because viable cells are fluorescent enough to be distinguished from dead cells and non-biological particles.

Images of bacterial cells on fabric swatches

The images of bacterial cells smaller than *C. albicans* were obtained with the UDF fluorescent microscope. A typical image of *P. fluorescens* environmental isolate that was inoculated on a fabric swatch is shown in Fig. 3. Many fluorescent spots with rod shape were speculated as single-cells of *P. fluorescens*. Non-specific fluorescence exists over the whole area of the swatch but this could be eliminated by a proper imaging program as described below. Figure 4 shows an image obtained with a swatch inoculated with *P. aeruginosa* ATCC 15442. In the magnified inset, respective single cells can be recognized. In this image, cells are more densely distributed than Fig. 3. In the case of *S. aureus* ATCC6538, the typical coccal shape could be clearly recognized as shown in Fig. 5 together with its magnified inset.

In the same manner, nine other species and strains (*B. cepacia* environmental isolate, *Pseudomonas* sp. environmental isolate (Zinc pyrithione resistant), *P. aeruginosa* environmental isolate, *C. freundii* environmental isolate, *S. marcescens* environmental isolate, *E. gergoviae* environmental isolate, *K. pneumoniae 2* environmental isolates, and *E. cloacae* environmental isolate (HCHO resistant)) could be visualized (Data not shown).

In spite of asperity of the fabric surface, microbial cells widely distributing throughout the fabric surface could be recognized.

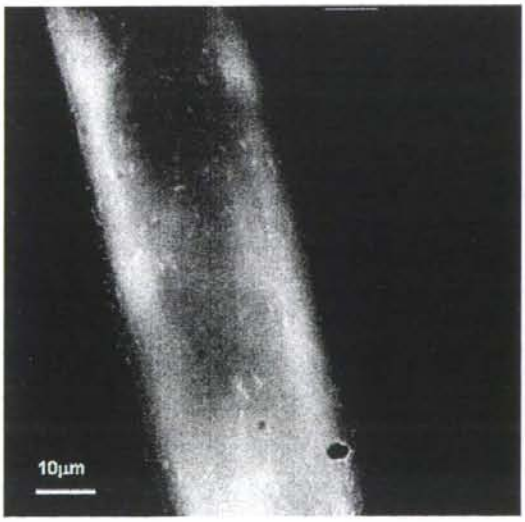

Fig. 3 Fluorescent image of a fabric swatch inoculated with *P. fluorescens* environmental isolate taken with the UDF fluorescent microscope

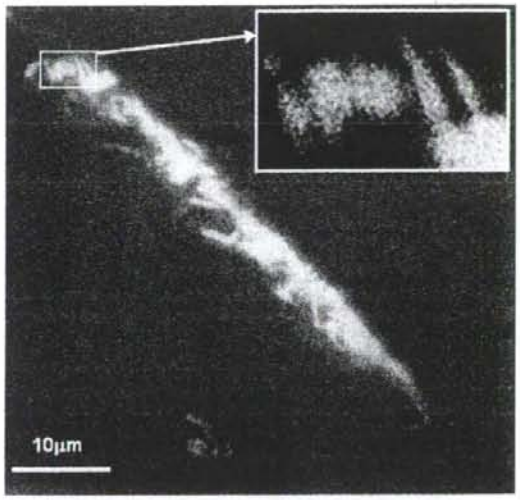

Fig. 4 Fluorescent image of a fabric swatch inoculated with *P. aeruginosa* ATCC15442 taken with the UDF fluorescent microscope

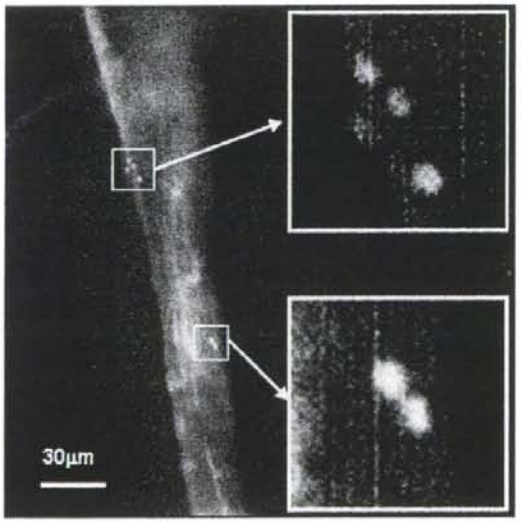

Fig.5 Fluorescent image of a fabric swatch inoculated with *S. aureus* ATCC6538 taken with the UDF fluorescent microscope

Observation of floating of microbial cells in damp-dried fabrics

In the course of this study, we have often observed the floating cells in damp-dried fabrics. Such water in damp-dried fabrics should contain salts and detergents. Their concentrations should be sufficiently low just after washing but they might be unexpectedly concentrated during successive drying process. Consequently such a residual

solution should be toxic to cells. The present method is based on the viable cell counting and therefore its possible influence on the un-removed viable cells should be considered.

To demonstrate that the developed method can detect viable cells even in such an environment, a damp-dried fabric was observed. A series of photographs from a to i of Fig. 6 were taken at every 1 s. If these photographs are carefully observed, it may be recognized that fluorescent spots are fluctuating during this observation period. In order to depict the cell fluctuation, one target cell was circled. In fact, the fluorescent spots were continually fluctuating in the microscopic view. Some spots disappeared probably because they moved to out-of-focus plane.

Demonstration of EMR evaluation

In order to demonstrate the evaluation of EMR of a laundry detergent, fabric swatches inoculated with *C. albicans* ATCC 10231 were observed before and after the washing treatment with the test detergent. Before the washing, viable cells on the swatch were observed as depicted in Fig. 7a. In contrast, after the washing, no fluorescent cell was observed (Fig. 7b). Therefore the EMR can potentially be evaluated.

In order to express EMR quantitatively, the image analysis program was optimized so that the non-specific fluorescent area may be discriminated from microbial spots. Figure 8 shows a typical result obtained after the image processing. The fluorescent spots in Fig. 8a were speculated as microbial cells. In this case, there are 39 cells. After the washing, no cell was detected (Fig. 8d). These analyses were repeated as triplicates.

Using another pair of test swatches, EMRs determined by the developed method and the conventional colony count method were compared. In the developed method, six square spots (each square $6.4\times10^{-5}\,cm^2$, total $3.8\times10^{-4}\,cm^2$) depicted in Fig. 9 were analyzed. Before the washing treatment with a detergent, the total number of viable cells detected in these six squares was 288 cells. After the washing, no cell was detected, indicating the total number of cells was smaller than 1. Therefore EMR may be determined as $\log[(288)/(<1)]$ = greater than 2.5. On the other hand, in the colony count method, the number of cells on the whole surface of the swatch ($0.5\times1.0\,cm^2$) before the washing was 2×10^6 cfu. After the washing, it decreased to be 4×10^2 cfu. Therefore, EMR may be determined as $\log[(2\times10^6)/(4\times10^2)]=3.7$. Both results are consistent with each other under the present definition of EMR.

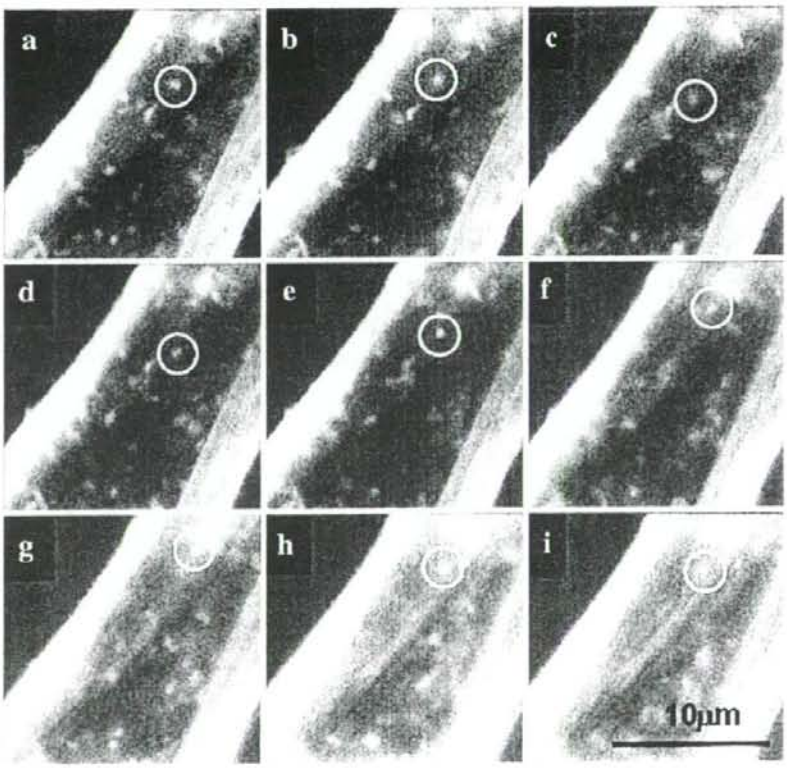

Fig. 6 Real time images of floating cells of *P. fluorescens* environmental isolate on a damp-dry fabric swatch. Photographs **a**–**i** were taken at every 1 s

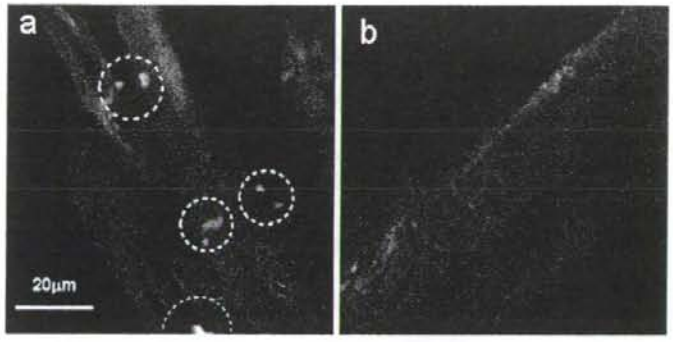

Fig. 7 Fluorescent images of a fabric swatch inoculated with *C. albicans* ATCC10231 taken before (**a**) and after (**b**) the washing treatment with a detergent

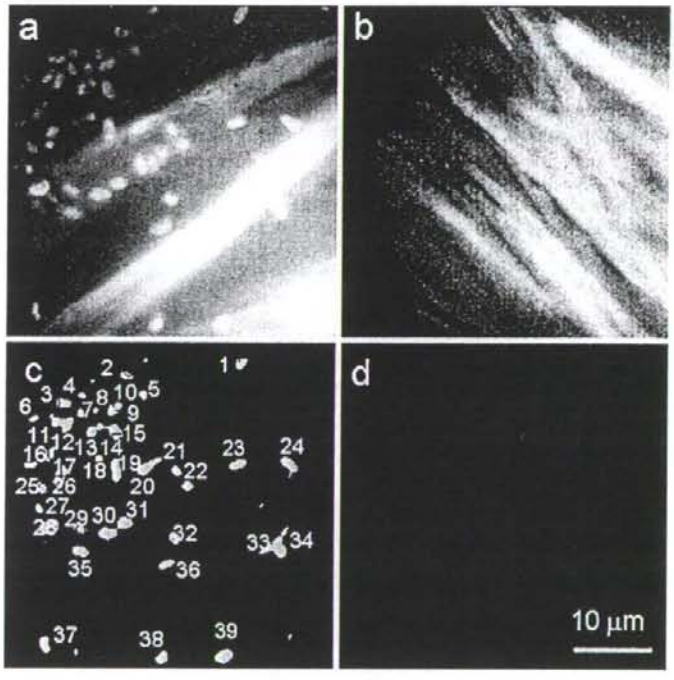

Fig. 8 Microbial cells on a fabric swatch numbered by image processing. A fabric swatch was inoculated with *C. albicans* ATCC 10231 and washed with a detergent. **a**, **c** Before washing; **b**, **d** after washing; **a**, **b** before image processing; **c**, **d** after image processing

Discussion

In the developed method, the observation area of a test fabric is only 3.8×10^{-4} cm^2 that is smaller than 0.1% of whole area (0.5 cm^2). Therefore, it should be of no use to compare the total number of cells localized in the observed area simply with the total number of cells washed out from whole area. What is worth discussing should be the relative number of cells before and after washing treatment, i.e., the indicator of EMR. In this sense, EMR estimated by the developed method was thought to be a reasonable value in comparison with EMR determined by the standard method [1]. In order to overcome a problem of statistics, it is essential to develop a more advanced system that can analyze much more observation spots at higher speed with higher spatial resolution.

The in-focus depth of an objective lens is a few micrometers (Fig. 10a), but the lens can be driven automatically in the Z-direction so that a fabric swatch with no greater than 1.0 mm surface roughness can be observed continuously in XY-plane (Fig. 10b). On the other hand, its working distance is 130 μm (Fig. 10a) and therefore the maximum focusing range in the fabric swatch is 130 μm. The cells entrapped in the indicated range can be detected by Z-scanning and displayed as a single image by image integration (Fig. 10c).

Cells entrapped in the fabric matrix beyond this range, if any, cannot be detected directly. These cells are less likely to be removed in the washing tests. To detect

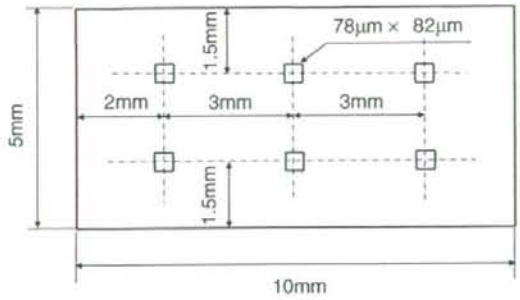

Fig. 9 Sketch of 6-square sampling points for EMR measurements

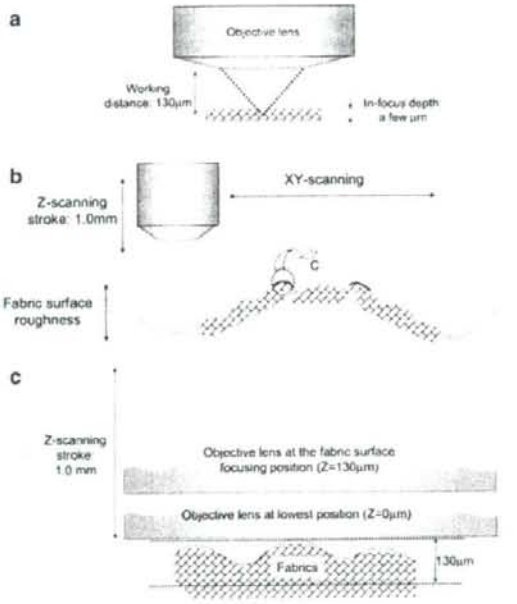

Fig. 10 Key dimensions of the developed apparatus. **a** Working distance and in-focus depth, **b** Z-scanning stroke and fabric surface roughness and **c** focusing range in a fabric swatch

these cells, it should be necessary to incubate the fabric in a culture medium for long time. For this purpose, the HCHO treatment after 2NBDG staining should be skipped to maintain the viability of these cells. The number of cells thus estimated together with the result of the developed method may provide us with useful information about the influence of fabric structure and degree of cell–substrate interaction.

The use of 2NBDG characterizes this study. 2NBDG can be taken only by the cells with high viability and concentrated in the cells [29]. This contrasts other dyes that enter the cells by passive diffusion [11, 13, 22]. In comparison with DNA staining dyes, 2NBDG is thought to be more specific to viable cells and therefore less liable to cause pseudo positive data. A typical case in which the use of 2NBDG is thought to be advantageous is biofilms on fabrics with antibacterial surface. The viable to dead cell ratio in biofilms is originally 70–95% [10] but it is reduced markedly when the surface maintains chemically modified antibacterial activity. In such a case, it is preferable to count only viable cells.

2NBDG cannot be taken by every species equally [15, 16]. Therefore, users should check if the microorganisms under consideration can take 2NBDG. 2NBDG can be taken by *E. coli* via mannose transporter as well as via glucose transporter (unpublished data). However, 2NBDG cannot stain every species and strain. Whoever wants to use this technique should make sure first that the microorganism that he wants to study can be stained with 2NBDG efficiently. If it cannot be stained with 2NBDG, it is necessary to use alternative dye. 2NBDG could not stain viable cells of 6 out of 41 strains but these unstained six strains could be stained with NBD-modified amino acids such as NBD-Gly and/or NBD-Leu (16 and unpublished data).

In conclusion, the present study has shown a successful combination of a novel UDF fluorescent microscope and a viable staining method.

Acknowledgements This research was supported by the Microbial Visualization Community of Practice in Procter & Gamble Company. The authors would like to thank Dr. P. Geis and Dr. S. Donaldson in Procter & Gamble Company for the constructive discussions and for reviewing the manuscript and Mr. Tottori of Kogaku Inc. for the supports and inputs for the microscopic system designs. This work is partially supported by the know-hows developed in the research project: The High Throughput Creation of Disease Model Cells and the Analysis of Their Function, which is funded by Core Research for Evolutional Science and Technology (CREST) of Japan Science and Technology Agency.

References

1. ASTM E2274-03 (2004) Standard test method for evaluation of laundry sanitizers and disinfectants
2. Borkow G, Gabbay J (2004) Putting copper into action: copper-impregnated products with potent biocidal activities. FASEB J 18:1728–1730
3. Brannan D-K, Dille J-C, Kaufman D-J (1987) Correlation of in vitro challenge testing with consumer use testing for cosmetic products. Appl Environ Microbiol 53:1827–1832
4. Buda A, Sands C, Jepson M-A (2005) Use of fluorescence imaging to investigate the structure and function of intestinal M cells. Adv Drug Deliv Rev 57:123–134
5. Burton K (2003) An aperture-shifting light-microscopic method for rapidly quantifying positions of cells in 3D matrices. Cytometry A 54:125–131
6. Cen L, Neoh K-G, Kang E-T (2004) Antibacterial activity of cloth functionalized with *N*-alkylated poly(4-vinylpyridine). J Biomed Mater Res A 71:70–80
7. JIS L0803 (1998) Standard adjacent fabrics for straining of colour fastness test
8. JIS L0844 (1997) Test methods for colour fastness to washing and laundering
9. JIS L1902 (2002) Testing for antibacterial activity and efficacy on textile products
10. Klueh U, Wagner V, Kelly S, Johnson A, Bryers J-D (2000) Efficacy of silver-coated fabric to prevent bacterial colonization and

subsequent device-based biofilm formation. J Biomed Mater Res 53:621–631
11. Laflamme C, Lavigne S, Ho J, Duchaine C (2004) Assessment of bacterial endospore viability with fluorescent dyes. J Appl Microbiol 96:684–692
12. Larson E-L, Lin S-X, Gomez-Pichardo C, Della-Latta P (2004) Effect of antibacterial home cleaning and handwashing products on infectious disease symptoms: a randomized, double-blind trial. Ann Intern Med 140(5):321–329
13. Lepeuple A-S, Gilouppe S, Pierlot E, de Roubin M-R. (2004) Rapid and automated detection of fluorescent total bacteria in water samples. Inter J Food Microbiol 92:327–332
14. Lin J, Qiu S, Lewis K, Klibanov A-M (2003) Mechanism of bactericidal and fungicidal activities of textiles covalently modified with alkylated polyethylenimine. Biotechnol Bioeng 83:168–172
15. Matsuoka H., Kurokawa T, Oishi K, Saito M (2002) Rapid detection of a small number of viable cells by the fluorescent glucose method. J Food Safety 22:141–153
16. Matsuoka H, Oishi K, Watanabe M, Kozone I, Saito M, Igimi S (2003) Viable cell detection by the combined use of fluorescent glucose and fluorescent glycine. Biosci Biotechnol Biochem 67:2459–2462
17. McNally J-G, Karpova T, Cooper J, Conchello J-A (1999) Three-dimensional imaging by deconvolution microscopy. Methods 19:373–385
18. Norman K (2005) Techniques: intravital microscopy—a method for investigating disseminated intravascular coagulation? Trends Pharmacol Sci 26:327–332
19. Petrocci A-M, Clarke P (1969) Proposed test method for antimicrobial laundry additives. J AOAC 52:836–842
20. Roldán M, Thomas F, Castel S, Quesada A, Hernández-Mariné M (2004) Noninvasive pigment identification in single cells from living phototrophic biofilms by confocal imaging spectrofluorometry. Appl Env Microb 70:3745–3750
21. Staudt C, Horn H, Hempel D-C, Neu T-R (2004) Volumetric measurements of bacterial cells and extracellular polymeric substance glycoconjugates in biofilms. Biotechnol Bioeng 88:585–592
22. Sunamura M, Maruyama A, Tsuji T, Kurane R. (2003) Spectral imaging detection and counting of microbial cells in marine sediment. J Microbiol Method 53:57–65
23. Takai K, Ohtsuka T, Senda Y, Nakao M, Yamamoto K, Matsuoka J, Hirai Y (2002) Antibacterial properties of antimicrobial-finished textile products. Microbiol Immunol 46:75–81
24. USEPA 712-C-97-056 (1997a) General requirements for public health uses of antimicrobial agents. Product Performance Test Guidelines OPPTS 810.2000
25. USEPA 712-C-97-056 (1997b) Products for use on hard surfaces – Basic efficacy data requirements. Product Performance Test Guidelines OPPTS 810.2100
26. USEPA 712-C-97-091 (1997c) Products for use on fabrics and textiles. Product Performance Test Guidelines OPPTS 810.2300
27. Vermeersch G, Leloup G, Delmee M, Vreven J (2005) Antibacterial activity of glass–ionomer cements, compomers and resin composites: relationship between acidity and material setting phase. J Oral Rehabil 32:368–374
28. Yamada K, Nakata M, Horimoto N, Saito M, Matsuoka H, Inagaki N (2000) Measurement of glucose uptake and intracellular calcium concentration in single, living pancreatic β-cells. J Biol Chem 275:22278–22283
29. Yoshioka K, Takahashi H, Homma T, Saito M, Oh K.-B, Nemoto Y, Matsuoka H (1996) A novel fluorescent derivative of glucose applicable to the assessment of glucose uptake activity of *Escherichia coli*. Biochim Biophys Acta 1289:5–9

FULL PAPER *Bacteriology*

Biochemical and Molecular Characterization of Minor Serogroups of Shiga Toxin-Producing *Escherichia coli* Isolated from Humans in Osaka Prefecture

Kazuko SETO[1], Masumi TAGUCHI[1], Kazuhiro KOBAYASHI[1] and Shunji KOZAKI[2]*

[1]*Department of Infectious Diseases, Osaka Prefectural Institute of Public Health, 1–3–69 Nakamichi, Higashinari-ku, Osaka 537–0025 and* [2]*Department of Veterinary Science, Graduate School of Life and Environmental Sciences, Osaka Prefecture University, 1–1 Gakuen-cho, Naka-ku, Sakai, Osaka 599–8531, Japan*

(Received 28 May 2007/Accepted 2 August 2007)

ABSTRACT. We have investigated 37 minor serogroup Shiga toxin-producing *Eschrichia coli* (STEC) strains other than O157, O26, and O111 isolated from human specimens in Osaka prefecture to determine their serological and biochemical characteristics, virulence-associated genes, and clinical signs in patients. The same serotype strains were genotyped by pulsed-field gel electrophoresis (PFGE). The O antigen of 33 strains were typed into 10 serogroups; O28, O63, O65, O91, O103, O119, O121, O126, O165, and O177, and other 4 strains were not agglutinated with any serum. Four different Shiga toxin (Stx) types (1, 2, 2c, and 2f) were distributed in these isolates. The intimin gene was present in 83.8% of the strains and subtyped into intimin α, β, ε, and ζ. STEC O165, O177, and OUT isolated from hemolytic uremic syndrome (HUS) patients revealed atypical biochemical characters; negative reaction for lysine decarboxylase and gas production from glucose. Eleven strains including the isolates from HUS patients generated no colonies on cefixime-tellurite (CT)-sorbitol-MacConkey agar plates, since they showed high sensitivity (MIC $\leq$ 1.25 μg/ml) to potassium tellurite. The finding show supportive information for use the selective agar plates with and without CT supplement for the isolation of minor serogroup STEC. PFGE analysis revealed that the strains isolated from family cases were closely related within the respective family, and it was useful for epidemiological analysis of minor serogroup STEC.

KEY WORDS: biochemical characteristics, minor serogroup, PFGE, STEC, *stx*.

J. Vet. Med. Sci. 69(12): 1215–1222, 2007

Shiga toxin-producing *Escherichia coli* (STEC) is known as an important cause of gastrointestinal disease in developed countries [5, 36, 42]. The public health impact of STEC infections is high because of their ability to cause secondary infections and systemic complications, such as hemolytic uremic syndrome (HUS) [3, 15, 23]. In Japan, STEC infection is classified as a category III notifiable infectious disease under the National Epidemiological Surveillance of Infectious Diseases (NESID) in compliance with the Law Concerning the Prevention of Infectious Diseases and Medical Care for Patients of Infections (Infectious Diseases Control Law), and reporting by physicians is mandatory. More than 3,000 new symptomatic and asymptomatic cases of STEC infection are reported every year, although reports have decreased slightly when compared with the reports from 2004 [30]. In 2005, 1600 STEC isolates were reported from prefectural and municipal public health institutes (PHIs) to the Infectious Diseases Surveillance Center (IDSC) of the National Institute of Infectious Diseases (NIID). The ratio of O157 isolates was decreased from 75.4% in 1997 to 68.4% in 2005. O26 accounted for 22% and O111 for 4.6% of the total STEC isolates in 2005. In addition, serogroups other than O157, O26, and O111 increased from 33 isolates (9 serogroups) in 1997 to 52 isolates (14 serogroups) in 2005 [30, 31]. Recently there was an outbreak of STEC O121 infections among school children in Chiba Prefecture [1]. Many effective and selective media are widely used in routine laboratory examination for the isolation of STEC O157 that have sorbitol-nonfermenting and β-glucuronidase-negative characteristics. Rhamnose and sorbose are also utilized as indicators for the isolation of STEC O26 and STEC O111, respectively [16, 41]. The biochemical characteristics of various other STEC serogroups have not yet been reported. In Osaka Prefecture, some cases of HUS caused by non-O157 STEC have been reported since 1997. The development of rapid and effective methods for the isolation of 'minor serogroup' (serogroup other than O157, O26, and O111) STEC are desirable to prevent secondary infections and analyze the infectious route. Methods for the detection of Stx, the cardinal virulence factors, directly from human stool or colonies on selective agar plate have been described [18, 24, 34], but these have been failures due to low sensitivity and false positives [8]. Isolation of STEC is required not only for reporting to NESID but also for epidemiological investigation.

Symptoms range from mild diarrhea to hemorrhagic colitis, and the infection may be complicated with HUS. STEC has been isolated from healthy individuals as well. It was suggested that the clinical outcome of STEC infection is associated with the Stx type and intimin, an outer membrane protein responsible for the intimate adherence between the bacteria and the intestinal epithelial cell membrane [7, 10, 13]. The finding that enterohemorrhagic *E. coli* (EHEC) hemolysin, called enterohemolysin, might act as a virulence factor in STEC O157, O26, and O111 was reported [6]. We

* CORRESPONDENCE TO: KOZAKI, S., Department of Veterinary Science, Graduate School of Life and Environmental Sciences, Osaka Prefecture University, 1–1 Gakuen-cho, Naka-ku, Sakai, Osaka 599–8531, Japan.
e-mail: kozaki@center.osakafu-u.ac.jp

therefore investigated minor serogroup STEC that had been collected from human cases from 1996 to 2006 in Osaka Prefecture to characterize the isolates for their serotypes, virulence factors, and their correlation with disease. Furthermore, we applied pulsed-field gel electrophoresis (PFGE) to analyze the similarity of isolates in the same serotype. The aims of this study were also to describe the characteristics as indicator of effective detection of minor serogroup STEC and to evaluate the tools for epidemiological analysis.

MATERIALS AND METHODS

Strains: STEC identified to serogroups except O157, O26, and O111 were used in this study. These strains were isolated from 24 patients and 13 healthy carriers between January 1996 and December 2006. Their clinical features are summarized in Table 1.

Serotype identification: Serotyping of O antigen (lipopolysaccharide) and H antigen (flagellar) of motile strains was performed according to the agglutination test [32] using *Escherichia coli* antisera set 1 and set 2 (Denka Seiken, Tokyo, Japan). The strains untypeable with commercially available serum were identified at the National Institute of Infectious Disease. The H types of nonmotile strains were investigated for the flagellin genes (*fliC*) by PCR followed by *Hha*I digestion of *fliC* PCR products and evaluation of restriction fragment length polymorphism (RFLP) patterns, as previously described [28].

Biochemical characterization: The strains were examined for biochemical properties using conventional methods [11]. The carbohydrate-fermenting ability was determined with pepton water containing Andrade's indicator (1%) and one of the following 14 carbohydrates (1%) (Wako Pure Chemicals, Osaka, Japan): adonitol, arabinose, dulcitol, glucose, inositol, lactose, maltose, mannitol, rhamnose, salicin, sorbitol, sucrose, trehalose, or xylose, after 3 days incubation at 37°C. The activity of β-glucuronidase was observed by inoculating on CLIG medium (Kyokuto Pharmaceutical, Tokyo, Japan).

Typing of Shiga toxins and stx genes: Production of Stx1 and Stx2 was tested using a reverse passive latex agglutination test (VTEC-RPLA; Denka Seiken), according to the manufacturer's instructions. The detection and subtyping of *stx* genes was performed by *Hinc*II digestion of a 900 bp DNA product, which was obtained by PCR with primers Lin5' and Lin3' [2].

Detection and subtyping of intimin gene (eae): The *eae* genes were detected by PCR using primers eaek1 and EA2 [26]. The subtyping of *eae* genes into intimin α, β, γ, ε, and ζ was performed by PCR with primer SK1 in combination with primers LP2 to LP6B [44].

Hemolytic phenotype and detection of EHEC hemolysin gene (ehxA): The hemolytic activity was assayed on enterohemolysin agar plates (Kanto Chemical, Tokyo, Japan) containing washed sheep blood and 10 mM calcium chloride, and sheep blood agar plates (Kanto Chemical). Enterohemolysin is only observed on the enterohemolysin agar plate,

Table 1. Source and number of isolates and clinical features of individuals infected with minor serogroup STEC

Serotype	Number of			Symptoms[c]				
	Family[a]	Sporadic[b]	Persons	AP	D	BD	HUS	None
O28:H20		1	1	1	1			
O63:H6	1	2	4	1	2			2
O65:NM[d]	2		5	4	1	3		1
O91:H14		1	1					1
O103:H2	1	2	6	3	1	2		3
O103:H11		1	1	1		1		
O119:H4		1	1					1
O119:[H25][e]	1		3					3
O121:H19		4	4	3	1	3		
O126:H8		1	1		1			
O165:[HUT][f]		5	5	4	2	3	2	
O177:[HUT]		1	1	1		1	1	
OUT[g]:H2		1	1					1
OUT:H14		1	1					1
OUT:H25		1	1	1	1			
OUT:[HUT]		1	1	1		1	1	

a) Family cases.
b) Sporadic cases.
c) AD; abdominal pain, D;nonbloody diarrhea, BD; bloody diarrhea, HUS; hemolytic uremic syndrome, None; asymptomatic.
d) Nonmotile and negative in the *fliC*-specific PCR.
e) An H type in brackets indicates the presence of non-motile (NM) strains, which were analyzed for their *fliC* type by PCR-RFLP.
f) HUT means untypeable with PCR-RFLP of *fliC*.
g) OUT means untypeable with antisera specific for O1 to O181.

and α-hemolysin reveals hemolysis on both agar plates [6]. Detection of *ehxA* was performed with primers hlyAF and hlyAR [35].

Growth on CT-SMAC and MIC of potassium tellurite: The growth of the strains was evaluated by comparing the colonies on MacConkey sorbitol agar (SMAC; Nissui, Tokyo, Japan) with and without CT supplements (cefixime;final 0.05 mg/liter and potassium tellurite; 2.5 mg/liter, ASKA Diagnostics, Tokyo, Japan). The bacterial solutions were adjusted to the 0.5 MacFarland standard (ca. 10^8 CFU/m*l*) with BBL Trypticase Soy Broth (Becton, Dickinson and Company, Sparks, MD, U.S.A.). Ten microliter of the solution was applied to SMAC and CT-SMAC, and incubated for 20 hr at 37°C. The MIC of potassium tellurite (Dynal A. S., Oslo, Norway) was measured by the agar dilution method [22] using SMAC as a substitute for Mueller Hinton agar.

Antimicrobial susceptibility testing: The antimicrobial susceptibilities were determined by the disk diffusion method [29] with the following 12 antimicrobial agents (Becton, Dickinson and Company): ampicillin, cefotaxime, chloramphenicol, ciprofloxacin, fosfomysin, gentamicin, kanamycin, nalidixic acid, ofloxacin, streptomycin, sulfamethoxazole-trimethoprim, and tetracycline.

PFGE: PFGE was performed according to the method of PulseNet Japan [43], using *Xba*I and *Bln*I (Roche Diagnostics, Mannheim, Germany). *Salmonella* Braenderup H9812 PulseNet Standard Strain was kindly provided by the Centers for Disease Control and Prevention (CDC) [19]. FingerprintingII Version 3 (Bio-Rad Laboratories, Hercules, CA, U.S.A.) was used for calculating the Dice similarity indices (tolerance 1.2%, unweighted pair group method using arithmetic averages) in the cluster analysis.

RESULTS

Serological diversity of STEC isolates: Of a total of 1,705 STEC strains, 1,519 O157 strains (89.1%), 136 O26 strains (8.0%), and 13 O111 strains (0.8%) were isolated during the period from January 1996 to December 2006. A total of 37 minor serogroup STEC strains were isolated from 5 family cases and 23 sporadic cases, and classified into 16 different O:H serotypes. Twenty-two motile strains belonged to the following 11 serotypes: O28:H20, O63:H6, O91:H14, O103:H2, O103:H11, O119:H4, O121:H19, O126:H8, O untypeable (OUT):H2, OUT:H14, and OUT:H25, and 15 other nonmotile strains were analyzed for their H type by *fliC*-specific PCR. Three strains belonging to O119 were identified as O119:[H25]. Although the same restriction pattern digested by *Hha*I was present in 7 strains belonging to serogroup O165, O177, and OUT, the pattern was not identical with any patterns that had been reported previously. Therefore these strains were designated as O165:[HUT], O177:[HUT], and OUT:[HUT]. Only serogroup O65 (5 isolates) was classified as O65:NM, because no amplification product was detected with the *fliC*-specific PCR (Table 1).

Clinical features: Twenty-four strains were isolated from patients, and the other strains were obtained from asymptomatic carriers (Table 1). HUS developed in 4 patients, and abdominal pain was the dominant symptoms in 20 patients. Bloody diarrhea and nonbloody diarrhea appeared in 14 and 10 patients, respectively. The serotypes isolated from HUS patients were O165:[HUT] (2 isolates), O177:[HUT], and OUT:[HUT]. The strains belonging to serotypes O91:H14, OUT:H2, and OUT:H14 were isolated from food-providing workers who showed no symptoms. The remaining asymptomatic carriers were family members of patients. O63:H6 (2 isolates) and O119:H4 were isolated from family members of STEC O157 patients. O119:[H25] (3 isolates) was isolated from family members of STEC O26 patients.

Biochemical characterization of isolates: The strains belonging to same serotypes showed the same results except for some carbohydrate fermentation results. The serotype O165:[HUT], O177:[HUT], and OUT:[HUT] strains revealed the most atypical phenotype, negative reaction for lysine decarboxylase and gas production from glucose. The serotype O119:[H25] and OUT:H25 strains were lysine decarboxylase-negative, and the serotype O65:NM strains did not produce gas from glucose. Sorbitol was not fermented in 5 serotypes; O63:H6, O119:[H25], O177[HUT], OUT:H14, and OUT:H25. Four serotypes (O103:H11, O165[HUT], O177[HUT], OUT:[HUT]) and two serotypes (O119:H4, O165:[HUT]) were rhamnose-negative and xylose-negative, respectively. Although it is well known that STEC O157 is β-glucuronidase negative, the minor serogroup STEC strains were positive except for O65:NM, which was positive on day 2 (Table 2).

Characterization of virulence factors: The production of Stx1 and Stx2 was examined with the VTEC-RPLA assay, and *stx* genotypes of the strains were determined by PCR-RFLP (Table 2). The serotype O65:NM strains produced both Stx1 and Stx2. Either Stx1 or Stx2 was detected in the other strains. The *stx* types of all strains were identical to the Stx types. The production of Stx2 and presence of *stx2* were founded in 16 strains of 6 serotypes (O28:H20, O63:H6, O121:H19, O165:[HUT], O177:[HUT], and OUT:[HUT]). O28:H20 (1 strain), O165:[HUT](3 strains), and OUT:[HUT] (1 strain) had *stx2c* in addition to *stx2*. Stx2 was also detected in O63:H6 (4 strains) carrying *stx2f*.

The *eae* gene was detected in 31 strains (83.8%) belonging to 10 serotypes. In the *eae*-positive strains, four intimin types, namely α, β, ε, and ζ, were detected (Table 2). Intimin ε was most frequent and detected in serotypes O103:H2, O121:H19, O165:[HUT], O177:[HUT], and OUT:[HUT]. Intimin α was found in O63:H6, β in O65:NM and O103:H11, and ζ in O119:[H25] and OUT:H25. The *ehxA* gene was detected in 31 strains including all except for 3 serotypes (O63:H6, O119:H4, and O126:H8), but eight strains, O165:[HUT], O177:[HUT], OUT:H14, and OUT:[HUT] showed no enterohemolytic activity. The 6 *ehxA* negative strains were negative for enterohemolytic activity (Table 2). There were no strains demonstrating α-hemolysis.

Table 2. Biochemical and virulence-associated characteristics of minor serogroup STEC isolated from human in Osaka Prefecture

Serotype	Number of strains	Biochemical characteristics[a]						Toxin type(s)		*eae*	Intimin type	Hemolytic activity[b]	*ehxA*
		LDC	Gas	GUR	Rhamnose	Sorbitol	Xylose	RPLA	PCR-RFLP				
O28:H20	1	+[c]	+	+	+	+	+	2	2+2c	–		+	+
O63:H6	4	+	+	+	+	–	+	2	2f	+	α	–	–
O65:NM[d]	5	+	–	(+)	(+)	+	+	1+2	1+2	+	β	+	+
O91:H14	1	+	+	+	+	+	+	1	1	–		+	+
O103:H2	6	+	+	+	d	+	+	1	1	+	ε	+	+
O103:H11	1	+	+	+	–	+	+	1	1	+	β	+	+
O119:H4	1	+	+	+	+	+	–	1	1	–		–	–
O119:[H25][e]	3	–	+	+	+	–	(+)	1	1	+	ζ	+	+
O121:H19	4	+	+	+	+	+	d	2	2	+	ε	+	+
O126:H8	1	+	+	+	+	+	+	1	1	–		–	–
O165:[HUT][f]	5	–	–	+	–	d	–	2	2+2c, 2[g]	+	ε	–	+
O177:[HUT]	1	–	–	+	–	–	+	2	2	+	ε	–	+
OUT[h]:H2	1	+	+	+	+	+	+	1	1	–		+	+
OUT:H14	1	+	+	+	+	–	+	1	1	–		–	+
OUT:H25	1	–	+	+	+	–	+	1	1	+	ζ	+	+
OUT:[HUT]	1	–	–	+	–	+	+	2	2+2c	+	ε	–	+

a) LDC; lysine decarboxylase, Gas; gas production from glucose, GUR; β-glucuronidase.
b) Enterohemolytic activity observed on enterohemolysin agar plate only.
c) +; positive within 1 day, (+); positive after 2days , –; no reaction in 3days, d; different reactions.
d) Nonmotile and negative in the *fliC*-specific PCR.
e) An H type in brackets indicates the presence of non-motile (NM) strains, which were analysed for their *fliC* type by PCR-RFLP.
f) HUT means untypeable with PCR-RFLP of *fliC*.
g) Three isolates have *stx2* and *stx2c*. Two isolates have only *stx2*.
h) OUT means untypeable with antisera specific for O1 to O181.

Growth on CT-SMAC and MIC of potassium tellurite: In 11 strains of 7 serotypes (O28:H20, O91:H14, O126:H8, O165:[HUT], O177:[HUT], OUT:H2, and OUT:[HUT]), there were few colonies on CT-SMAC that was used as a selective agar plate for STEC O157. MICs of potassium tellurite for these strains were shown to be below 1.25 μg/ml (Table 3).

Antimicrobial resistance: Antimicrobial resistance patterns are shown in Table 4. Fifteen (40.5%) of the strains showed resistance to one or more antibiotics. The 5 strains belonging to serotype O65:NM that were isolated from 2 individual families showed identical resistance patterns. On the other hand, in one family case, the strain O103:H2 isolated from a patient showed multiple resistance, but the other strains from the family members were susceptible. There were no strains resistant to fosfomysin or ciprofloxacin (Table 4).

PFGE: A dendrogram of the *Xba*I digest pattern is shown in Fig. 1 for discrimination of the strains within the same serotype isolated from independent cases. The strains isolated from family cases were closely related (96% to 100% similarity) within the respective family. In the cluster of O65:NM the similarity of the strains isolated from Family B and Family C was 97%. In sporadic cases, the two O121:H19 strains isolated in 2003 showed the identical PFGE profile, and the two O63:H6 strains isolated in 2003 and 2004 showed 97% similarity as high as the strains of Family D. In the cluster of O165:[HUT], five strains were heterogeneous but three strains isolated in 2006 were related (93% similarity). The strains with the identical patterns by *Xba*I digestion revealed also high similarity by *Bln*I digestion (data not shown).

Table 3. Growth on CT-SMAC and MIC of potassium tellurite of minor serogroup STEC isolates

Growth on CT-SMAC	Serotype	Number of strains	MIC of potassium tellurite (mg/ml)
+	O63:H6	4	5
	O65:NM	5	>20
	O103:H2	6	>20
	O103:H11	1	>20
	O119:H4	1	5
	O119:[H25][a]	3	>20
	O121:H19	4	5
	OUT[b]:H14	1	5
	OUT:H25	1	>20
–	O28:H20	1	≤1.25
	O91:H14	1	≤1.25
	O126:H8	1	≤1.25
	O165:[HUT][c]	5	≤1.25
	O177:[HUT]	1	≤1.25
	OUT:H2	1	≤1.25
	OUT:[HUT]	1	≤1.25

a) An H type in brackets indicates the presence of non-motile (NM) strains, which were analysed for their *fliC* type by PCR-RFLP.
b) OUT means untypeable with antisera specific for O1 to O181.
c) HUT means untypeable with PCR-RFLP of *fliC*.

Table 4. Antimicrobial resistance pattern of minor serogroup STEC isolates

Resistance pattern[a]	Number of strains	Serotype
SM, TC, CP	1	O103:H2
SM, TC, KM	1	O119:H4
ABPC, SM	1	O103:H11
SM, KM	1	O103:H2
SM, TC	6	O65:NM(5)[b], O165:[HUT][c]
TC, CP	1	O165:[HUT]
CP	2	O121:H19, OUT[d]:[HUT]
SM	1	O165:[HUT]
TC	1	O126:H8
Total	15 (40.5%)	

a) SM; streptomycin, TC; tetracycline, CP; chloramphenicol, KM; kanamycin, ABPC; ampicillin.
b) Number in parentheses are number of isolates.
c) HUT in blackets means untypeable with PCR-RFLP of *fliC* of non-motile strains.
d) OUT means untypeable with antisera specific for O1 to O181.

DISCUSSION

Reports of STEC isolation from PHIs to IDSC have totalled 12,477 since 2000. Although O157 was the predominant serogroup, over 100 strains belonging to serogroups O103 or O121 that were untypeable with commercially available antisera until August 2005 were isolated [20]. In Osaka Prefecture, STEC O103 and STEC O121 were sometimes isolated, but the STEC identified as rare serogroups such as O65, O165, and O177, were found among clinical isolates. O177 is a new serogroup that was designated in 2004 [38]. The earliest clinical isolate of serotype O177:NM was *stx2*, *eae*, and *ehxA* positive and was provided from CDC in USA in 1998. The first STEC O177 isolate in Osaka Prefecture was isolated from a girl (4-years-old) who complicated with HUS following bloody diarrhea in July 2003, and it was also *stx2*, *eae*, and *ehxA* positive. Another O177 was isolated from a HUS patient (2-years-old) living in Osaka City in same month, but these two isolates showed different PFGE patterns [40]. Since the 5 isolates of STEC O65:NM used in this study were only reported to IDSC, they were isolated from five individuals of two families eating together at a *yaki-niku* restaurant. STEC O65:NM strains were isolated from swine feces in North America [9, 12]. The strains from humans in Osaka Prefecture were widely different from swine isolates, because the human isolates had *stx1*, *stx2*, and *eae* (intimin *β*) although most of swine isolates were *stx2e* positive [12], and *eae* negative [25]. STEC O165:[HUT] was isolated from two children in 1998 and 2001 who presented complicated HUS and three children in 2006 who presented diarrhea or bloody diarrhea. It became easier to detect *E. coli* O165 because seven antiserum, including O165 antisera, came onto the market in August in 2005. STEC belonging to serotypes O165:NM, O165:H19, and O165:H25 were isolated in Europe and Australia [13, 37]. The PCR-RFLP patterns of *fliC* of STEC O165 isolated in Osaka Prefecture was identical to the patterns of neither H19 nor H25, whereas they were the same with those of O177 and OUT isolated from HUS patients. It is of interest whether any strain of nonmotile STEC reported to IDSC shows the same pattern. Although ten isolations of STEC O63:H6 were reported from 2000 to 2006 in Japan, there are no reports of isolation of O63 from other countries [37]. The four O63:H6 strains in Osaka Prefecture produced Stx2, and had *stx2f* and intimin α genes. STEC O128 strains harboring *stx2f* were isolated from pigeons and infant patients [14, 21, 39]. In addition, intimin α was found frequently in enteropathogenic *E. coli* (EPEC) [33] and it was detected in only two STEC O177:H7 isolates in Germany [5]. In this study, we isolated and characterized the unique STEC O63:H6 haboring the *stx2f* and intimin α genes.

It is probable that rhamnose, sorbitol, or xylose can be used as a discriminative marker, because 80% of *E. coli* fermented these carbohydrates [11]. The serotypes O63:H6, O119:[H25], OUT:H14, OUT:H25, and O103:H11 can be detected on CT-SMAC or CT-RMAC used as selective medium for STEC O157 or STEC O26, because they were sorbitol- or rhamnose-negative and resistant to potassium tellurite. We attempted to prepare medium without potassium tellurite to detect STEC O165:[HUT], O177:[HUT], and OUT:[HUT] that did not ferment sorbitol and/or rhamnose, because these serotypes were susceptible to the compound. Since it is impossible to detect all minor serogroup STEC by using one media, we should pick up sorbitol-fermenting colonies on CT-SMAC, and use the medium without CT supplements, for example desoxycholate-hydrogen sulfide-lactose (DHL). When public health agencies investigate follow-up cultures, MacConkey agar base (Becton, Dickinson and Company) and DHL agar base (Nihon Pharmaceutical, Osaka, Japan) supplemented with a discriminative carbohydrate are useful. It is widely known that enteroinvasive *E. coli* reveal characteristics similar to *Shigella*. Since some of the minor serogroup STEC produced a negative reaction for lysine decarboxylase and gas production from glucose, we performed Stx examination of the atypical *E. coli*.

It was reported previously that enterohemolysin agar plates were useful as screening media for detection of non-O157 STEC [4]. In this study the serogroups O103 and O121 that were reported to IDSC frequently showed enterohemolytic activity, but 14 strains of 7 serotypes including isolates from HUS patients were negative. In addition, *ehxA* was not associated with disease severity [10], so that it is suggested that enterohemolysin agar plates are not suitable to detect minor serogroup STEC.

The National Veterinary Assay Laboratory reported that antimicrobial resistance was more frequent in serogroups O26 and O145 than serogroup O157 among bovine STEC strains [25]. Similar results were found in our study, where the resistance rate to antibiotics of minor serogroup STEC (40.5%) was higher than STEC O157 (10.8%) isolated in Osaka Prefecture in 2006. Although there were no strains

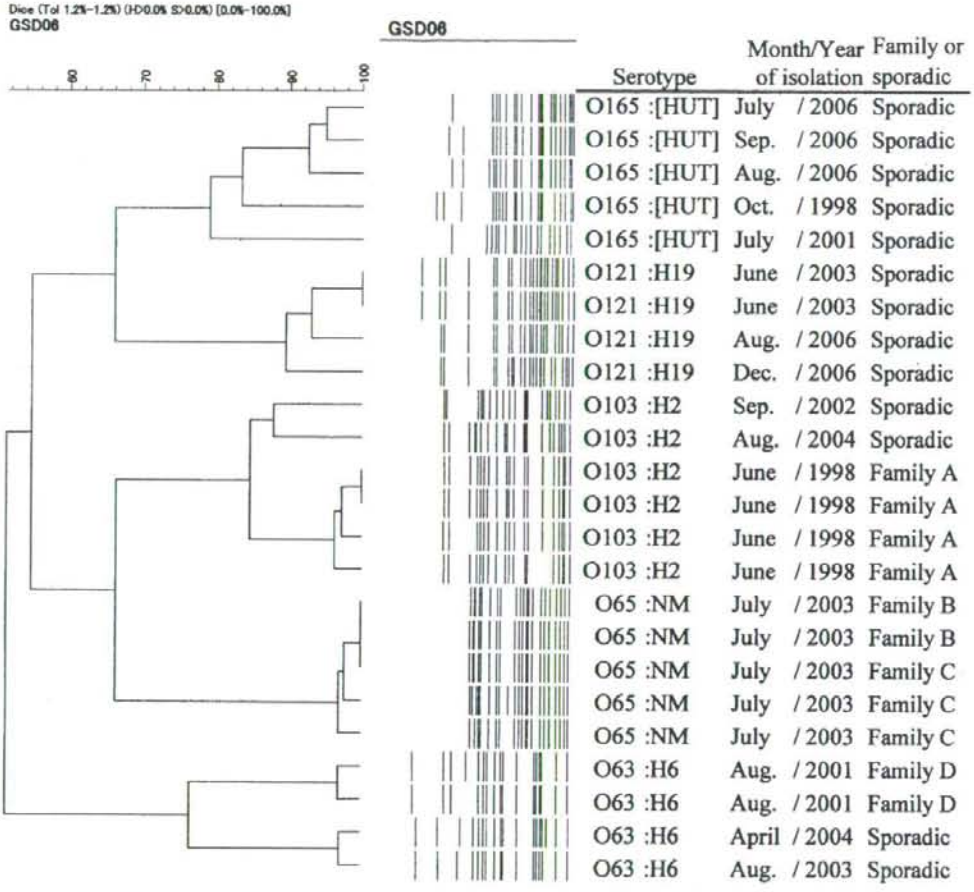

Fig. 1. Dendrogram of *Xba*I-digested PFGE profiles of STEC O63:H6, O65:NM, O103:H2, O121:H19, and O165:[HUT] strains. Fingerprinting II Version 3 (Bio-Rad Laboratories) was used for calculating the Dice similarity indices (tolerance 1.2%, unweighted pair group method using arithmetic averages) in the cluster analysis. The serotype of the strain, month and year of isolation, and whether the strain was isolated from a family case or sporadic case was indicated on the right.

resistant to the drugs for therapy of STEC infection, an outbreak due to multiple-drug resistant O26:H11 and the isolation of O26:H11 producing extended-spectrum β-lactamase have been reported in Japan[17, 27], so that the careful monitoring of antimicrobial resistance of minor serogroup STEC should be continued.

PFGE analysis revealed that all of the O65:NM strains isolated from Family B and Family C were closely related, which confirmed epidemiologically and genotypically that they had been exposed to the same infectious source. In sporadic cases, the PFGE profiles of some strains suggested a genomic relationship, but there was no epidemiological information to support these relationships. Since high diversity was shown in O63:H6, O103:H2, and O165:[HUT], the PFGE profiles of these strains should be compared with the strains of the domestic PFGE network.

An epidemiological study for 6 years in Denmark indicated that risk factors for HUS were the combined presence of *stx2* and *eae* rather than serogroup O157 [10]. The present study shows that the strains isolated from HUS patients were harboring both *stx2* and *eae*. Ten of 13 strains isolated from asymptomatic carriers were Stx1-positive and Stx2-negative, whereas 10 of 14 strains isolated from patients with bloody diarrhea produced Stx2, supporting the previous reports that Stx2 was associated with severity of disease [7, 10].

The current methods for the detection of STEC have been developed to isolate serogroups O157 and O26. We should consider that some of minor serogroup STEC strains present atypical phenotypes and form no colonies on CT-SMAC. In addition, only 50 serogroups are typeable with commercially available antiserum, while there are over 130 serogroups reported as STEC [37]. Our results indicate that it is important to examine Stx production of all isolates on selective agar plates with and without CT supplement for the detection of various serogroups of STEC.

ACKNOWLEDGMENTS. We are grateful to Dr. Sunao Iyoda, Department of Bacteriology, National Institute of Infectious Diseases (Japan), for typing of the strains untypeable with commercially available diagnostic *E. coli* antiserum. We also thank Dr. Teizo Tsukamoto for his helpful advice, and all of the investigators throughout Osaka Prefecture for providing us with samples and information. The PFGE standard strain *Salmonella enterica* serovar Braenderup H9812 was obtained from PulseNet program, Enteric Diseases Laboratory Branch, Centers for Disease Control and Prevention, USA through National Institute of Infectious Diseases (Japan). This study was supported by a grant-in-aid from Ministry of Health, Labour, and Welfare for Research on Emerging and Re-emerging infectious Diseases (H18-Shinkou-Ippan-16).

REFERENCES

1. Akiba, Y., Kimura, T., Takagi, M., Akimoto, T., Mitsui, Y., Ogasawara, Y. and Omichi, M. 2005. Outbreak of enterohemorrhagic *Escherichia coli* O121 among school children exposed to cattle in a ranch for public education on dairy farming. *Jpn. J. Infect. Dis.* **58**: 190–192.
2. Bastian, S. N., Carle, I. and Grimont, F. 1998. Comparison of 14 PCR systems for the detection and subtyping of *stx* genes in Shiga-toxin-producing *Escherichia coli*. *Res. Microbiol.* **149**: 457–472.
3. Belongia, E. A., Osterholm, M. T., Soler, J. T., Ammend, D. A., Braun, J. E., MacDonald, K. L. 1993. Transmission of *Escherichia coli* O157:H7 infection in Minnesota child daycare facilities. *JAMA* **269**: 883–888.
4. Beutin, L., Zimmermann, S. and Gleier, K. 1996. Rapid detection and isolation of a Shiga-like toxin (verocytotoxin)-producing *Escherichia coli* by direct testing of individual enterohemolytic colonies from washed sheep blood agar plates in the VTEC-RPLA assay. *J. Clin. Microbiol.* **34**: 2812–2814
5. Beutin, L., Krause, G., Zimmermann, S., Kaulfuss, S. and Gleier, K. 2004. Characterization of Shiga toxin-producing *Escherichia coli* strains isolated from human patients in Germany over a 3-year period. *J. Clin. Microbiol.* **42**: 1099–1108.
6. Beutin, L., Montenegro, M. A., Orskov, I., Orskov, F., Prada, J., Zimmermann, S. and Stephan, R. 1989. Close association of verotoxin (Shiga-like toxin) production with enterohemolysin production in strain of *Escherichia coli*. *J. Clin. Microbiol.* **27**: 2559–2564.
7. Boerlin, P., McEwen, S. A., Boerlin-Petzold, F., Wilson, J. B., Johnson, R. P. and Gyles, C. L. 1999. Associations between virulence factors of Shiga toxin-producing *Escherichia coli* and disease in humans. *J. Clin. Microbiol.* **37**: 497–503.
8. Centers for Disease Control and Prevention. 2001. University outbreak of calicivirus infection mistakenly attributed to shiga toxin-producing *Escherichia coli* O157:H7 - Virginia, 2000. *MMWR* **50**: 489–491.
9. DesRosiers, A., Fairbrother, J. M., Johnson, R. P., Desautels, C., Letellier, A. and Quessey, S. 2001. Phenotypic and genotypic characterization of *Escherichia coli* verotoxin-producing isolates from human and pigs. *J. Food Prot.* **64**: 1904–1911.
10. Ethelberg, S., Olsen, K. E. P., Scheutz, F., Jensen, C., Schiellerup, P., Enberg, J., Petersen, A. M., Olsen, B., Gerner-Smidt, P. and Molbak, K. 2004. Virulence factors for hemolytic uremic syndrome, Denmark. *Emerg. Infect. Dis.* **10**: 842–847.
11. Ewing, W. H. 1986. The Genus Escherichia. pp. 93–134. *In*: Identification of *Enterobacteriaceae*, 4th ed. (Ewing, W. H. and Edwards, P. R. eds.), Elsevier Science Publishing, New York.
12. Fratamico, P. M., Bagi, L. K., Bush, E. J. and Solow, B. T. 2004. Prevalence and characterization of Shiga toxin-producing *Escherichia coli* in swine feces recovered in the national animal health monitoring system's swine 2000 study. *Appl. Environ. Microbiol.* **70**: 7173–7178.
13. Friedrich, A. W., Bielaszewska, M., Zhang, W. L., Pulz, M., Kuczius,T., Ammon, A. and Karch, H. 2002. *Escherichia coli* harboring Shiga toxin 2 gene variants: frequency and associated with clinical symptoms. *J. Infect. Dis.* **185**: 74–84.
14. Gannon, V. P., Teerling, C., Marsi, S. A. and Gyles, C. L. 1990. Molecular cloning and nucleotide sequence of another variant of the *Escherichia coli* Shiga-like toxin II family. *J. Gen. Microbiol.* **136**: 1125–1135.
15 Gerber, A., Karch, H., Allerberger, F., Verweyen, H. M. and Zimmerhackl, L. B. 2002. Clinical course and the role of shiga toxin-producing *Escherichia coli* infection in the hemolytic-uremic syndrome in pediatric patients, 1997–2000, in Germany and Austria: a prospective study. *J. Infect. Dis.* **186**: 493–500.
16. Hiramatsu, R., Matsumoto, M., Miwa, Y., Suzuki, Y., Saito, M. and Miyazaki, Y. 2002. Characterization of Shiga toxin-producing *Escherichia coli* O26 strains and establishment of selective isolation media for these strains. *J. Clin. Microbiol.* **40**: 922–925.
17. Hiruta, N., Murase, T. and Okamura, N. 2000. An outbreak of diarrhoea due to multiple antimicrobial-resistant Shiga toxin-producing *Escherichia coli* O26:H11 in a nursery. *Epidemiol. Infect.* **127**: 221–227.
18. Holland, J. L., Louie, L., Simor, A. E. and Louie, M. 2000. PCR detection of *Escherichia coli* O157:H7 directry from stools: evaluation of commercial extraction methods for purifying fecal DNA. *J. Clin. Microbiol.* **38**: 4108–4113.
19. Hunter, S. B., Vauterin, P., Lambert-Fair, M. A., Van Duyne, M. S., Kubota, K., Graves, L., Wringley, D., Barrett,T. and Ribot, E. 2005. Establishment of a universal size standard strain for use with the PulseNet standardized pulsed-field gel electrophoresis protocols: converting the national databases to the new size standard. *J. Clin. Microbiol.* **43**: 1045–1050.
20. Infectious Disease Surveillance Center [database on the Internet]. Japan: Isolation of bacteria in the past years. [updated 2007 Jan 28; cited 2007 Feb 10]. Available from: http://idsc.nih.go.jp/iasr/virus/pbacteria-e.html
21. Isobe, J., Kimata K., Shimojima, M., Hosorogi, S., Tanaka, D. and Gyobu, Y. 2004. Isolation of *Escherichia coli* O128:HNM harboring *stx2f* genes from diarrhea patients. *J. Jpn. Assoc. Infect. Dis.* **78**: 1000–1005 (in Japanese).
22. Jorgensen, J. H. and Turnidge, J.D. 2003. Susceptibility test

methods: dilution and disk diffusion methods. pp. 1108–1127. *In*: Manual of Clinical Microbiology, 8th ed. (Murray, P. R., Baron, E. J., Jorgensen, J. H., Pfaller, M. A. and Yolken, R. H. eds.), ASM Press, Washington, DC.

23. Karch, H., Bielaszewska, M., Bitzan, M. and Schmidt, H. 1999. Epidemiology and diagnosis of Shiga toxin-priducing *Escherichia coli* infections. *Diagn. Microbiol. Infect. Dis.* **34**: 229–243.
24. Kehl, K. S., Havens, P., Behnke, C. E., Acheson, D. W. K. 1997. Evaluation of the Premier EHEC assay for detection of Shiga toxin-producing *Escherichia coli*. *J. Clin. Microbiol.* **35**: 2051–2054.
25. Kijima-Tanaka, M., Ishihara, K., Kojima, A., Morioka, A., Nagata, R., Kawanishi, M., Nakazawa, M., Tamura, Y. and Takahashi, T. 2005. A national surveillance of Shiga toxin-producing *Escherichia coli* in food -producing animals in Japan. *J. Vet. Med.* **B52**: 230–237.
26. Kobayashi, K., Seto, K., Yatsuyanagi, J., Saito, S., Terao, M., Kaneko, M., Serizawa, T., Kuramoto, S., Fujisawa, T., Suzuki, R., Yamazaki, M., Hayashi, K., Matsune, W., Yasuoka, T., Horikawa, K., Murakami, K., Kawano, K., Yamada, T. and Ito, K. 2002. Presence of the genes regarding adherence factors of *Escherichia coli* isolates and a consideration of the procedure for detection of diarrheagenic strain. *J. Jpn. Assoc. Infect. Dis.* **76**: 911–920 (in Japanese).
27. Kon, M., Kurazono, T., Ohshima, M., Yamaguchi, M., Morita, K., Watanabe, N., Kanamori, M. and Matsushita, S. 2005. Cefotaxime-resistant Shiga toxin-producing *Escherichia coli* O26:H11 isolated from a patient with diarrhea. *J. Jpn. Assoc. Infect. Dis.* **79**: 161–168 (in Japanese).
28. Machado, J., Grimont, F. and Grimont, P. A. 2000. Identification of *Escherichia coli* flagellar types by restriction of the amplified *fliC* gene. *Res. Microbiol.* **151**: 535–546.
29. National Committee for Clinical Laboratory Standards. 2000. Performance standards for antimicrobial disk susceptibility tests; approved standard, 7th ed. NCCLS Document M2-A7, National Committee for Clinical Laboratory Standards, Pennsylvania.
30. NIID (National Institute of Infectious Diseases) and Tuberculosis and Infectious Diseases Control Division, Ministry of Health, Labor and Welfare of Japan. 2006. Enterohemorrhagic *Escherichia coli* infection in Japan as of May 2006. *Infect. Agents Surv. Rep.* **27**: 141–143.
31. NIID (National Institute of Infectious Diseases) and Tuberculosis and Infectious Diseases Control Division, Ministry of Health, Labor and Welfare of Japan. 2000. The status of enterohemorrhagic *Escherichia coli* infection in Japan, 1998-March 2000. *Infect. Agents Surv. Rep.* **21**: 92–93.
32. Orskov, F. and Orskov, I. 1984. Serotyping of *Escherichcia coli*. pp. 43–112. *In*: Methods Microbiol. Vol. 14 (Bergan, T. ed.), Academic Press, London.
33. Oswald, E., Schmidt, H., Morabito, S., Karch, H., Marches, O. and Caprioli, A. 2000. Typing of intimin genes in human and animal enterohemorrhagic and enteropathogenic *Escherichia coli*: characterization of a new intimin variant. *Infect. Immun.* **68**: 64–71.
34. Park, C. H., Kim, H. J., Hixon, D. L. and Bubert, A. 2003. Evaluation of the duopath verotoxin test for detection of shiga toxins in cultures of human stools. *J. Clin. Microbiol.* **41**: 2650–2653.
35. Paton, A. W. and Paton, J. C. 1998. Datection and characterization Shiga toxigenic *Escherichia coli* by using multiplex PCR assays for *stx1*, *stx2*, *eaeA*, enterohemorrhagic *E. coli hlyA*, rfb_{O111}, and rfb_{O157}. *J. Clin. Microbiol.* **36**: 598–602.
36. Rangel, J. M., Sparling, P. H., Crowe, C., Griffin, P. M. and Swerdlow, D. L. 2005. Epidemiology of *Escherichia coli* O157:H7 Outbreaks, United States, 1982–2002. *Emerg. Infect. Dis.* **11**: 603–609.
37. Scheutz, F. and Strockbine, N. A. 2005. Genus I. Escherichia. pp. 607–624. *In*: Bergey's Manual of Systematic Bacteriology, 2nd ed., vol. 2, Part B (Garrity, G. M., Brenner, D. J., Kreig, N. R. and Staley, J. T. eds.), Springer-Verlag, New York.
38. Scheutz, F., Cheasty, T., Woodward, D. and Smith, H. R. 2004. Designation of O174 and O175 to temporary O groups OX3 and OX7, and six new *E. coli* O groups that include verocytotoxin-producing E. coli (VTEC): O176, O177, O178, O179, O180, O181. *APMIS* **112**: 569–584.
39. Schmidt, H., Scheef, J., Morabito, S., Caprioli, A., Wieler, L. H. and Karch, H. 2000. A new Shiga toxin 2 variant (Stx2f) from *Escherichia coli* isolated from pigeons. *Appl. Environ. Microbiol.* **66**: 1205–1208.
40. Takahara, T., Iyoda, S., Asada, J., Mizumoto, H., Uematsu, A., Hata, A., Watanabe, H., Tamura K. and Hata. D. 2005. A case of haemolytic uremic syndrome precede by only one day of mild diarrhea without bloody stool due to enterohemorragic *Escherichia coli* O177:NM. *J. Jpn. Pediatr. Soc.* **109**: 54–57 (in Japanese).
41. Tanaka, H., Yatsuyanagi, J., Uchimura, M., Saito,M., Kobayashi, K., Horikawa, K. and Mori, R. 1999. Evaluation of sorbose MacConkey medium containing cefixime and tellurite for isolation of enterohemorrhagic *Escherichia coli* O111. *J. Jpn. Clin. Microbiol.* **9**: 48–50 (in Japanese).
42. Terajima, J., Izumiya, H., Wada, A., Tamura, K. and Watanabe, H. 1999. Shiga Toxin-Producing *Escherichia coli* O157:H7 in Japan. *Emerg. Infect. Dis.* **5**: 301–302.
43. Terajima, J., Izumiya, H. and Watanabe, H. 2004. Identification in bacterial strain levels: subtyping of enteric bacterium by pulsed-field gel electrophoresis. *J. Intest. Microbiol.* **18**: 117–122 (in Japanese).
44. Zhang, W.L., Kohler, B., Oswald, E., Beutin, L., Karch, H., Morabito, S., Caprioli, A., Suerbaum, S. and Schmidt, H. 2002. Genetic diversity of intimin genes of attaching and effacing *Escherichia coli* strains. *J. Clin. Microbiol.* **40**: 4486–4492.

〔Bokin Bobai Vol.35, No.6, pp.401～410, 2007〕

講座

食品の微生物検査法と食中毒発生時の疫学調査法[2]
大腸菌群，糞便系大腸菌群，大腸菌

浅尾　努

〈掲載予定内容，著者，掲載巻号〉

（注）本講座は，納期以降のものについては受付順で掲載する。

1．はじめに

食品の細菌学的な安全性を評価するもっとも確実な方法は，当該食品に関係する食中毒菌を直接検査することである。しかし，すべての食中毒菌を検査対象にするのは，経済的，時間的，技術的な制約のために現実的には不可能である。食中毒菌のうちで，赤痢菌，コレラ菌，サルモネラなどの腸管系食中毒菌と由来や挙動を共にする可能性が高いとされる菌群は，衛生指標菌あるいは汚染指標菌と呼ばれている。衛生指標菌の代表として大腸菌群（coliforms），糞便系大腸菌群（fecal coliforms），大腸菌（*Escherichia coli*）があり，いずれも食品の衛生的な取り扱いの良否や，食品への腸管系食中毒菌汚染の有無を推定するためのツールとして世界的に広く利用されている。わが国でも，衛生指標菌を標的とした成分規格，加工基準，保存基準をはじめとして，衛生規範や指導基準などが告示・通知されてきた。

本稿では，まず衛生指標菌試験法に関する日本の現状や問題点などについて説明する。次に試験法の国際的な調和が求められている現状に即して，日本の告示法・通知法とともに，米国FDA/BAM（Bacterial Analytical Manual）法やISO（International Organization for Standardization）法などの衛生指標菌試験法の概要を紹介する。なお実際の手技については食品衛生検査指針などの成書に譲ることとした。

大阪府立公衆衛生研究所　〒537－0025　大阪市東成区中道1－3－69　☎06－6972－1321

0385－5201/2007/0610－0401　$02.00/0

2．日本の衛生指標菌試験法の現状と問題点

食品衛生法には乳等省令の28種類の食品（表1）と，それ以外の18種類の一般食品（表2）に対して，大腸菌群陰性あるいはE. coli陰性（一部でMPN限界値や菌数限界値）の成分規格が定められている。食品の規格基準への適否の判定は，乳及び乳製品の成分規格等に関する省令（いわゆる乳等省令），氷雪の成分規格，冷凍食品の成分規格に記載された試験法などに従わなければならない。しかし，これらの試験法は長年改訂されていないために，国際的な趨勢から取り残された感は否めない。国内的にも，既存の衛生指標菌試験法が見直されないままに新たな試験法が告示・通知されてきた弊害により，例えば以下のような問題点が生じている。

①食品の希釈液が統一されていない。告示された時期が古い試験法では希釈液に生理食塩水が指定されたが，その後はリン酸緩衝液になり，最近ではペプトン加生理食塩水へと変遷してきた（表1，表2）。ペプトンは食品中の損傷菌の回復に効果があるといわれている。

②培養温度の表示方法が統一されていない。告示時期の新しい一般食品では，35±1℃の厳密な培養条件が設定されているが，告示時期の古い乳等省令関連の食品に対しては，32～35℃と幅のある培養条件が許されている。しかし，両方の培養条件を満たす共通の温度帯がわずかに異なるため，これらの食品を1台の培養器で同時に試験できない。なお，欧米の乳製品試験法の培養温度は，30±1℃あるいは32±1℃に設定されている。

③試料の採取法が非現実的である。食肉製品や魚肉ねり製品等は，“切断すべき表面をアルコール綿花でよくふいた後，滅菌した器具を用いて無菌的に切断し，その断面の中央部から25gを無菌的に採り試料とする”と指示されている。しかし，中心部のみを採取しなければならない理由が明確ではなく，しかも食品の形状によっては，この指示通りに試料を採取するのは困難であるか事実上不可能な場合もある。可食部で

表1．食品別の規格試験法の比較（乳および乳製品）

	食品	スタートの培地・培養条件
1	牛乳	培地：BGLB 培養温度：32～35℃ 培養時間：24±2時間 および48±3時間 希釈液 1～9：規定なし 10～19：生理食塩水
2	殺菌山羊乳	
3	加工乳	
4	成分調整牛乳	
5	低脂肪牛乳	
6	無脂肪牛乳	
7	特別牛乳	
8	クリーム	
9	乳飲料	
10	加糖れん乳	
11	加糖脱脂れん乳	
12	全粉乳	
13	脱脂粉乳	
14	クリームパウダー	
15	ホエイパウダー	
16	たんぱく質濃縮ホエイパウダー	
17	バターミルクパウダー	
18	加糖粉乳	
19	調整粉乳	
20	バター	培地：デソ寒天 培養温度：32～35℃ 培養時間：20±2時間 希釈液：生理食塩水
21	バターオイル	
22	プロセスチーズ	
23	濃縮ホエイ	
24	アイスクリーム	
25	アイスミルク	
26	ラクトアイス	
27	発酵乳	
28	乳酸菌飲料	

表2．食品別の規格試験法の比較（一般食品）

	食品	スタートの培地	希釈液
29	氷菓	デソ寒天	生理食塩水
30	生食用かきの原料生産海域海水（加工基準）	EC	希釈不要
31	直接食品に接触させて食品を保存する氷雪（保存基準）	BTB-LB	規定なし
32	氷雪	BTB-LB	
33	清涼飲料水	BTB-LB	
冷凍食品			
34	無加熱摂取	デソ寒天	リン酸緩衝液
35	加熱後摂取（凍結直前加熱）	デソ寒天	
36	生食用冷凍鮮魚介類	デソ寒天	
37	加熱後摂取（凍結直前非加熱）	EC	
38	冷凍ゆでだこ	デソ寒天	
39	冷凍ゆでがに	デソ寒天	
40	生食用かき	EC	
41	粉末清涼飲料	BTB-LB	
42	鯨肉製品	BGLB	
43	魚肉ねり製品	BGLB	
食肉製品			
44	非加熱	EC	ペプトン加生理食塩水
45	特定加熱	EC	
46	包装後加熱	BGLB	
47	殺菌後包装	EC	
48	乾燥	EC	

デソ寒天（デソキシコレート寒天）：35±1.0℃，20±2時間
EC培地：44.5±0.2℃，24±2時間
BTB加LB培地：35±1.0℃，24±2時間および48±3時間
BGLB培地：35±1.0℃，24±2時間および48±3時間

ある食品表面を除外した検査では，衛生指標菌検査の意味合いが希薄になると思われる。

④食品の10倍乳剤作製法が一様ではない。アイスクリーム類や氷菓等では，秤量した検体10gに希釈液を90ml 加えるが，他の食品では希釈液を加えて100ml までメスアップすることになっている。冷凍食品や食肉製品等では，25gの検体に225ml の希釈液を加える。食品の乳剤作製機器として，古い告示法ではホモジナイザーが，最近ではストマッカーが指定されている。

⑤氷雪や清涼飲料水等の推定試験には，pH 指示薬である BTB を加えた乳糖ブイヨン（BTB-LB 培地）を使用しなければならない（表２）。ところが完全試験では，氷雪や清涼飲料水等も含むすべての食品に対して BTB 不含の乳糖ブイヨン（LB 培地）が指定されている（図１）。法律を忠実に遵守するならば，氷雪や清涼飲料水等の試験には２種類の乳糖ブイヨンを使い分けなければならない。なお FDA/BAM や ISO の衛生指標菌試験法では，LB 培地や BTB-LB 培地は使われていない。

⑥乳等省令には“検体採取後４時間以内に試験に供しなければならない”との非現実的な規定がある。使用培地は，今では有名無実となった“間けつ滅菌”することになっている。

⑦試験遂行上の問題はないと思われるが，食品衛生法には２種類の異なる大腸菌群の定義が存在する。乳等省令では乳糖を分解して“ガス”を産生するものとされ，その他の一般食品では乳糖を分解して“酸とガス”を産生するものと定義されている。［参考１］に乳糖分解により酸とガスが産生される機序を示した。

図１．日本の汚染指標菌試験法の概略図（液体培地法）

以上のように，日本の衛生指標菌試験法（告示法）には実効性に欠ける記述があるだけではなく，試験法の国内的な調和すらとられていないことも明白である。一貫性のない試験法は検査員に無用のストレスを与え，結果として検査ミスを誘発する要因にもなりかねない。

３．衛生指標菌名の混乱

日本の食品衛生法では，腸管系病原菌に対する衛生指標菌として，大腸菌群と“E. coli”（イタリックではない）の２種類のみが使用されているだけで，諸外国のような大腸菌を対象とした規格基準などはない。平成10年の「生食用食肉等の安全性確保について」の通知で，厚生労働省は初めて糞便系大腸菌群の用語を使用し，これは食肉製品等の成分規格に使用されている E. coli と同じものであると定義した。E. coli は日本の食品衛生法上の独特の用語であり，欧米で使用されている fecal coliforms（FDA/BAM：糞便系大腸菌群）あるいは presumptive *E. coli*（ISO：推定大腸菌）とは，試験法は異なるが性状は類似する菌群と考えられる。大腸菌以外の名称は食品衛生学領域でのみ使用される用語であり，細菌学の分類に基づくものではない。近年日本でも使用されるようになってきた糞便系大腸菌群の用語は，大腸菌よりも糞便汚染の可能性がより高いことを示す指標菌のような間違ったイメージを与えかねない。糞便系大腸菌群とは，大腸菌群のうち44℃～45.5℃の高温域でも発育可能な菌群であるという，単なる培養手技上の名称である。元来の標的である大腸菌以外にも，自然界に常在するクレブジェラ，エンテロバクター，サイトロバクターなどがこのような培養条件でも発育するので，糞便系大腸菌群は必ずしも糞便汚染の特異的な指標菌とはいえない。糞便系大腸菌群に代わる用語として，thermotolerant coliforms が，さらには

thermotrophic coliforms がより相応しいとの考え方もある。

4．日本の告示法・通知法，FDA/BAM，ISO の衛生指標菌試験法の比較

日本のほとんどの衛生指標菌試験法では，得られる結果は陰性か陽性かの定性的なものである。これに対して，FDA/BAM 法および ISO 法は，定量的な結果が得られる MPN 法を採用している点で日本の試験法とは基本的に異なる。なお本文中に記載した培地名などの略語は表 3 に示したので，詳細は各メーカーの説明書を参考にされたい。

4-1. 大腸菌群および糞便系大腸菌群（日本では E. coli）試験法

①日本の告示法・通知法

大腸菌群および E. coli 試験法は，基本的には推定試験，確定試験，完全試験の 3 つのステップで構成されている。大腸菌群の推定試験には BGLB 培地，BTB-LB 培地（図 1）あるいはデソキシコレート寒天培地（図 2）が，E. coli の推定試験には EC 培地が指定されている（図 1）。BGLB 培地は栄養素に富む牛乳類や食肉製品試験用に，BTB-LB 培地は栄養素の少ない氷雪や清涼飲料水の試験用にと使い分けているようである（表 1，表 2）。氷雪や清涼飲料水のような貧栄養の食品中の大腸菌群は，飢餓ストレスを受けて損傷菌になっている可能性が高い。損傷菌の回復をはかる目的で，最初のステップに非選択性の BTB-LB 培地を採用したと推察される。EC 培地は生食用カキの E. coli の MPN 法，冷凍食品や食肉製品の E. coli 推定試験用に指定されている。

デソキシコレート寒天培地中に暗赤色集落が発生した場合，あるいは BGLB 培地などの液体培地でガス産生が認められた場合は推定試験陽性と判定する（参考 1，参考 2 を参照）。推定試験の培地は異なっても，次の確定試験では一律に EMB 培地に画線培養を行う（図 1，図 2）。EMB 培地に発生した大腸菌群あるいは E. coli 様集落は，LB 培地と普通寒天斜面培地へ移植して完全試験を実施する。LB 培地でガスを産生し，かつ普通寒天培地上の菌がグラム陰性の無芽胞桿菌であることを確認すれば完全試験陽性と判定する（図 1，図 2）。MPN 法では例外的に推定試験の EC 培地のみで最終判定し，ガス陽性の試験管数から MPN 表を使用して菌数を算定する。

わが国の衛生指標菌試験法は，定性的な成分規格に軽重の差をつけるために，培地に接種する試料量を変えている。例えば，食肉製品では10倍乳剤10ml ずつを 3 本の BGLB 培地に接種する（3 g）。牛乳では原液，10倍および100希釈液 1 ml ずつを，それぞれ 2 本のBGLB培地に接種する（2.22 ml）。冷凍食品では100倍乳剤 1 ml ずつを 3 本のEC培地に接種する（0.03g）。アイス

表 3．図表，本文中に記載した培地名などの略語

	液体培地
EC	(Escherichia coli)
BGLB	(Brilliant green lactose bile)
LB	(Lactose broth)
EE	(Enterobacteriaceae enrichment)
	またはBufferd brilliant green lactose bile glucose)
MMG	(Minerals Modified glutamate)
BPW	(Bufferd peptone water)
	寒天培地
TBG	(Tryptone bile glucuronic)
EMB	(Eosin methylene blue)
VRBA	(Violet red bile agar)
VRBG	(Violet red bile glucose)
TSA	(Tryptic soy agar)
	酵素基質
BCIG	(5-bromo-4-chloro-3-indolyl-beta-D-gluculonic acid)
	またはX-GLUC
MUG	(4-methylumbeliferyl-beta-D-glucuronide)

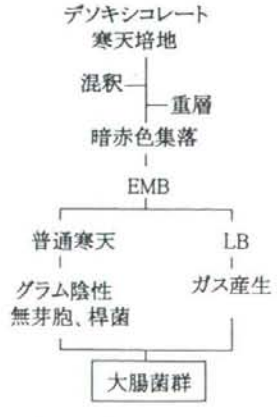

図 2．日本の大腸菌群試験法の概略図（平板法）

クリーム類では10倍乳剤を1 ml（0.1g）ずつ，冷凍食品では100倍乳剤を1 ml（0.01g）ずつ，それぞれ2枚のデソキシコレート寒天培地で混釈培養するようになっている。このような食品ごとに異なる試料の希釈倍率や，推定試験に使用する試験管数の相違が，わが国の衛生指標菌試験法を複雑にしている大きな原因の一つでもある。

② FDA/BAM 法および ISO 法との比較

FDA/BAM と ISO の大腸菌群および糞便系大腸菌群試験法は，わが国の方法とは異なり，試験のスタート時に使用する液体培地は LST 培地にほぼ一本化されている。FDA/BAM の大腸菌群試験法（図3）ではガス陽性の LST 培地から BGLB 培地へ，糞便系大腸菌群試験法（図3）ではガス陽性の LST 培地から EC 培地へ移植し，いずれも一定時間培養後にガスの産生が認められた試験管を陽性として MPN 値を算出する。ISO の大腸菌群試験法（図4）は FDA/BAM 法と類似はするが，LST 培地でガス産生が認められたものだけではなく，ガス産生なしに混濁したものも推定試験陽性として BGLB 培地へ移植する。2倍濃度の LST 培地にはダーラム管はいれないことも異なる。このため，ガス産生性とは関係なく，単に混濁した LST 培地の培養液を次のステップの BGLB 培地に移植することになる。

ISO の糞便系大腸菌群試験法（図5）には，FDA/BAM 法にはないインドール試験を実施するためのペプトン水培養が追加されている。Health Protection Agency（英国食品安全庁）の糞便系大腸菌群（正確には presumptive *E. coli*）試験法は，ガス陽性の EC 培地に 1 N NaOH を添加後にインドルール試験を実施する。この便法はペプトン水培養を省略できるため，ISO 法（図5）に比べて試験時間の大幅な短縮（48時間）が可能となる。なお EC 培地の培養温度は，日本：44.5±0.2℃，FDA/BAM 法：貝類は44.5±0.2℃でその他の食品では45.5±0.2℃，ISO 法：44±1.0℃と若干異なっている。EC 培地は空調式恒温槽ではなく，厳密な温度制御が可能である恒温水槽で培養しなければならない。

大腸菌群試験に使用する平板培地は，日本ではデソキシコレート寒天培地であるが，FDA/BAM 法と ISO 法ではいずれも VRBA 培地が指定されている。ISO 法（図6）では，ピンク・赤・紫色で直径が0.5mm 以上の大きさの典型集落は，集落の形態や色調だけで大腸菌群陽

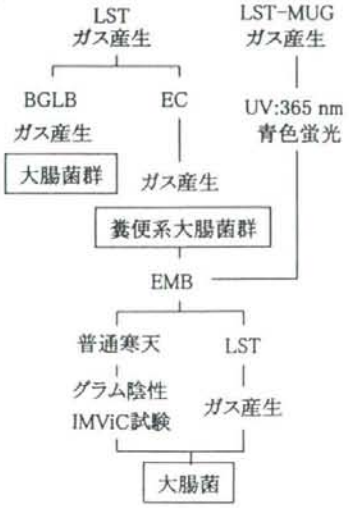

図3．FDA/BAM の汚染指標菌試験法の概略図（MPN法）

図4．ISO の大腸菌群試験法概略図（MPN 法）

図5．ISO の糞便系大腸菌群試験法概略図（MPN 法）

性菌と判定して菌数測定を行うが，非典型集落ではBGLB培地でガス産生性を確認することになっている。VRBA培地上の集落の大きさが0.5mm以上という大腸菌群陽性菌の基準は，一応の目安と考えるのが適切であろう。FDA/BAM法（図7）は，典型集落でもBGLB培地でガス産生性を確認して大腸菌群陽性菌と判定する。FDA/BAM法やISO法は，日本の告示法・通知法のようなEMB培地での確定試験と，それに続くグラム染色やLB培地での完全試験がないので，より簡便・迅速化された試験法といえる。

日本の告示法・通知法，FDA/BAM法，ISO法ともに，乳製品試験法の培養温度（32～35℃，32±1℃，30±1℃）は一般食品（35±1℃，ISO法のみ37±1℃）に比べて低く設定されている。ISO法では，ヒトや動物由来の衛生指標菌を標的とする場合は37±1℃培養を行い，製造工程での環境汚染や低温性菌を標的とする場合は30±1℃培養が採用されているようである。

4-2. 大腸菌試験法

わが国には大腸菌を標的とした規格基準がないので，当然大腸菌試験法に関する告示・通知法はない。ISOの平板法（図8）は，BCIG（酵素基質：X-GLUCと同一）を添加したTBX培地と試料液を混釈して，44±1℃で18～24時間培養する。ISOの液体法（図9）は，MMG培地を用いて37±1℃で24±2時間培養した後，乳糖分解により酸が産生された（pH指示薬で判定）培養液をTBX培地に画線培養する。TBX培地に大腸菌が発育すると，β-グルクロニダーゼ（大腸菌の約95%が保有）がBCIGを加水分解し，その結果生成される色素によって青～青緑色に着色された集落を形成する。ただし腸管出血性大腸菌O157などはβ-グルクロニダーゼを保有せず，44℃の高温では発育もできない。FDA/BAMの液体法（図3）は，LST培地からEC培地へ，次にEMB培地へとガス産生性を指標として移植する。EMB培地で大腸菌様集落が発生すれば，再度LST培地でのガス産生性，および普通寒天培地で発育した菌のグラム染色性およびIMViC

図6．ISOの大腸菌群試験法の概略図（平板法）

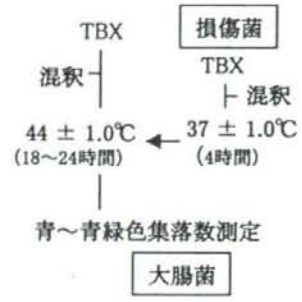

図8．ISOの *E. coli* 試験法概略図（平板法）

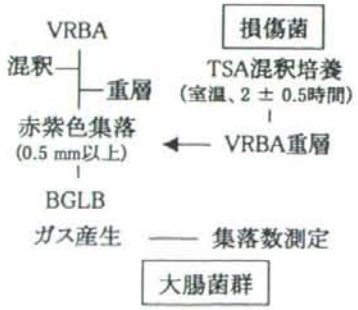

図7．FDA/BAMの大腸菌群試験法の概略図（平板法）

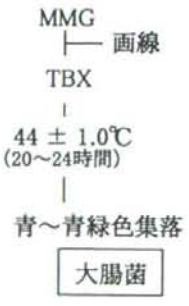

図9．ISOの *E. coli* 試験法概略図（MPN法）

試験により大腸菌を同定する（参考3を参照）。IMViC試験のかわりにAPI20EやVITEKなどの簡易同定法も使用可能である。試験の最初のステップでLST培地の代わりに酵素基質MUGを添加したLST-MUG培地を使用すれば，EC培地を省略して直接EMB培地への画線培養が可能になる。貝類は内因性のβ-グルクロニダーゼ活性を有するため，疑陽性反応を起こす恐れのあるLST-MUG培地を最初のステップでは使用できない。まずLST培地で培養し，次のステップでEC-MUG培地を使用すれば疑陽性反応は防止できるとされている。FDA/BAMの平板法（図10）は，試料をVRBA培地で混釈した後，VRBA-MUG培地を重層して35±1℃で18～24時間培養する。本法では365nmの長波長UVの照射下で青色蛍光色の集落を大腸菌として計測するが，可視光線下でのピンク・赤色・紫色の大腸菌群の同時測定も可能である。

4-3. *Enterobacteriaceae*（仮訳：腸内細菌科菌群）試験法

腸内細菌科菌群とはグラム陰性の無芽胞桿菌で，ブドウ糖を分解して酸を産生し，オキシダーゼ反応陰性の通性嫌気性菌であると定義されている。腸内細菌科菌群を衛生指標菌とした試験法では，乳糖非分解菌である赤痢菌，サルモネラ，エルシニアのような食品衛生上重要な腸管系食中毒菌も検出可能である。大腸菌や大腸菌群試験が陰性であったアイスクリームが，サルモネラ食中毒の原因となった事例が知られている。ブドウ糖分解性を指標とする腸内細菌科菌群は，乳糖分解性を指標とする大腸菌群よりもより広い腸管系食中毒菌をカバーできる衛生指標菌といえる。衛生指標菌として腸内細菌科菌群が大腸菌群よりも適切であるとの考え方は，米国よりはむしろ欧州で広く採用されてきたようである。2006年1月1日から効力を発揮している“New EU microbiological criteria”（参考4で説明した新しい欧州連合の食品規格）では，いくつかの食品の大腸菌群の規格が腸内細菌科菌群の規格へと移行した。

ISO法の腸内細菌科菌群の分離培地には，大腸菌群用のVRBA培地の乳糖をブドウ糖に置き換えたVRBG培地が使用されている。VRBG培地で混釈・重層し，37±1℃あるいは30±1℃で24±2時間培養後に発生した集落のうち，ブドウ糖を分解しかつオキシダーゼ陰性の菌を腸内細菌科菌群とする（図11）。水やそれに関連する食品には，元来は水系の常在菌であって糞便汚染とは関係がない，オキシダーゼ陽性のエロモナス属菌がしばしば存在する。オキシダーゼ試験は，本来の標的ではないエロモナス属菌を腸内細菌科菌群から容易に除外できる有力な武器である。腸内細菌科菌群は，VRBG培地で脱色された非典型集落を形成する場合もあるので，白色集落であっても典型的集落と同様に5個ないしは10個の集落を選択して，ブドウ糖分解試験およびオキシダーゼ試験を実施することになっている。ISOの液体培地法（図12）では，まず選択剤を含まない

図10. FDA/BAMの*E. coli*試験法概略図（平板法）

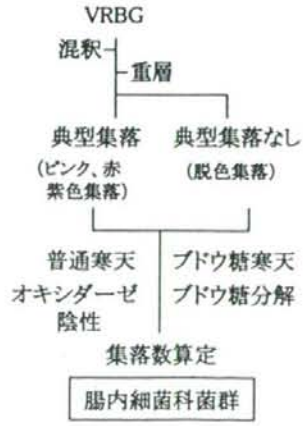

図11. ISOの腸内細菌科菌群試験法概略図（平板法）