio* and is one of the most important food-borne pathogen worldwide. In the "gold standard" selective cultivation test, the same enrichment and selective media are usually used for isolations of *V. cholerae* and *V. parahaemolyticus*. Twelve *lt* gene-positive ETEC isolates and 7 *V. parahaemolyticus* isolates were cultured under AKI-SW condition, and the resultant culture supernatants were examined by CT-IC. As shown in Figure 3, although no strong signals could be detected, 3 out of 12 ETEC isolates gave weak false-positive signals. On the other hand, no false-positive signals were observed in all *V. parahaemolyticus* strains examined.

4. Discussion

Analysis of CT production is critical for accurate diagnosis of cholera because, even if *V. cholerae* is isolated from patient, we cannot attribute the symptoms to the isolated *V. cholerae* bacterium without verification of ability to produce CT. In this study, we constructed CT-IC with the lower detection limit of 10 ng/mL CT which is comparable to the detection limit of commercially available RPLA (1–3 ng/mL) [10]. Although both IC and RPLA are the simple and rapid detection techniques, the detection limits of them are not sufficient for direct detection of CT from stool sample. It was reported that CT concentrations in most of the patient stools were lower than 10 ng/mL, even though the concentrations of CT in patient stools vary, ranging from 26 pg/mL to >100 ng/mL [15]. For some toxigenic bacteria, including thermostable direct hemolysin- (TDH-) producing *V. parahaemolyticus* and Shiga toxin (Stx-) producing *E. coli*, it had been reported that precultivation by using enrichment media is helpful for immunochromatographic identification of toxigenic bacteria from stool or food sample [16, 17]. In the case of CT detection, alkaline peptone water (APW) is expected to be useful as the enrichment media. APW is most commonly used media for

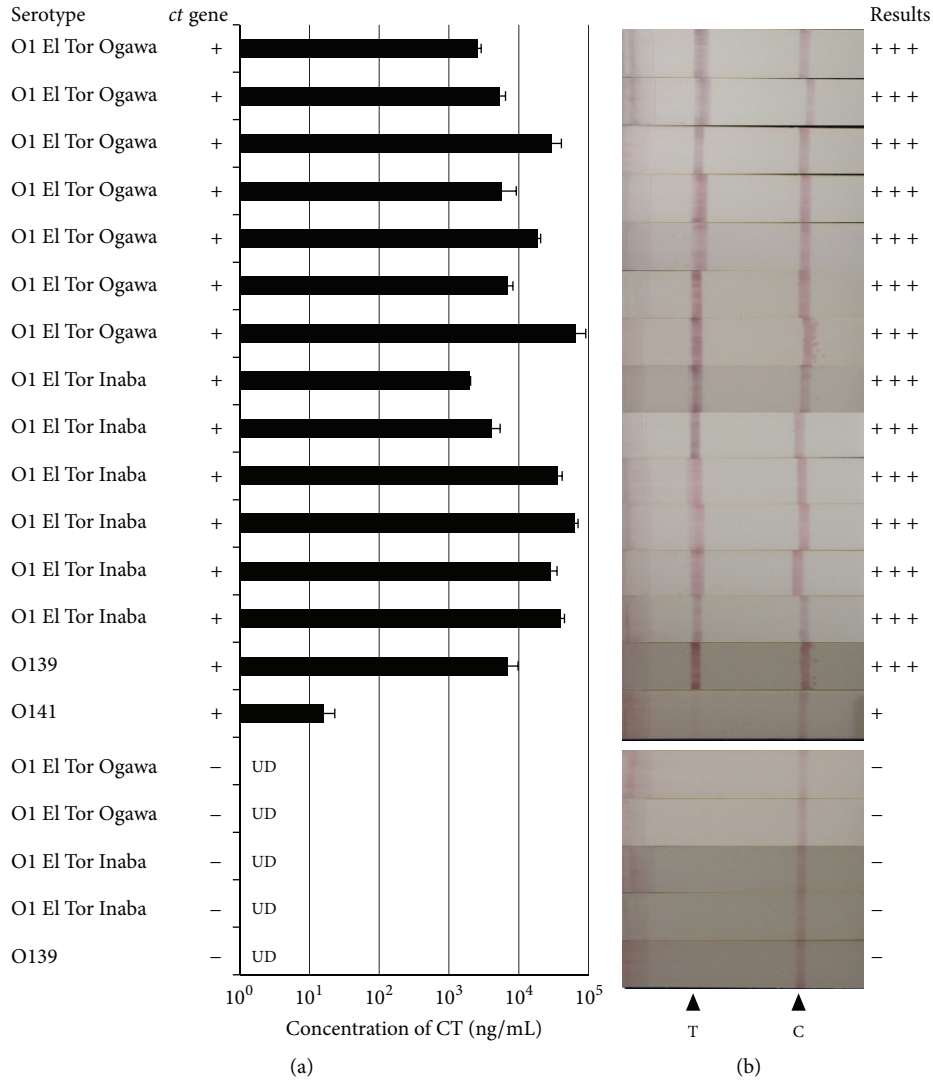

Figure 2: Ability of CT-IC to detect the toxigenic *V. cholerae* strains. Fifteen *ct* gene-positive *V. cholerae* isolates and 5 *ct* gene-negative *V. cholerae* isolates were cultured under AKI-SW condition, and then obtained supernatants of each culture were examined by quantitative Bead-ELISA specific for CT (a) or CT-IC (b). For quantitative analysis, data are means ± SD of values from three independent experiments. UD: undetectable. For IC, the "+++", "+", or "−" symbols were placed on the left side of the strips developing "strong," "faint," or "no" bands at test lines, respectively. T: test line, C: control line.

enrichment step in *Vibrio* spp. isolation [18]. In APW, *Vibrio* spp. can grow rapidly whereas growth of *E. coli* is inhibited or slow. This selectivity of APW has an important implication for CT detection because some ETEC have the ability to develop false-positive signal on CT-IC (Figure 3). So, if APW is employed at enrichment precultivation step, the occurrence of false-positive results might be reduced. In addition, it is evidence to the value of APW that *V. parahaemolyticus* did not give false-positive result in CT-IC. That is, even if *V. parahaemolyticus*, which is the most frequently isolated species among genus *Vibrio*, grow during enrichment cultivation in APW, it might have no effect on the results of CT-IC detection.

Various methods including immunoassay, bioassay, and DNA-based assay for investigation of toxigenicity of *V. cholerae* isolates had been established. Within them, immunoassay and bioassay require some degree of CT expression. Optimal *in vitro* CT expression in the El Tor biotype of *V. cholerae* serogroup O1 which is causative bacterium of ongoing 7th cholera pandemic had been persistently discussed because optimal condition for CT expression in El Tor biotype was significantly different from that for classical biotype which was responsible for the earlier cholera pandemics. Cultivation procedures with AKI media had been developed as a powerful tool for investigation of toxigenicity of *V. cholerae* isolates [3, 11–14]. It had been reported that most of *V. cholerae* El Tor could express considerably high amount of CT in the AKI media. In addition to AKI media, yeast extract-peptone water (YEP) media were reported to be able to stimulate CT expression, and the expression level of CT in YEP media tended to be higher than in AKI media [11]. YEP media differ from AKI media in the lack of sodium bicarbonate.

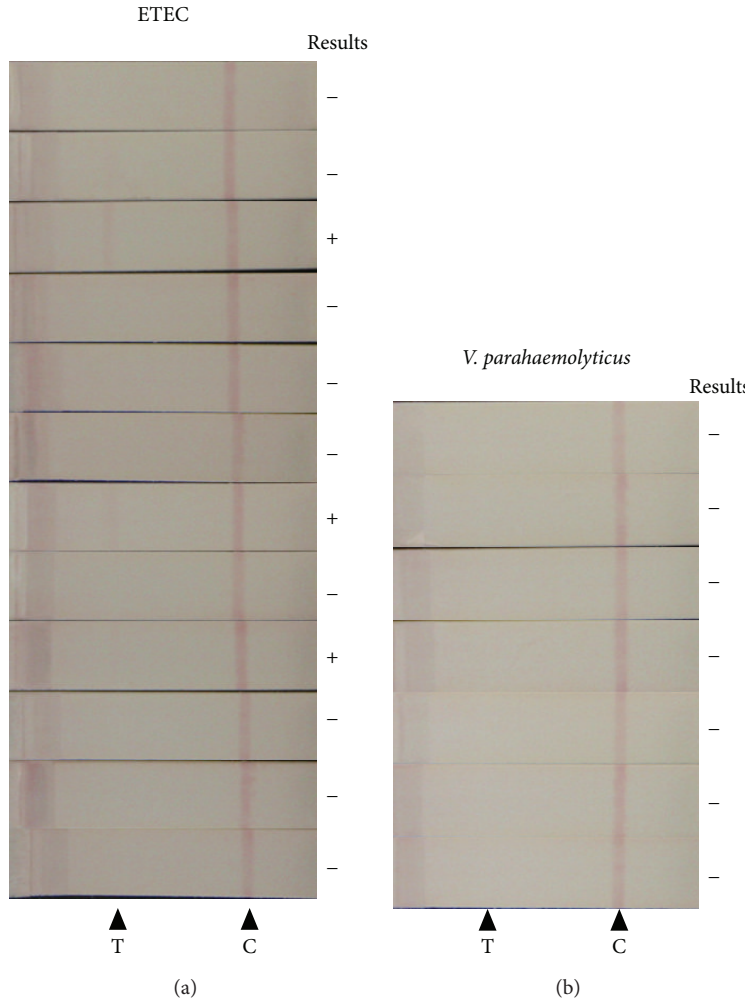

Figure 3: Reactivity of CT-IC with non-*V. cholerae* strains. Twelve ETEC isolates (a) and 7 *V. parahaemolyticus* isolates (b) were cultured under AKI-SW condition, and then obtained supernatants of each culture were examined by CT-IC. The "+" or "–" symbols were placed on the left side of the strips developing "faint" or "no" bands at test lines, respectively. T: test line, C: control line.

Bicarbonate is an important component of small-intestinal fluid to protect intestine from acid arriving from stomach and is present in upper small intestine where *V. cholerae* colonizes at the almost the same concentration as in AKI media. Although some differences in molecular mechanism regulating CT expression between *in vitro* and *in vivo* had been reported, it was supposed that the expression level of CT in AKI media more closely mimic the situation in small intestine than in other media [19–21]. For this reason, we used AKI media in the present study. In addition, we employed "complex" biphasic AKI-SW condition to induce CT expression because CT expression under AKI-SW condition is considerably higher than that under monophasic culture condition (i.e., 20 h cultivation in a stationary test tube at 37°C). On the other hand, previous reports indicated that almost all of the *ct* gene-positive *V. cholerae* strains could express CT at concentration higher than the detection limit of CT-IC (10 ng/mL) even under the monophasic culture condition [11, 13]. In addition, we have confirmed that CT-IC could detect CT from 14 out of 15 toxigenic *V. cholerae* strains even under monophasic culture condition (data not shown). Based on these results, there is a strong possibility that CT-IC can detect almost all of toxigenic strain even under the monophasic culture condition. However, we think that cultivation with AKI-SW condition is indispensable for accurate judgment of toxigenicity because, for example, in our case, 1 out of 15 toxigenic strains was missed with monophasic culture condition. So, we suggest that, especially when a lot of samples must be examined, the "simple" monophasic stationary culture condition may be used for preliminary screening to reduce the number of samples. And only for the isolates gave negative results in the primary screening, further elaborate investigation must be done with "complex" AKI-SW condition to judge toxigenicity as accurately as possible.

Taken together, combined use of APW and AKI medium is predictably beneficial at the sample preparation for CT-IC. APW may help reduce false-positive result and AKI may help give accurate judgment of toxigenicity. Further studies are needed to investigate the ability of CT-IC to identify toxigenic *V. cholerae* from patient stools.

5. Conclusions

In the case of *V. cholerae* identification, typing based on toxigenicity is critical. In this study, we established a toxigenic *V. cholerae*-specific immunochromatographic test strip. Because polyclonal antibodies were employed, the established test strip might have an ability to detect CT in various isolates even if some minor mutation in antigen was occurred in it. In addition to the adaptive capacity for polymorphism, the established test strip has high specificity; that is, it can discriminate CT from LT. We conclude that this high specific immunochromatographic strip is valuable in preventing the risk of failing to detect toxigenic *V. cholerae*.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Staphylococcus aureus Clinical Isolates: Antibiotic Susceptibility, Molecular Characteristics, and Ability to Form Biofilm

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Periodic monitoring of *Staphylococcus aureus* characteristics in a locality is imperative as their drug-resistant variants cause treatment problem. In this study, antibiograms, prevalence of toxin genes (*sea-see, seg-ser, seu, tsst-1, eta, etb*, and *etd*), PFGE types, accessory gene regulator (*agr*) groups, and ability to form biofilm of 92 *S. aureus* Thailand clinical isolates were investigated. They were classified into 10 drug groups: groups 1–7 (56 isolates) were methicillin resistant (MRSA) and 8–10 (36 isolates) were methicillin sensitive (MSSA). One isolate did not have any toxin gene, 4 isolates carried one toxin gene (*seq*), and 87 isolates had two or more toxin genes. No isolate had *see, etb*, or *tsst-1*; six isolates had *eta* or *etd*. Combined *seg-sei-sem-sen-seo* of the highly prevalent *egc* locus was 26.1%. The *seb, sec, sel, seu*, and *eta* associated significantly with MSSA; *sek* was more in MRSA. The *sek-seq* association was 52.17% while combined *sed-sej* was not found. Twenty-three PFGE types were revealed, no association of toxin genes with PFGE types. All four *agr* groups were present; *agr* group 1 was predominant (58.70%) but *agr* group 2 strains carried more toxin genes and were more frequent toxin producers. Biofilm formation was found in 72.83% of the isolates but there was no association with antibiograms. This study provides insight information on molecular and phenotypic markers of Thailand *S. aureus* clinical isolates which should be useful for future active surveillance that aimed to control a spread of existing antimicrobial resistant bacteria and early recognition of a newly emerged variant.

1. Introduction

Staphylococcus aureus, a gram positive coccal bacterium, is either commensal that colonizes healthy nasal mucosa [1] or pathogen of humans. As a pathogen, the bacteria cause a variety of community and hospital acquired diseases including skin abscess [2], food poisoning [3], pneumonitis [4], sepsis [5], and toxic shock syndrome [6]. This bacterium produces several virulent factors including adhesins (colonization factors), toxic proteins/enzymes (e.g., DNase for bacterial spread, coagulase, and catalase for host immunity evasion) and exotoxins including exfoliative toxins (ExTs), staphylococcal enterotoxins (SEs), and toxic shock syndrome toxin-1 (TSST-1). Patients infected with the ExT producing *S. aureus* may develop scalded-skin syndrome [7]. The SEs and TSST-1, besides causing food poisoning, are also superantigens (SAg) that can stimulate a relatively large fraction of peripheral blood T cells to release massive amounts of proinflammatory

cytokines and T-cell stimulating factors leading to toxic shock syndrome which may be fatal [8, 9]. The enterotoxicity and superantigenicity are distinct properties of the toxin molecule [6]. SEs are classified into two types based on their emetic activity in the toxin fed modeled primate. Toxins that induce vomiting in the primate are placed in the classical SE type while those that lack the emetic activity or have not been tested are allocated in the SE-like (SE*l*s) type [10, 11]. Members of the classical SEs are SEA-SEE and the more recently recognized SEG, SEH, SEI, SER, SES, and SET. The SE*l*s members include SE*l*J, SE*l*K, SE*l*L, SE*l*M, SE*l*N, SE*l*O, SE*l*P, SE*l*Q, SE*l*U, SE*l*U2 or SEW, and SE*l*V [11]. The staphylococcal enterotoxin F (SEF) which lacks emetic activity but is associated with toxic shock syndrome is presently called toxic shock syndrome toxin-1 (TSST-1) [12]. The SEs and the TSST-1 as well as the bacterial resistance to drugs are encoded by genes on the mobile genetic elements including prophages, plasmids, pathogenicity islands, genomic islands, and antibiotic resistance cassette [13]; thus they are transmitted horizontally rather easily. Expression of *S. aureus* virulence factors and metabolism of metabolic pathways during growth are coordinated/regulated by a quorum-sensing operon named accessory gene regulator (*agr*) [14, 15]. Based on the amino acid sequence polymorphisms of the *agr*-encoding autoinducing peptides and their responding receptors, *S. aureus* strains can be divided into four major *agr* groups (groups 1–4) [16].

During the last five decades, *S. aureus* clones that resist methicillin (methicillin-resistant *S. aureus*, MRSA) disseminated and caused medical and public health problem worldwide [17, 18]. These strains are not only resistant to methicillin, but also resistant to all other β-lactams, such as cephalosporin [18, 19]. In Thailand, MRSA infections were reported from 23 hospitals from 1988 to 1998 [20, 21]. The proportions of MRSA to MSSA in the northeast, central, and southern regions of the country during the studied period increased from 11 to 23.4%, 16 to 30.5%, and 21 to 30.3%, respectively [22]. Moreover, methicillin-resistant *S. aureus* with reduced susceptibility to vancomycin was recognized [23]. However, data on genotypic characteristics and other attributes of the *S. aureus* isolates in Thailand are relatively rare. Therefore, this study investigated the prevalence of virulence toxin genes coding for enterotoxins (*sea-see, seg-ser*, and *seu*), toxic shock syndrome toxin-1 (*tsst-1*), and exfoliative toxins (*eta*, *etb,* and *etd*) among *S. aureus* Thailand clinical isolates. Molecular diversity of the isolates regarding their endonuclease-restricted patterns of genomic DNA (PFGE), *agr* types, and antimicrobial susceptibility as well as their ability to produce biofilm were also investigated.

2. Materials and Methods

2.1. Bacterial Strains. Ninety-two strains of *S. aureus* isolated from clinical specimens were obtained from three hospitals. They were 43 strains (S1–S43) isolated in 2007 from patients of Prince of Songkla University Teaching Hospital and kept at the Department of Microbiology, Faculty of Science, Prince of Songkla University, Songkhla province, southern Thailand; 36 strains (P1–P36) from the patients of Prasat Neurological Institute, Bangkok, in 2010, and 13 strains (T1–T13) isolated in 2010 from patients of the Hospital for Tropical Diseases, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand. The bacteria were reconfirmed by Gram staining, biochemical testing (catalase, coagulase, and DNase), and mannitol fermentation. Their ability to produce protein A was detected by agglutination assay.

2.2. Antimicrobial Susceptibility Testing. Disc diffusion method was used for antimicrobial susceptibility testing of the *S. aureus* isolates which was done according to CLSI guidelines [24]. Antibiotic discs were cefoxitin, ciprofloxacin, clindamycin, erythromycin, gentamycin, oxacillin, penicillin G, rifampin, tetracycline, sulfamethoxazole plus trimethoprim, and teicoplanin (Oxoid, UK). Cefoxitin disc (30 μg) and oxacillin disc (1 μg) were used for detecting methicillin-resistant isolates. *S. aureus* ATCC 25923 was used as control. Reduction of vancomycin susceptibility of the isolates was also determined by observing the minimum inhibitory concentration (MIC) by agar dilution according to the CLSI guidelines [24].

2.3. Detection of Genes Coding for Staphylococcal Enterotoxins, TSST-1, and ExTs. Genomic DNA was extracted from each *S. aureus* isolate by DNA extraction kit (Geneaid, Taiwan) following the protocol for Gram-positive bacteria. Quality of each DNA preparation was assessed by determining the ratio of $OD_{260\,nm}/OD_{280\,nm}$. Twenty-two virulence genes were amplified including *sea-see*, *seg-ser* and *seu*, *tsst-1* and *eta*, *etb* and *etd*, using specific oligonucleotide primer sequences listed in Table 1 [25, 26]. The PCR reaction mixture (25 μL) is composed of 1 mM of each primer, 1x *Taq* buffer PCR, 0.2 mM dNTP, 2 mM $MgCl_2$, 1 unit of *Taq* DNA polymerase (Fermentas, Germany), and 100 ng of DNA template. The PCR reaction mixture was subjected to the thermal cycles: an initial denaturation of DNA at 95°C for 10 min prior to 35 cycles of denaturation at 95°C for 30 sec, 55°C for 30 sec, and 72°C for 30 sec, followed by a final extension of 10 min at 72°C using the Lifecycler (BioRad, USA). The amplified products were analyzed by 1.5% agarose gel electrophoresis and ethidium bromide staining. The DNA bands were observed under an UV transilluminator (Syngene, England). Control bacteria for the PCR included strains ATCC 19095 (*sea, sec*, *seh*, *seg*, *sei*, *sel*, *sem*, *sen*, *seo*, *seu,* and *tst*), ATCC 14458 (*seb* and *sek*), ATCC 23235 (*sed*, *sej*), and ATCC 27664 (*see*, *seq,* and *sea*). For *eta*, *etb,* and *etd*, the PCR amplicons were verified by DNA sequencing and the nucleotide sequences were aligned with the staphylococcal *eta, etb,* and *etd* sequences of the database (accession numbers: L25372.1, M17348.1, and AB057421.1, resp.).

2.4. Detection of SEs, TSST-1, and ExTs. The bacterial isolates which carried *sea, seb, sec* and *sed; eta* and *etb*; *tsst-1* were tested for their ability to express the respective proteins by the reversed-passive latex agglutination (RPLA) using commercially available kits: SET-RPLA, TST-RPLA, and EXT-RPLA (Denka Seiken, Japan), respectively. Other toxin detections were not done due to lack of available test kits.

Table 1: The primer sequences for amplification of the *S. aureus* enterotoxin genes.

Target gene		Primer sequence 5′ → 3′	Size of PCR product (bp)	Reference
sea	(F)	GAAAAAAGTCTGAATTGCAGGGAACA	560	[26]
	(R)	CAAATAAATCGTAATTAACCGAAGGTTC		
seb	(F)	ATTCTATTAAGGACACTAAGTTAGGGA	404	[26]
	(R)	ATCCCGTTTCATAAGGCGAGT		
sec	(F)	CTTGTATGTATGGAGGAATAACAAAACATG	275	[26]
	(R)	CATATCATACCAAAAAGTATTGCCGT		
sed	(F)	GAATTAAGTAGTACCGCGCTAAATAATATG	492	[26]
	(R)	GCTGTATTTTTCCTCCGAGAGT		
see	(F)	CAAAGAAATGCTTTAAGCAATCTTAGGC	482	[26]
	(R)	CACCTTACCGCCAAAGCTG		
seg	(F)	ACCTGAAAAGCTTCAAGGA	204	[26]
	(R)	CGCCAACGTAATTCCAC		
seh	(F)	CAATCACATCATATGCGAAAGCAG	376	[26]
	(R)	CATCTACCCAAACATTAGCACC		
sei	(F)	CTYGAATTTTCAACMGGTAC	461	[26]
	(R)	AGGCAGTCCATCTCCTG-3		
sej	(F)	TCAGAACTGTTGTTCCGCTAG	138	[26]
	(R)	GAATTTTACCAYCAAAGGTAC		
sek	(F)	ATGCCAGCGCTCAAGGC	134	[26]
	(R1)	AGATTCATTTGAAAATTGTAGTTGATTAGCT		
	(R2)	TGCCAGCGCTCAAGGTG		
sel	(F)	GCGATGTAGGTCCAGGAAAC	234	[26]
	(R)	CATATATAGTACGAGAGTTAGAACCATA		
sem	(F)	CTATTAATCTTTGGGTTAATGGAGAAC	326	[26]
	(R)	TTCAGTTTCGACAGTTTTGTTGTCAT		
sen	(F)	CGTGGCAATTAGACGAGTC	474	[26]
	(R)	GATTGATYTTGATGATTATKAG		
seo	(F)	AGTTTGTGTAAGAAGTCAAGTGTAGA	180	[26]
	(R)	ATCTTTAAATTCAGCAGATATTCCATCTAAC		
sep	(F)	GAATTGCAGGGAACTGCT	182	[26]
	(R)	GGCGGTGTCTTTTGAAC		
seq	(F)	ACCTGAAAAGCTTCAAGGA	204	[26]
	(R)	CGCCAACGTAATTCCAC		
ser	(F)	AGCGGTAATAGCAGAAAATG	363	[26]
	(R)	TCTTGTACCGTAACCGTTTT		
seu	(F)	AATGGCTCTAAAATTGATGG	215	[26]
	(R)	ATTTGATTTCCATCATGCTC		
tst	(F)	TTCACTATTTGTAAAAGTGTCAGACCCACT	180	[26]
	(R)	TACTAATGAATTTTTTTATCGTAAGCCCTT		
eta	(F)	ACTGTAGGAGCTAGTGCATTTGT	190	[26]
	(R)	TGGATACTTTTGTCTATCTTTTTCATCAAC		
etb	(F)	CAGATAAAGAGCTTTATACACACATTAC	612	[25]
	(R)	AGTGAACTTATCTTTCTATTGAAAAACACTC		
etd	(F)	CAAACTATCATGTATCAAGGATGG	358	[26]
	(R)	CCAGAATTTCCCGACTCAG		

2.5. Pulsed-Field Gel Electrophoresis (PFGE). PFGE patterns of chromosomal DNA of all *S. aureus* isolates were determined by digesting each DNA preparation with *Sma*I. The digested DNA preparations were subjected to electrophoretic separation in a CHEF-DR II system (BioRad, USA) as described previously [27]. DNA fragment patterns were analyzed in the GeneDirectory Application Version 2.01.00 Copyright 2000–2008 Synoptics Ltd. Percent similarities were identified on dendrogram derived from the unweighted pair group method with arithmetic averages (UPGMA) and based on Dice coefficients. Band position tolerance was set at 1.0%. A coefficient similarity of 70% was selected to define cluster of the PFGE types.

2.6. The Agr Alleles. Genomic DNA of the 92 *S. aureus* isolates was used as templates for amplification of *agr* alleles using

the group specific primers [16]. The common forward (pan) primer: (5′-ATGCACATGGTGCACATGC-3′) and reversed primers including: agr1 (5′-GTCACAAGTACTATAAGC-TGCGAT-3′), agr2 (5′-TATTACTAATTGAAAAGTGCC-ATAGC-3′), agr3 (5′-GTAATGTAATAGCTTGTATAA-TAATACCCAG-3′), and agr4 (5′-CGATAATGCCGTAAT-ACCCG-3′) were used. These primers allowed amplification of 439-, 572-, 320-, and 657-bp DNA fragments of the *agr* groups 1–4, respectively.

2.7. Biofilm Formation. Ability of the *S. aureus* isolates to form biofilm was determined according to the protocol described previously [28] with modification. Individual bacterial isolates were cultured in TSB (Oxoid) supplemented with 0.25% glucose at 35°C until the turbidity reached McFarland no. 0.5. Approximately 100 cfu of each culture were applied in triplicate into wells of 96-well flat-bottomed microplate containing 200 μL of the TSB and 0.25% glucose. Wells added with cultured *S. epidermidis* (ATCC12228) served as negative controls. The plate was incubated for 24 h. The content of each well was then discarded and the wells were washed five times with sterile 0.9% NaCl solution. Each well surface was stained by adding 100 μL of 0.3% (w/v) crystal violet (Merck) in water and kept for 5 min. After five washing with sterile distilled water and air dried. The biofilm fixed on each well surface was extracted with 100 μL of 70% ethanol and measured the absorbance at $OD_{570\,nm}$. The isolates with $OD_{570\,nm}$ values above the mean $OD_{570\,nm}$ values plus three standard deviations of the negative control ($mean_{neg} + 3\,SD_{Neg}$) were considered positive for biofilm formation.

2.8. Statistical Analyses. SPSS Statistics 16.0 was used for statistical analysis. Chi-squared (χ^2) test and t-test were used to analyze the data sorted by MRSA and MSSA groups and frequencies of virulence genes and biofilm formation, respectively. A probability value (P) < 0.05 was considered different significantly.

3. Results

3.1. Antimicrobial Susceptibility. All of the 92 bacterial isolates from culture stocks were verified as *S. aureus* strains according to their phenotypic characteristics determined by the conventional microbiological method. After testing with the 30 μg cefoxitin disc, 56/92 isolates (60.87%) were MRSA (37 isolates from the Prince of Songkla hospital and 19 isolates from Prasat Neurological Institute), and 36 isolates (39.13%) were MSSA (5 isolates from the Prince of Songkla hospital, 17 isolates from the Prasat Neurological Institute, and 19 isolates from the Hospital for Tropical Diseases). The 92 *S. aureus* Thailand isolates were arbitrarily classified into 10 drug groups. Groups 1–7 were MRSA and groups 8–10 were MSSA. Data on susceptible and intermediate sensitivity to the 11 antibiotics tested (cefoxitin, ciprofloxacin, clindamycin, erythromycin, gentamicin, oxacillin, penicillin, rifampin, trimethoprim/sulfamethoxazole (T/S), tetracycline, and teicoplanin) were group 1 (16 isolates): susceptible (9 isolates) and intermediate (7 isolates) to rifampin and susceptible to teicoplanin; group 2 (2 isolates): susceptible to gentamicin and teicoplanin, intermediate to rifampin; group 3 (7 isolates): susceptible to gentamicin and teicoplanin; group 4 (1 isolate): susceptible to tetracycline and teicoplanin; group 5 (7 isolates): susceptible to rifampin, trimethoprim/sulfametoxazole, tetracycline, and teicoplanin and susceptible to gentamicin (1 isolate); group 6 (10 isolates): susceptible to rifampin, trimethoprim/sulfametoxazole (10 isolates), intermediate to trimethoprim/sulfametoxazole (1 isolate), and susceptible to teicoplanin; group 7 (13 isolates): susceptible to teicoplanin; group 8 (3 isolates): susceptible to oxacillin (2 isolates), cefoxitin, gentamicin, gentamicin, and teicoplanin; group 9 (28 isolates): resistant to penicillin and tetracycline (13 isolates), intermediate to erythromycin (1 islates); group 10 (5 isolates): resistant to gentamicin (1 isolate), ciprofloxacin (2 isolates), erythromycin (2 isolates), and clindamycin (2 isolates). All of the isolates were sensitive to vancomycin according to the MIC testing. The methicillin susceptibility and drug groups of the 92 isolates are shown in Table 2.

3.2. Prevalence of Toxin Genes in Individual S. aureus Isolates. Among the 92 isolates, 1 isolate (1.08%) did not have any toxin gene (S38), 4 (4.35%) isolates (S16, S33, S40, and P33) carried one toxin gene (*seq*), and the remaining 87 isolates (94.57%) carried two or more toxin genes (Table 2). There were only 6/92 isolates that carry the *etx* genes either *eta* or *etd* (P28, P31, T3, T8, T9, and T13). The prevalence of toxin genes among the isolates is shown in Figure 1. The predominant enterotoxin gene was *seq* which was presented in 91/92 isolates (98.91%), followed by *sea* (65.22%) and *sek* (54.35%). There was no isolate with *see*, *tsst-1* (*sef*), or *etb*. The prevalence of *sea, sec, sed, seg, seh, sei, sej, sem, sen, seo, sep, seq, ser, eta,* and *etd* among the MRSA and MSSA isolates were not different. However, the prevalence of *seb, sel,* and *seu* among isolates of the two methicillin groups was different significantly.

3.3. Determination of Toxin Production. The bacterial isolates which carried *sea*, *seb*, *sec*, *sed*; *eta* and *etb*; *tsst-1* were determined for their ability to produce the respective toxins by using SET-RPLA, TST-RPLA, and EXT-RPLA, respectively, and 35 isolates were toxin producers (Table 2). There were 21/60 *sea* strains (35%) that produced SEA; 9/13 *seb* isolates (69.23%) produced SEB; 4/7 *sec* isolates (57.14%) produced SEC; and 3/5 *sed* isolates (60%) produced SED. One of the three *eta* positive strains (33.33%) could produce ETA. None of the four isolates with *etd*-positive strains produced ETD. Among the MRSA, 24/56 isolates (42.86%) produced toxins (17 SEA and 7 SEB), whereas 11/36 (30.55%) of the MSSA isolates produced toxins (SEA 4 isolates, SEB 1 isolate, SEC 3 isolates, SED 2 isolates, and SEB and ETA 1 isolate). There were 3 MSSA isolates that produced more than one toxin: S41 produced SEB and SED, P23 produced SEA and SEC, and T3 produced SEB and ETA.

3.4. PFGE Types. The 92 *S. aureus* isolates could be classified according to the PFGE results into 23 genotypes, genotypes 1–23 (Figure 2). Information on the PFGE types of individual

Table 2: Characteristics of the 92 *S. aureus* Thailand isolates.

Isolate no.	Methicillin susceptibility	Drug group	Enterotoxin gene(s)	ExT gene	RPLA toxin	PFGE type	Agr group	Biofilm (OD)
S1	R	1	*sek*, *seq*	—	ND	1	1	+ (0.831)
S2	R	1	*sea*, *sek*, *seo*, *seq*	—	ND	1	1	+ (0.828)
S3	R	1	*sek*, *seq*	—	ND	1	1	+ (0.039)
S4	R	1	*sek*, *seq*	—	ND	1	1	+ (0.181)
S5	R	1	*sea*, *sek*, *seq*	—	—	1	1	+ (0.701)
S6	R	1	*sek*, *seq*	—	ND	2	1	+ (1.566)
S7	R	1	*sea*, *sed*, *sek*, *seq*	—	—	1	2	+ (1.841)
S8	R	1	*sea*, *sek*, *seq*	—	SEA	6	1	+ (1.701)
S9	R	1	*sea*, *sek*, *seq*	—	SEA	21	1	+ (0.996)
S10	R	1	*sea*, *sek*, *seq*	—	SEA	21	1	+ (1.219)
S11	R	1	*sea*, *sek*, *seo*, *seq*	—	SEA	21	1	+ (1.749)
S12	R	1	*sea*, *sek*, *seq*	—	SEA	21	1	+ (1.377)
S13	R	1	*sea*, *sek*, *seq*	—	SEA	21	1	+ (1.687)
S14	R	1	*sea*, *sek*, *seq*	—	SEA	21	1	+ (0.796)
S15	R	2	*sea*, *sek*, *seq*	—	—	1	1	+ (0.097)
S16	R	2	*seq*	—	ND	3	1	+ (0.132)
S17	R	3	*sea*, *sek*, *seq*	—	—	1	1	+ (0.085)
S18	R	3	*sea*, *sek*, *seq*	—	—	4	1	+ (0.230)
S19	R	3	*sea*, *sek*, *seq*	—	—	4	1	+ (0.080)
S20	R	3	*sek*, *seq*	—	ND	6	1	+ (0.417)
S21	R	3	*sek*, *seq*	—	ND	6	1	+ (1.103)
S22	R	3	*sek*, *seq*	—	ND	6	1	+ (1.835)
S23	R	3	*sek*, *seq*	—	ND	21	1	+ (0.097)
S24	R	4	*sea*, *sek*, *seq*	—	—	2	1	+ (0.552)
S25	R	7	*sea*, *sek*, *seq*	—	—	1	1	+ (0.569)
S26	R	7	*sek*, *seq*	—	ND	1	1	+ (1.000)
S27	R	7	*sek*, *seq*	—	ND	1	1	+ (1.155)
S28	R	7	*sea*, *sek*, *seq*	—	—	1	1	+ (0.715)
S29	R	7	*sek*, *seq*	—	ND	2	1	+ (1.061)
S30	R	7	*sek*, *seq*	—	ND	2	1	+ (1.131)
S31	R	7	*sek*, *seq*	—	ND	2	1	+ (0.774)
S32	R	7	*sea*, *sec*, *sek*, *sel*, *seq*	—	—	9	1	+ (1.796)
S33	R	7	*seq*	—	ND	21	1	+ (2.481)
S34	R	7	*sea*, *sec*, *sel*, *seq*	—	—	21	1	+ (1.000)
S35	R	7	*sea*, *sek*, *seq*	—	—	21	1	+ (1.792)
S36	R	7	*sek*, *seq*	—	ND	21	1	+ (1.184)
S37	R	7	*sek*, *seq*	—	ND	21	1	+ (2.332)
S38	S	8		—	ND	4	1	− (−0.052)
S39	S	8	*sej*, *sek*, *seq*	—	ND	4	2	+ (0.367)
S40	S	8	*seq*	—	ND	21	1	+ (0.508)
S41	S	9	*seb*, *sed*, *sej*, *sek*, *seq*, *ser*, *etd*	—	SEB, SED	21	3	+ (0.074)
S42	S	9	*seg*, *sei*, *sem*, *sen*, *seo*, *seq*, *seu*	—	—	19	3	− (−0.007)
S43	S	9	*seg*, *sei*, *sek*, *sem*, *sen*, *seo*, *seq*	—	—	7	2	− (−0.008)
P1	R	1	*sea*, *seq*	—	SEA	21	1	+ (0.317)
P2	R	1	*sea*, *seg*, *sei*, *sek*, *sen*, *seo*, *seq*	—	SEA	9	2	+ (0.700)

Table 2: Continued.

Isolate no.	Methicillin susceptibility	Drug group	Enterotoxin gene(s)	ExT gene	RPLA toxin	PFGE type	Agr group	Biofilm (OD)
P3	R	5	*sea*, *seg*, *sei*, *sem*, *sen*, *seo*, *seq*	—	SEA	9	2	+ (0.098)
P4	R	5	*sea*, *seg*, *sei*, *sem*, *sen*, *seo*, *seq*	—	SEA	9	2	− (−0.194)
P5	R	5	*sea*, *sei*, *sek*, *sen*, *seo*, *seq*	—	SEA	9	2	+ (0.543)
P6	R	5	*sea*, *seg*, *sei*, *sem*, *sen*, *seo*, *seq*	—	SEA	9	2	− (−0.144)
P7	R	5	*sea*, *seg*, *sei*, *sek*, *sem*, *sen*, *seo*, *seq*	—	SEA	9	2	− (−0.095)
P8	R	5	*sea*, *seg*, *sei*, *sem*, *sen*, *seo*, *seq*	—	SEA	13	2	+ (0.451)
P9	R	5	*sea*, *sed*, *seg*, *sei*, *sej*, *sem*, *sen*, *seo*, *sep*, *seq*, *ser*	—	SED	16	2	− (−0.05)
P10	R	6	*sea*, *sek*, *seq*	—	SEA	21	1	+ (0.817)
P11	R	6	*sea*, *seb*, *seg*, *sei*, *sem*, *sen*, *seo*, *seq*	—	SEB	22	2	+ (0.141)
P12	R	6	*seb*, *seg*, *sei*, *sem*, *sen*, *seo*, *seq*	—	SEB	22	2	+ (0.179)
P13	R	6	*seb*, *seg*, *sei*, *sem*, *sen*, *seq*	—	SEB	22	2	− (−0.176)
P14	R	6	*sea*, *seb*, *seg*, *sei*, *sem*, *sen*, *seo*, *seq*	—	—	22	2	+ (0.182)
P15	R	6	*sea*, *seb*, *seg*, *sei*, *sem*, *sen*, *seo*, *seq*	—	SEB	22	2	− (−0.084)
P16	R	6	*sea*, *seb*, *seg*, *sei*, *sem*, *sen*, *seo*, *seq*	—	SEB	22	2	− (−0.249)
P17	R	6	*seb*, *seg*, *sei*, *sem*, *sen*, *seo*, *seq*	—	SEB	22	2	− (−0.051)
P18	R	6	*seb, seg, sei, sem, sen, seo, seq*	—	SEB	22	2	− (−0.137)
P19	R	6	*sea*, *seb*, *seg*, *sei*, *sem*, *sen*, *seo*, *seq*	—	—	22	2	− (−0.117)
P20	S	9	*sea*, *sek*, *sel*, *seq*	—	—	1	1	+ (1.311)
P21	S	9	*sea*, *sec*, *sel*, *seq*	—	—	2	1	− (−0.173)
P22	S	9	*sea*, *seo*, *seq*	—	—	8	1	+ (0.300)
P23	S	9	*sea*, *sec*, *sel*, *seq*	—	SEA, SEC	9	1	− (−0.204)
P24	S	9	*sea*, *seq*	—	—	9	1	+ (2.210)
P25	S	9	*sea*, *sek*, *seq*	—	—	10	4	+ (0.484)
P26	S	9	*sea*, *sek*, *seo*, *seq*	—	—	17	1	+ (1.156)
P27	S	9	*sea*, *seh*, *sek*, *seq*	—	SEA	17	3	− (−0.058)
P28	S	9	*sea*, *sed*, *sei*, *seq*	*etd*	SED	18	1	− (−0.225)
P29	S	9	*sea*, *seq*	—	—	18	2	+ (0.098)
P30	S	9	*sea*, *sec*, *seg*, *sei*, *sel*, *sem*, *sen*, *seo*, *seq*	—	SEC	18	2	− (−0.390)
P31	S	9	*sea*, *seb*, *seg*, *sei*, *sem*, *sen*, *seo*, *seq*, *seu*	*eta*	—	18	2	− (−0.147)
P32	S	9	*sea*, *sec*, *seg*, *sei*, *sel*, *sen*, *seo*, *seq*	—	SEC	20	3	+ (0.128)
P33	S	9	*seq*	—	ND	20	3	− (−0.245)
P34	S	9	*seo*, *seq*	—	ND	22	1	− (−0.326)
P35	S	9	*sea*, *seo*, *seq*	—	—	23	1	+ (0.054)
P36	S	9	*sed*, *sek*, *seq*	—	ND	23	1	+ (1.107)
T1	S	9	*sea*, *sen*, *seq*	—	—	5	2	− (−0.073)
T2	S	9	*sea*, *seg*, *sem*, *sen*, *seo*, *seq*	—	SEA	5	2	+ (0.046)
T3	S	9	*seb*, *seg*, *sei*, *sem*, *sen*, *seo*, *seq*, *seu*	*eta*	SEB, ETA	8	4	+ (3.872)

Table 2: Continued.

Isolate no.	Methicillin susceptibility	Drug group	Enterotoxin gene(s)	ExT gene	RPLA toxin	PFGE type	Agr group	Biofilm (OD)
T4	S	9	*sea*, *seg*, *sen*, *seq*	—	—	9	2	+ (0.319)
T5	S	9	*sea*, *seg*, *sei*, *sek*, *sem*, *sen*, *seo*, *seq*, *seu*	—	—	9	4	− (−0.081)
T6	S	9	*seg*, *sen*, *seq*	—	ND	11	1	+ (0.736)
T7	S	9	*sea*, *seg*, *sei*, *sek*, *sen*, *seo*	—	—	15	2	+ (2.818)
T8	S	9	*sea*, *seg*, *sei*, *sem*, *sen*, *seo*, *seq*	*eta*	—	18	1	+ (0.156)
T9	S	10	*seq*	*etd*	ND	12	1	− (−0.114)
T10	S	10	*sea*, *seg*, *sei*, *sem*, *sen*, *seo*, *seq*	—	SEA	14	2	+ (0.086)
T11	S	10	*sea*, *seg*, *sei*, *sem*, *sen*, *seo*, *seq*	—	—	15	2	+ (0.808)
T12	S	10	*sec*, *seh*, *sel*, *seq*	—	SEC	15	3	− (−0.198)
T13	S	10	*sea*, *sek*, *seq*	*etd*	SEA	18	1	+ (0.235)

−: not detectable, +: produced biofilm.
ND: not done.

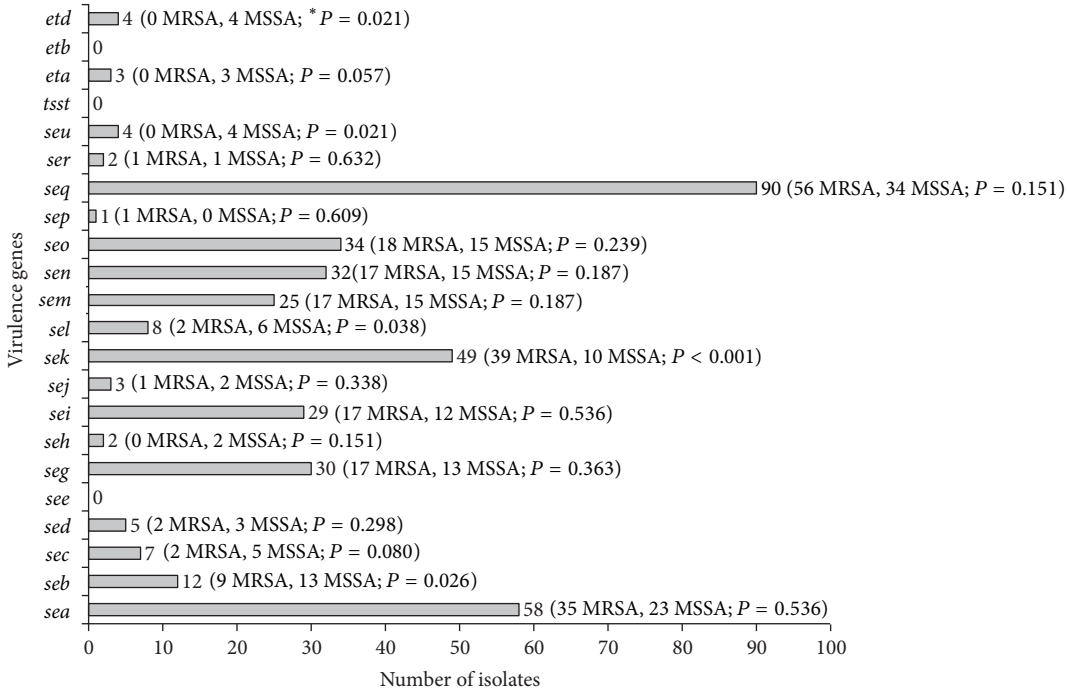

Figure 1: Prevalence of the enterotoxin and exfoliative toxin genes among the 92 *S. aureus* Thailand isolates. *P value between prevalence of MRSA compared to MSSA.

isolates is given in Table 2. PFGE type 21 was predominant (16 isolates), followed by types 1, 9, and 22 (13, 11, and 10 isolates, resp.); types 2 and 18 had 6 isolates each; types 4 and 6 had 4 isolates each; 3 isolates belonged to type 15; types 5, 8, 17, 20, and 23 had 2 isolates each, and types 3, 7, 10, 11, 12, 13, 14, 16, and 19 had 1 isolate each.

3.5. The Agr Groups. The predominant *agr* group among the 92 isolates was group 1 (54/92 isolates; 58.70%) followed by groups 2 (29 isolates; 31.52%), 3 (6 isolates; 6.52%), and 4 (3 isolates; 3.26%).

3.6. Biofilm Formation. There were 67/92 isolates (72.83%) that produced biofilm; 21/36 (58.33%) were MSSA and 46/56 isolates (82.14%) were MRSA. The prevalence of the biofilm formation of the MRSA and MSSA was not different ($P > 0.05$).

4. Discussion

Diseases caused by *S. aureus* are health hazard to human worldwide. Since the first recognition of methicillin-resistant *S. aureus* in 1961 [29], there has been an upsurge of infections

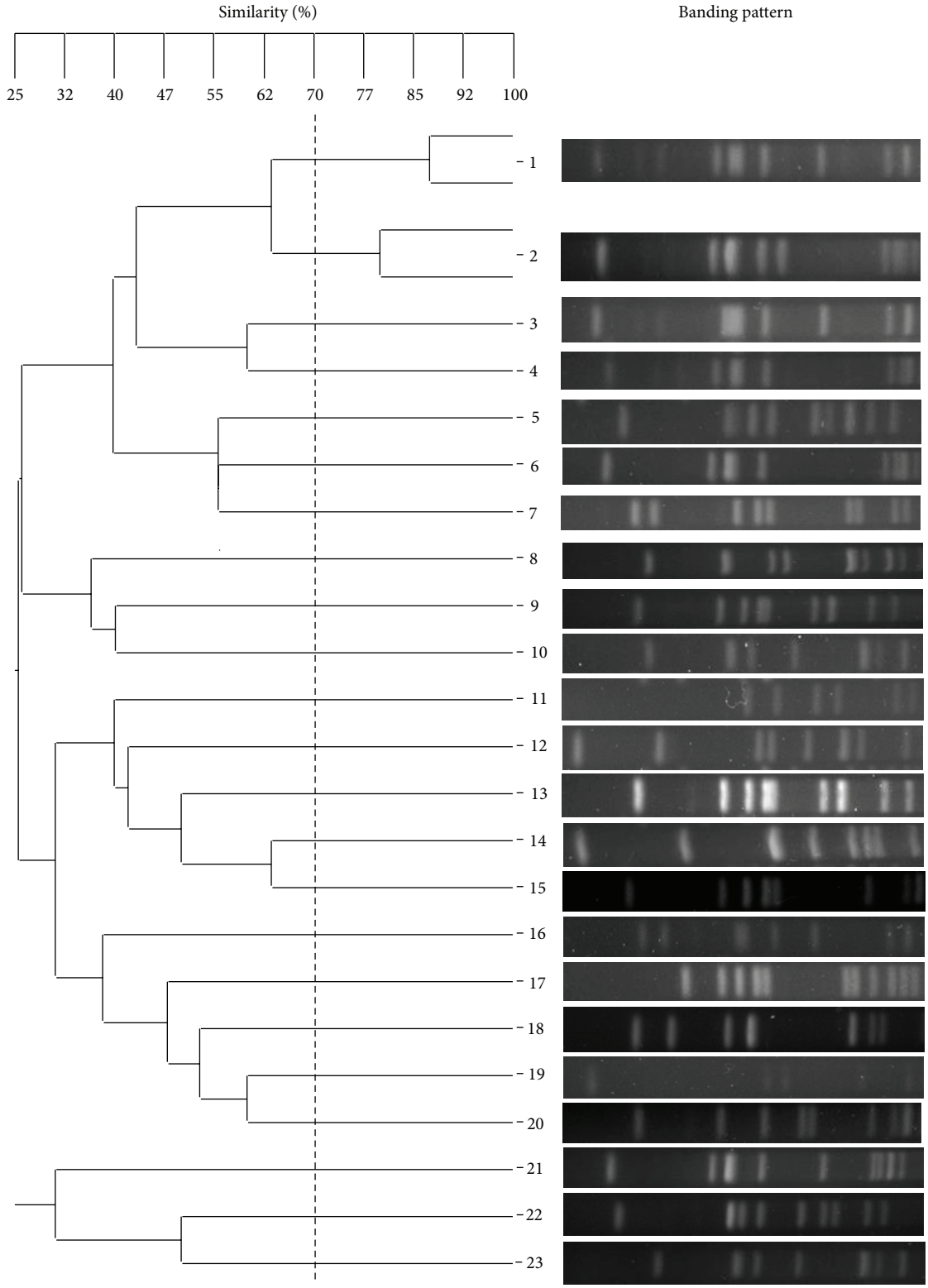

Figure 2: Dendrogram of PFGE patterns the 92 *S. aureus* Thailand isolates.

caused by the *S. aureus* variants that resist not only methicillin, but also other β-lactams and vancomycin, which are therapeutic drugs of choice [30–32], leading to treatment failure and increased case fatality rate. The methicillin and vancomycin resistance of the *S. aureus* are encoded by staphylococcal cassette chromosome *mec* (SCC*mec*) and *vanA*, respectively [30, 31]. Association of the presence of *S. aureus* toxin genes with methicillin sensitivity and resistance

among *S. aureus* has been reported previously [28, 33–35]. The association was found also in the present study; the prevalence of the *seb, sec, sel, seu,* and *eta* was associated significantly ($P < 0.05$) with the MSSA while *sek* was found more in MRSA.

The toxin genes carried by the 92 Thailand isolates varied from none to as many as 11 genes (Table 2). Five of the *S. aureus* enterotoxin genes, that is, *seg, sei, sem, sen,* and *seo*, belonged to the highly prevalent *egc* locus [36, 37]; thus, their coexistence was frequently reported. Coexistence of *seg-sei* in the same strain, either alone or in more combination with other toxin gene(s) (*sea*, *sec*, *sed*, *seh*, *sej,* and/or *tst*) was found in 55% of the 429 *S. aureus* isolates from Germany [38]. In Japan, the *seg-sei* alone or with *seb, sec,* or *sed* were 24, 2.7, 6.8, and 2.0%, respectively [39]. The combined *seg-sei-sem-sen-seo* with *seu* was 15.1% among the Chinese isolates [26]. In the present study, the combined *seg-sei-sem-sen-seo* with other toxin genes including *sea, seb, sed, sej, sek, sel, sep, seq, ser,* and/or *eta* was found in 24/92 isolates (26.1%). There were 3 isolates that carried *seg-sei-sen-seo* with *sea, sec, sek, sel,* and/or *seq* and 1 isolate with *seg-sei-sem-sen* and *seb*. The previously reported fixed association of *sed-sej* [38] was not found among the 92 Thailand isolates. The combined *sek-seq* with other toxin gene(s), that is, *sea* and/or *seb*, was 45.5% among the Chinese isolates [26]. In the present study, the *sek-seq* association was found in 48 of the 92 isolates (52.17%), either the two genes alone (16.3%) or with the other toxin genes (35.86%).

The ability of the isolates to produce SEA, SEB, SEC, and SED and ETA, ETB, and TSST-1 was examined by using SET-RPLA, TST-RPLA, and EXT-RPLA test kits, respectively. Not all isolates harboring the genes expressed the respective toxins. The results were similar to the finding reported previously among *S. aureus* isolates from milk and milk products from Morocco [40]. The unconformed results between genotypes (by PCR) to phenotypes (by RPLA) could be due to the fact that toxin production of the bacteria can be affected by the growth conditions including temperature, pH, and water activity. The so-produced toxin levels might be lower than the detection limits of the immunoassay [40, 41]. Alternatively, the toxin gene may not be expressed due to mutation either in the coding region or in a regulatory region, for example, *agr* [42, 43]. No annotated data are available in the literature on association of the ability of toxin production and antibiograms of the *S. aureus.* Nevertheless, in this study, the frequency of toxin production is higher among the MRSA (48.86%) than the MSSA (30.55%) ($P < 0.05$).

There was no association between PFGE patterns with the MRSA and MSSA of the 92 Thai strains which conformed to the results reported elsewhere [44, 45]. However, PFGE patterns 21 and 22 of MRSA strains predominated among isolates from Prince of Songkla Hospital and Prasat Neurological Institute, that is, 32.5 and 27.8%, respectively. Among the 7 isolates of PFGE pattern 21 of Songkla that could produce enterotoxins, 6 strains (85.7%) produced SEA. All 7 isolates of PFGE type 22 of Prasat Neurological Institute isolates produced SEB.

The polymorphism in the *agr* locus was first described by Ji et al. in 1997 [46]. To date, *S. aureus* isolates were classified into four different *agr* groups [25, 46]. In this study, all *agr* groups were found; large proportion (58.6%) of the isolates was *agr* group 1 which was similar to the data reported previously [16]. Moreover, majority (38/54 isolates, 70%) of the *agr* group 1 were MRSA which conformed also to the previous report [47]. However, it is noteworthy that isolates of the *agr* group 2 in this study carried more number of enterotoxins genes, and most of the toxin producing strains belonged to this *agr* group. The data were different from elsewhere which showed that most toxin producing *S. aureus* strains were either *agr* groups 3 [46] or 4 [48].

Biofilm formation contributes to bacterial pathogenesis and resistance to antibiotics and harsh environment. *S. aureus* isolates did form biofilms [28, 49]. More strains of MSSA produced biofilm compared to MRSA strains [28]. In this study, 72.83% of the *S. aureus* isolates formed biofilm but there was no association with their antibiotic patterns.

In conclusion, the results of this study provide insight information on molecular and phenotypic markers of *S. aureus* clinical isolates in Thailand which should be useful for future active surveillance that aimed to control a spread of existing antimicrobial resistant bacteria as well as early recognition of a newly emerged variant.

Conflict of Interests

The authors have declared that no conflict of interests exists.

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Research Article

Characterization of *Legionella pneumophila* Isolated from Environmental Water and Ashiyu Foot Spa

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Hot springs are the most common infectious source of *Legionella pneumophila* in Japan. However, little is known about the association between *L. pneumophila* and environmental waters other than hot springs. In this study, water samples from 22 environmental water sites were surveyed; of the 22 samples, five were *L. pneumophila* positive (23%). *L. pneumophila* was mainly isolated from ashiyu foot spas, a type of hot spring for the feet (3/8, 38%). These isolates had genetic loci or genes that encoded the virulence factors of *L. pneumophila*. Moreover, these isolates showed higher intracellular growth and stronger cytotoxicity compared with the reference strain. These results suggest that ashiyu foot spa can be the original source for *L. pneumophila* infection.

1. Introduction

Legionella pneumophila is the causative agent of legionellosis. In humans, *L. pneumophila* can induce Legionnaires' disease and Pontiac fever. Legionnaires' disease is a form of severe pneumonia, while Pontiac fever produces acute flu-like symptoms without pneumonia [1]. A number of factors including type II and type IV secretion systems, a pore-forming toxin, type IV pili, flagella, and heat shock proteins [2–7] contribute to *L. pneumophila* virulence. *L. pneumophila* is a facultative intracellular Gram-negative bacterium that can reside and multiply within free-living amoebae in environmental waters. *L. pneumophila* can withstand temperatures of 0–68°C and a pH range of 5.0–8.5 and survive in most environments for long periods [8]. *L. pneumophila* mainly lives in natural and man-made aquatic environment such as ponds, hot springs, fountains, cooling towers, and portable waters [8]. Hot springs and public baths are known to be most common source of *L. pneumophila* outbreaks in Japan [9–11]. Abundance information about the relationship between *L. pneumophila* and hot springs and public baths has been accumulated, but there is little information regarding *L. pneumophila* in environmental waters other than hot springs and public baths.

In this study, 22 environmental water places were surveyed in Yamaguchi Prefecture, Japan, and *L. pneumophila* was isolated from five sites.

2. Materials and Methods

2.1. Bacteria and Culture Conditions. *Legionella pneumophila* Lp02 and the Δ*dotA* mutant, Lp03 [2, 5], were maintained as frozen glycerol stocks and cultured on N-(2-acetamido-) 2-aminoethanesulphonic acid (ACES)-buffered charcoal-yeast extract broth containing 1.5% agar (CYET) or liquid ACES-buffered yeast extract broth (AYET) supplemented with 100 μg/mL thymidine.

Isolation of *L. pneumophila* was performed using CYET supplemented with glycine (Wako, Osaka, Japan, 3 mg/mL), vancomycin HCl (Wako, 1 μg/mL), polymyxin B (Sigma, MD, USA, 79.2 IU/mL), and sulfate cycloheximide (Wako,

80 μg/mL) (GVPC agar) [12]. Isolated bacteria were grown on CYET at 37°C or in AYET with shaking [13].

2.2. Specimen Collection and Preparation. Samples were collected from 22 environmental water sites. Eight samples were collected from ashiyu foot spas, seven were from water fountains, four were from basins of shrine, and three were from ponds (Table 1). Five hundred milliliters of sample was collected from each site in sterile bottles or small plastic containers and centrifuged at 3000 rpm for 20 min at 4°C. The deposits were resuspended in 500 μL distilled water as concentrates. Concentrated samples were heated at 50°C for 30 min and spread onto the surface of GVPC agar. Plates were incubated at 37°C and they were inspected daily.

2.3. PCR Analysis. The primers used for PCR analysis are summarized in Table 2. After denaturation of the bacterial chromosomal DNA template at 95°C for 5 min, 35 cycles of PCR amplification were performed using expand high fidelity PCR system (Roche, Basel, Switzerland).

2.4. Serotyping. Serotypes of isolated bacteria were determined based on their reactions during the immunoagglutination serotyping with *Legionella* immune sera (Denka Seiken, Tokyo, Japan).

2.5. Cell Lines and Culture Conditions. HeLa cells were grown at 37°C and 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM, Sigma) containing 10% heat-inactivated fetal bovine serum (FBS, Biowest, Paris, France). A human monocytic cell line, THP-1 cells, was grown at 37°C and 5% CO_2 in RPMI 1640 medium (Sigma), containing 10% heat-inactivated FBS. THP-1 cells were differentiated with 100 nM phorbol 12-myristate 13-acetate (PMA, Sigma) at 48 h prior to use.

2.6. Intracellular Invasion and Growth Assays. Bacteria were added to a monolayer of HeLa cells or THP-1 cells in 48-well tissue culture dishes at multiplicity of infection (MOI) of 100 or 1, respectively. These plates were centrifuged for 5 min at 900 ×g and incubated for 1 h at 37°C. Extracellular bacteria were killed by gentamicin (50 μg/mL) treatment for 1 h. To measure the invasion efficiency, cells were washed twice with phosphate-buffered saline (PBS) and lysed with cold distilled water. To measure the intracellular growth, the cells were incubated in fresh medium at 37°C for particular time and washed three times with PBS, followed by lysis with cold distilled water. Colony forming units (CFU) were determined by serial dilution on CYET.

2.7. Cytotoxicity Measurement. Bacteria were added to a monolayer of HeLa cells or THP-1 cells in 48-well tissue culture dishes at MOI of 100 or 1, respectively. These plates were centrifuged for 5 min at 900 ×g and incubated for 1 h at 37°C. Extracellular bacteria were killed by gentamicin (50 μg/mL) treatment for 1 h. Cells were washed twice with PBS and incubated in fresh medium at 37°C. At 24 or 48 h after incubation, the supernatants of infected cells were collected. Cytotoxicity was determined by measuring LDH release using a Cytotoxicity Detection Kit^{PLUS} (LDH) (Roche) according to the manufacturer's instructions.

Table 1: Detection of *Legionella pneumophila* from environmental waters.

Place	No. of collected points	No. of positive points	Positive rate (%)
Water fountain	7	1	14
Ashiyu foot spa	8	3	38
Basin	4	0	0
Pond	3	1	33
Total	22	5	23

Table 2: Oligonucleotides.

Name/region	Sequence (5′-3′)	Reference
lvh1/*lvhB3*	attgggagcttctggcaata	This study
lvh2/*lvhB3*	gctggggtgacctttgaata	This study
rtx1/*rtxA*	gctgcaaccacctctttgat	This study
rtx2/*rtxA*	caggggctggttatgttgat	This study
dot1/*dotA*	caaatccggcattcaaaatc	This study
dot2/*dotA*	ctattgtcgccttgggtgtt	This study
hsp1/*hsp60*	gcgaatcgttgttaccaaagaaaac	[15]
hsp2/*hsp60*	caatttgacgcattggagattcaatag	[15]
mip1/*mip*	ggtgactgcggctgttatgg	[16]
mip2/*mip*	ggccaataggtccgccaacg	[16]

2.8. Statistical Analysis. Data are expressed as the mean of triplicate samples from three identically performed experiments, and the error bars represented the standard deviations. Statistical analyses were performed using Student's t-test. Statistically significant differences are indicated by asterisks ($*$, $P < 0.05$).

3. Results

3.1. Isolation and Identification. Twenty-two samples were collected from environmental water sites in Yamaguchi Prefecture, Japan. Samples were concentrated and spread on GVPC agar. Five possible colonies were obtained. Three were isolated from ashiyu foot spas, one was isolated from a water fountain, and the other was isolated from a pond.

To confirm whether these isolates were *L. pneumophila* or not, the presence of *L. pneumophila* specific gene, *mip* [14], was tested by PCR. The *mip* gene was detected in all isolates, indicating that these isolates were *L. pneumophila*. We named these isolates Twr292, Ymt294, Ofk308, Ymg289, and Bnt314 (Tables 1 and 3).

The serotypes of these five isolates were then determined by immunoagglutination serotyping. Twr292, Ymt294, and Ymg289 were classified into serotype I, and Ofk308 and Bnt314 were classified into serotype IV (Table 3).

3.2. Growth in Liquid Medium. We compared the growth of the five isolates in AYET medium with that of the virulent

Table 3: Isolation of *Legionella pneumophila* from PCR-positive sites.

Strain	Place	CFU/100 mL	Serotype
Ymg289	Water fountain	1	I
Twr292	Ashiyu foot spa	128	I
Ymt294	Ashiyu foot spa	2	I
Ofk308	Ashiyu foot spa	2	IV
Bnt314	Pond	4	IV

reference strain Lp02 and the avirulent Δ*dotA* mutant Lp03, which lacks a functional Dot/Icm secretion system. Twr292, Ofk308, Ymg289, and Bnt314 showed comparable growth with Lp02 and Lp03. In contrast Ymt294 had shown lower growth rate. After 48 h, the number of Ymt294 was almost one-tenth of Lp02 and Lp03 (Figure 1).

3.3. Invasion, Intracellular Growth, and Cytotoxicity in HeLa Cells. To investigate the intracellular behavior of the isolates, their invasion, growth, and cytotoxicity in HeLa cells were examined. HeLa cells were infected with the isolates, and the number of invaded *L. pneumophila* was counted at 1 h after infection. Ymt294, Twr292, and Ymg289 invaded HeLa cells more than ten times higher than reference strain Lp02 (Figure 2(a)).

Intracellular growth of the isolates was measured by counting intracellular bacteria numbers at 24 and 48 h after infection. At 24 h after infection, Twr292, Ymg289, and Bnt314 showed higher growth and the bacterial number was more than ten times as compared with the reference strain Lp02. At 48 h after infection, the numbers of all isolates were decreased. The Δ*dotA* mutant Lp03 failed to replicate in HeLa cells, as previously reported [17] (Figure 2(b)).

The cytotoxicity of isolates was measured by LDH release assay and phase-contrast microscopy. At 24 and 48 h after infection, Ymt294, Twr292, and Ymg289 showed high cytotoxicity (Figure 2(c)). At 24 h after infection with isolates, cells were damaged and detached from the culture plates (Figures 4(a)–4(c) and data not shown).

3.4. Intracellular Growth and Cytotoxicity in THP-1 Cells. *L. pneumophila* resides predominantly in macrophages after infection; therefore, the growth and cytotoxicity of isolates were examined in a human macrophage cell line, THP-1 cells. At 24 h and 48 h after infection, all isolates showed potent growth. The numbers of these isolates were ten times higher than the reference strain Lp02. The Δ*dotA* mutant Lp03 failed to grow in THP-1 cells (Figure 3(a)). Moreover, all isolates showed higher cytotoxicity than the reference strain in THP-1 cells. Particularly Twr292 induced strong cytotoxicity (Figure 3(b)). Damaged and detached THP-1 cells were observed with phase-contrast microscopy after cells were infected with Twr292 (Figures 4(d)–4(f)).

3.5. Detection of Loci and Genes Related to Virulence Factor. To estimate whether these isolates are pathogenic to humans, the presence of genetic loci of *dot*, *lvh*, and *rtx* that encode

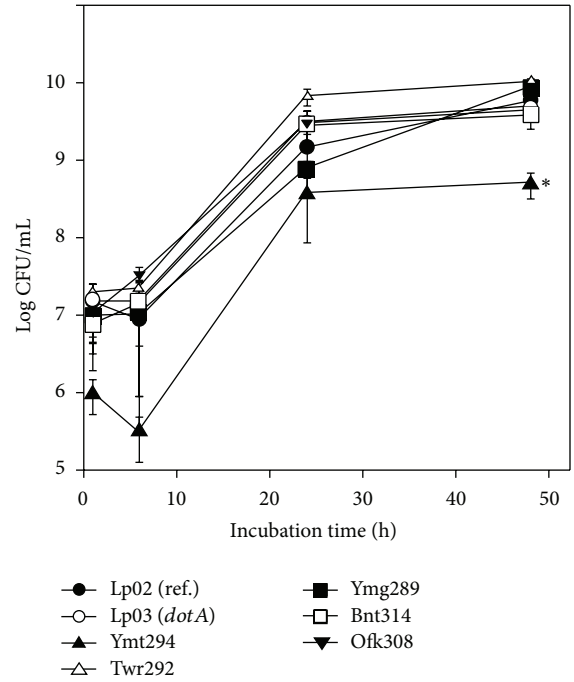

Figure 1: Growth of *L. pneumophila* isolates in liquid medium. Bacteria were grown in AYET. After 1, 24, and 48 h of incubation, samples were diluted with PBS and spread on CYET. All values represent the average and the standard deviation for three identical experiments. Statistically significant differences compared with the control are indicated by asterisks (∗, $P < 0.05$).

Table 4: Detection of loci and genes related to virulence factor.

Region	Lp02	Lp03	Ymg289	Twr292	Ymt294	Ofk308	Bnt314
lvhB3	+	+	+	+	+	+	+
rtxA	+	+	+	+	+	+	+
dotA	+	−	+	+	+	+	+
hsp60	+	+	+	+	+	+	+

typical virulence factors of *L. pneumophila* was examined. Loci of *dot* and *lvh* encode components of type IV secretion system that play an important role in intracellular growth [18]. Locus *rtx* encodes proteins involved in adherence, cytotoxicity, and pore formation [19]. The presence of *dot*, *lvh*, and *rtx* loci was tested by detecting *dotA*, *lvhB3*, and *rtxA* genes located in these loci, respectively, by PCR. The presence of the *hsp60* gene was also examined. *hsp60* encodes a 60 kDa heat shock protein (Hsp60) that enhances invasion and elicits cytokine expression in macrophages [20, 21]. These genes were detected in all five isolates (Table 4), indicating that these isolates are human pathogenic.

4. Discussion

In Japan, hot springs are reported to be the major infectious source for *L. pneumophila* [9–11]. However, there is little information about *L. pneumophila* in environmental waters other than hot springs. In this study, we tested

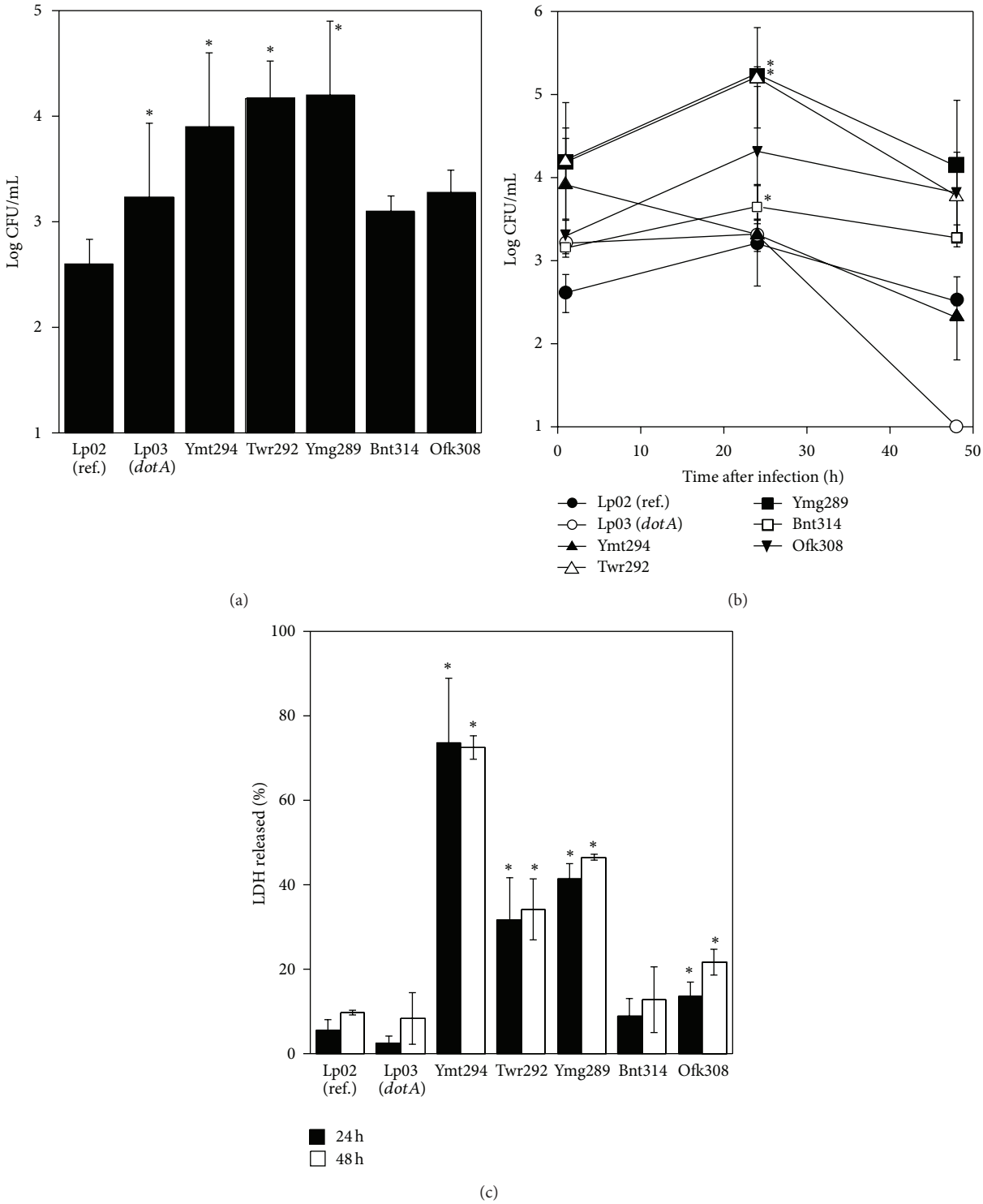

Figure 2: Invasion, intracellular growth, and cytotoxicity in HeLa cell. (a) HeLa cells were infected with *L. pneumophila* strains for 1 h. The infected cells were cultured in the presence of 50 μg/mL gentamicin. After 1 h of incubation, the infected cells were washed with PBS and lysed with cold distilled water. CFU were determined by serial dilution on CYET. (b) HeLa cells were infected with *L. pneumophila* strains at MOI of 100 for 1 h. The infected cells were cultured in the presence of 50 μg/mL gentamicin. The infected cells were cultured for 1, 24, and 48 h and washed with PBS followed by lysis with cold distilled water. CFU were determined by serial dilution on CYET. (c) HeLa cells were infected with *L. pneumophila* strains for 1 h. The infected cells were cultured in the presence of 50 μg/mL gentamicin for 1 h. After 24 or 48 h incubation, the cells were washed and cultured in fresh medium. The supernatants of infected cells were collected, and the release of LDH was measured. All values represent the average and the standard deviation for three identical experiments. Statistically significant differences compared with the control are indicated by asterisks ($*$, $P < 0.05$).

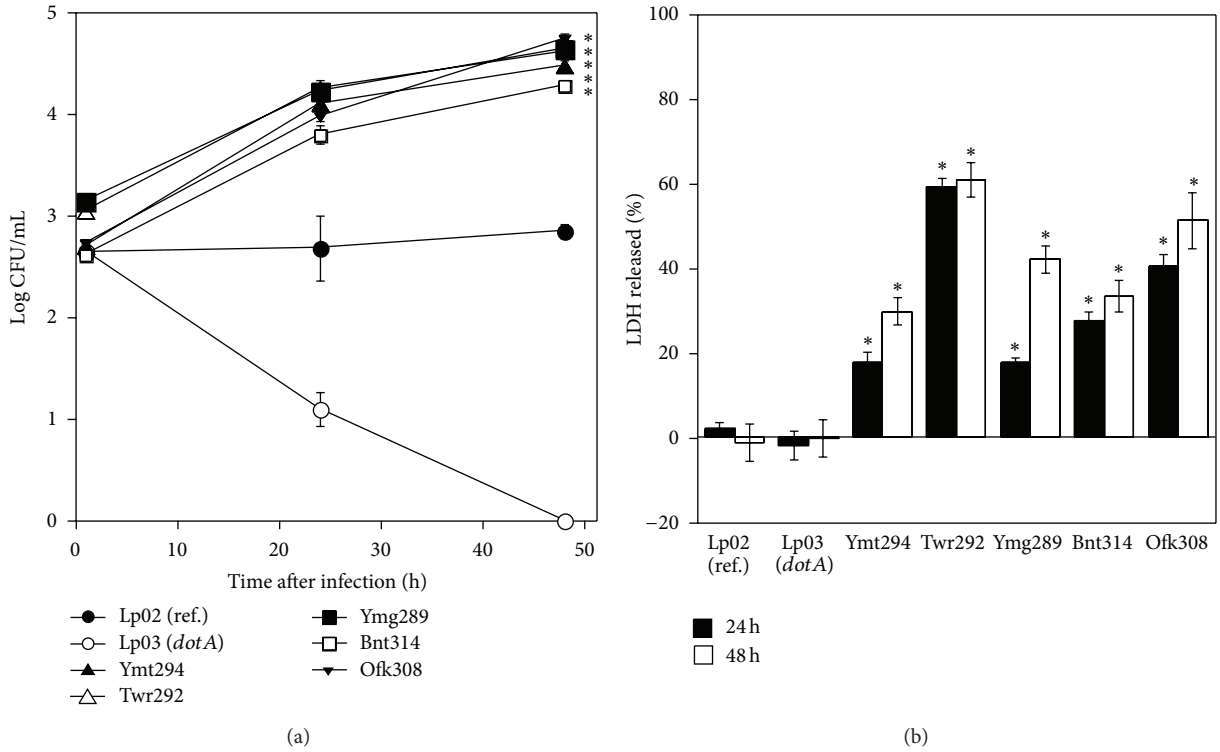

Figure 3: Intracellular growth and cytotoxicity in THP-1 cell. (a) THP-1 cells were infected with *L. pneumophila* strains at MOI of 1 for 1 h. The infected cells were cultured in the presence of 50 μg/mL gentamicin. The infected cells were cultured for 1, 24, and 48 h and washed with PBS followed by lysis with cold distilled water. CFU were determined by serial dilution on CYET. (b) THP-1 cells were infected with *L. pneumophila* strains for 1 h. The infected cells were cultured in the presence of 50 μg/mL gentamicin for 1 h. After 24 or 48 h of incubation, the cells were washed and cultured in fresh medium. The supernatants of infected cells were collected, and LDH release was measured. All values represent the average and the standard deviation for three identical experiments. Statistically significant differences compared with the control are indicated by asterisks ($*$, $P < 0.05$).

22 environmental water sites in Yamaguchi Prefecture, Japan. Eight were from ashiyu foot spas, seven were from water fountains, four were from basins, and three were from ponds. *L. pneumophila* was isolated from five sites (23%) (Table 1). Three were isolated from ashiyu foot spas (38%), one was isolated from a water fountain (14%), and the other was isolated from pond (33%). Interestingly, *L. pneumophila* was isolated mostly from ashiyu foot spas. Ashiyu foot spa is a type of hot spring where people bathe their feet. Ashiyu foot spa is usually in open air and freely available. Its temperature is generally controlled around 45°C. Older people often use this facility. For these people, *L. pneumophila*-containing aerosols generated from environmental waters could be a source of *L. pneumophila* infection. To the best of our knowledge, this is the first report related to isolation of *L. pneumophila* from ashiyu foot spa. Previous surveys of hot springs have demonstrated that around 30% of hot springs or public bathes were *L. pneumophila* positive [22, 23]. In this study, *L. pneumophila* was isolated from three of the eight sites (38%) of ashiyu foot spa sampled. These results may suggest an equivalent risk of contracting *L. pneumophila* at ashiyu foot spa as compared with hot spring. However, a more extensive survey is required to obtain more accurate epidemiological relevance and to analyze the risk of *L. pneumophila* infection from ashiyu foot spa.

The growth of the *L. pneumophila* isolates in liquid medium was almost the same as reference strain Lp02, but Ymt294 showed lower growth rate (Figure 1). Since the number of Ymt294 was not increased from 24 to 48 h, the growth of Ymt294 seemed to be saturated at one-tenth of final concentration of other strains. Intracellular growth of these isolates was different in HeLa and THP-1 cells. In HeLa cells, growth of isolates was significantly higher than Lp02 at 24 h after infection. However, the numbers of intracellular bacteria were decreased at 48 h after infection (Figure 2(b)). Some isolates such as Ymt294, Twr292, and Ymg289 showed strong cytotoxicity in HeLa cells, and cells were detached from culture plate at 48 h (Figures 2(c) and 4). This detachment may be a dominant factor of decrease in intracellular growth of those isolates. In THP-1 cells, the numbers of intracellular bacteria were increased from 24 to 48 h, despite the high cytotoxicity of those isolates (Figures 3(a) and 3(b)). Consistent with the strong preference of *L. pneumophila* for macrophages, these results indicate that macrophages are more suitable for *L. pneumophila* growth than epithelial cells.

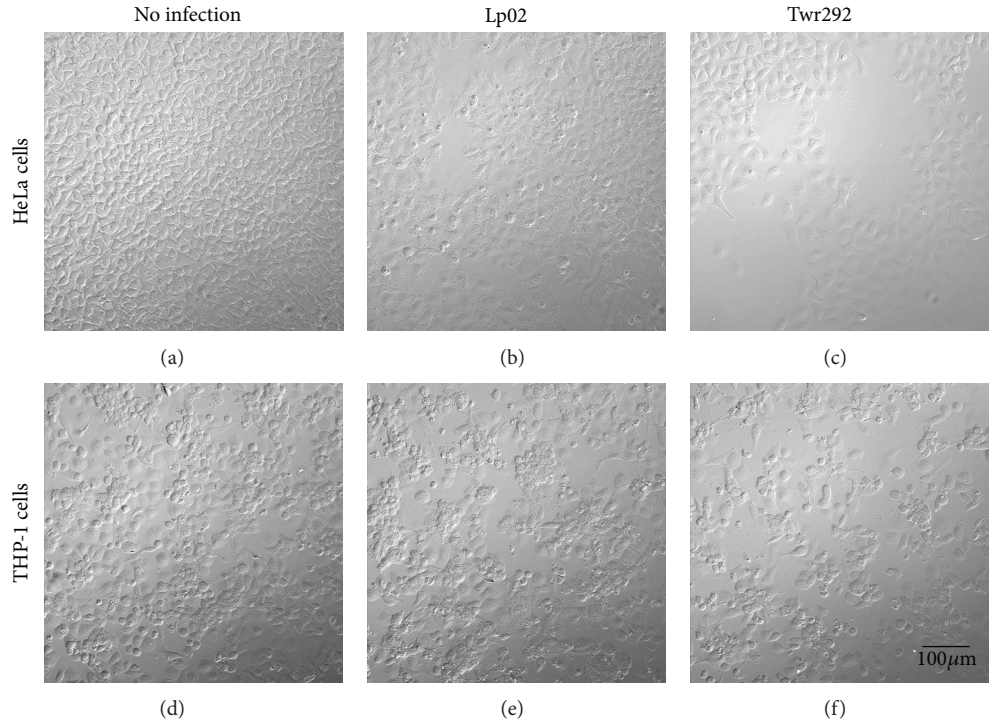

Figure 4: Cytotoxicity in HeLa and THP-1 cells. HeLa cells ((a)–(c)) and THP-1 cells ((d)-(e)) were infected with *L. pneumophila* strains Lp02 ((b) and (e)) or Twr292 ((c) and (f)) for 1 h. The infected cells were cultured in the presence of 50 μg/mL gentamicin for 1 h. The cells were washed and cultured in fresh medium. After 24 h of incubation, the condition of cells was observed using phase-contrast microscope.

Since all isolates harbored genes of well-characterized virulence factors including *dot*, *lvh*, *rtx*, and *hsp60*, the relationship between virulence factors and cytotoxicity or intracellular growth was not clear. However, the existence of genes of the virulence factors may suggest that those isolates can be human pathogenic. In particular, the Twr292 isolate from ashiyu foot spa showed high intracellular growth and strong cytotoxicity in HeLa and THP-1 cells. In addition, the contamination level of Twr292 was very high (128 CFU/100 mL). According to the guidelines of Japan's Ministry of Health, Labour and Welfare, the concentration of *L. pneumophila* should be maintained below 10 CFU/100 mL in hot springs or public bathes. The concentration of Twr292 was more than ten times that of the defined standard.

Overall, our results strongly suggest that ashiyu foot spa is a possible source of *L. pneumophila* infection.

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