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Rapid detection of coliform bacteria using a lateral flow test strip assay

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ABSTRACT

Coliform bacteria in foods are enumerated at food processing plants and are used as sanitary and quality indicators. To detect coliform bacteria rapidly, seven Lateral Flow Test Strips (LFTSs) that can detect the genera *Aeromonas, Citrobacter, Enterobacter, Hafnia, Klebsiella/Raoultella, Pantoea* and *Serratia* were developed. For 55 tested food isolates, the detection rate of each individual LFTS assay was only 38% to 76%, but the detection rate of the 7 combined assays was 100%. For 38 culture collection strains, including clinical isolates, each individual LFTS assay had a detection rate of only 18% to 76%, but the 7 assays in combination had a detection rate of 89%. A feasibility study conducted on 20 types of meat (beef, chicken and pork) indicated that the LFTS assays detected coliform bacteria from 3 types of meat without incubation and from all other meats after 8 h of incubation. LFTS assays showed a positive signal when the meat was spoiled by more than 4.9 $\log_{10}$ (cfu/g) coliform bacteria. A longer incubation time led to increased bacterial counts, more positive LFTSs (1.8 at 8 h and 4.6 at 24 h) and a greater maximal signal intensity (1, 366 at 8 h and 2, 678 at 24 h). Thus, LFTSs of coliform bacteria have great potential for the rapid determination of food freshness as well as food sanitation status.

1. Introduction

Food poisoning from bacteria continues to be a worldwide problem. The Foodborne Disease Outbreak Surveillance System of the United States received reported 5, 760 outbreaks and 100,939 illnesses from 2009 to 2015 (Dewey-Mattia et al., 2018). From 2003 to 2012, there were 390 *Escherichia coli* O157 outbreaks in the United States, 78 of which were from beef (Heiman et al., 2015). There are also many reports of yersiniosis outbreaks (due to *Yersinia enterocolitica*) from pasteurized milk and pork (Gupta et al., 2015). Improper handling of food was the cause of these outbreaks.

To prevent such outbreaks, food manufacturers conduct inspections of products in which they target indicator microorganisms such as coliform bacteria to achieve hygiene control. Coliform bacteria are Gram-negative, non-spore-forming rod-shaped cells that are aerobic or facultatively anaerobic and produce acids and gases by decomposing lactose within 48 h (JFHA, 2015). The term coliform is used in the field of food hygiene bacteriology and includes many *Enterobacteriaceae*, such as species in the genera *Citrobacter*, *Enterobacter*, *Escherichia* and *Klebsiella* (JFHA, 2015). Historically, coliform bacteria were used as indicators of fecal contamination. However, because these bacteria are widely distributed in nature regardless of feces, they are now considered sanitary indicators. In addition, contaminated coliforms produce off-flavors, so they are also used as quality indicators (JFHA, 2015). Thus, coliform bacteria levels are commonly measured in meat products, seafood, frozen desserts, soft drinks, and dairy products (JFHA, 2015; Martin et al., 2016; Trmčić et al., 2016).

Culturing is the traditional method used to detect coliform bacteria. In this procedure, violet red bile agar (VRBA) and a food sample solutions are mixed and incubated for 24 to 48 h. Then, colonies presumed to be positive are selected and inoculated into a brilliant green lactose bile (BGLB) fermentation tube and cultured for another 24 h. Gas evolution indicates that the food is coliform-positive (JFHA, 2015). A major limitation of this method is that it takes 2 to 3 days to obtain the results. Another method uses agar medium with β-galactosidase (a lactose degrading enzyme) to determine the number of coliform bacteria, but this method takes approximately 1 day. The culture method has been used for many years and is considered the “gold standard”; however, this method is very time-consuming (Gupta et al., 2015). A PCR-based method was developed that can detect bacteria without culturing (Tominaga, 2007; Gokduman et al., 2016; Rawool et al., 2016). Using this method, *LacZ*, a gene encoding β-galactosidase, was targeted to detect coliform bacteria (Martín et al., 2010; Molina et al., 2015; Hu et al., 2016). Because this method does not require culturing, it can be completed in 2 to 4 h. However, this method requires dedicated equipment and trained personnel (Shan et al., 2015) and is therefore difficult to use at food production or retail sites.

Recent research has focused on Lateral Flow Test Strip (LFTS) analysis to quickly obtain on-site results (Ramos et al., 2017; Tominaga, 2017). In this method, bacterial cells in a droplet of sample solution are

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Table 1
LFTS assays of food isolates.

		Origin	pAb name used for LFTS							Coliform bacteria[a]
			A5	C4	E8	H3	KR8	P2	S9	
Aeromonas sp.	c1-2	chicken cartilage	+ + +	+ + +	+	+ +	+ + +	−	−	+
Aeromonas sp.	c2-2	chicken liver	+ +	−	+	+	−	−	+ +	+
Aeromonas sp.	p11-3	pork giblets	+ + + +	−	−	−	−	−	−	+
Aeromonas sp.	p12-1	pork gut	+ +	−	−	−	−	−	−	+
Aeromonas sp.	p13-3	pork liver	+ + +	−	−	+	−	−	−	+
Citrobacter sp.	b6-1	ground beef	−	+ + +	+	+	−	−	+	+
Citrobacter sp.	b15-1	sliced beef	−	+ +	−	+	+	−	−	+
Citrobacter sp.	c1-3	chicken cartilage	+	+ + + +	+ + + +	+ + + +	+ + + +	−	−	+
Citrobacter sp.	c4-2	chicken skin	+ +	+ + + +	+ + + +	+ + + +	+ + +	+ + +	−	+
Citrobacter sp.	s1-1	tofu	−	+ +	−	−	−	−	−	+
Enterobacter sp.	b6-4	ground beef	−	+	+ + +	+ +	+	−	+ +	+
Enterobacter sp.	b16-4	sliced beef	+ +	+ +	+ + +	+	+	+ +	+ +	+
Enterobacter sp.	c1-1	chicken cartilage	−	−	+ + +	+	+	+	+	+
Enterobacter sp.	c4-4	chicken skin	−	+ +	+ + + +	+ + +	+ +	+	+	+
Enterobacter sp.	c9-2	minced chicken	−	+ +	+ + + +	+ + +	+	+ +	+ +	+
Enterobacter sp.	c10-1	minced chicken	+	+ + + +	+ + +	+ +	+ +	−	+ + +	+
Enterobacter sp.	p7-2	ground pork	+	−	+ + + +	+	+ +	+	+	+
Enterobacter sp.	p13-2	pork liver	+ +	+ + +	+ + + +	+ + +	+ +	+ + +	+ +	+
Enterobacter sp.	a4-1	pastry	−	−	+	−	+	−	−	+
Enterobacter sp.	s7-1	pastry	−	−	+ + +	−	−	−	−	+
Enterobacter sp.	s9-1	pastry	+	−	+ + +	+	+	−	−	+
Enterobacter sp.	r3-1	pastry	+ +	+ + +	+ + +	+ +	+ +	−	−	+
Enterobacter sp.	sm1-1	ready-to-eat	+ + + +	+ + +	+ +	+ +	+ +	−	+	+
Enterobacter sp.	sm2-1	ready-to-eat	+ +	+ +	+ + +	+	+	+	+ +	+
Enterobacter sp.	xm1-1	ready-to-eat	+	+	−	−	−	−	−	+
Enterobacter sp.	xm2-1	ready-to-eat	+	+ +	+	−	−	−	+	+
Enterobacter sp.	s2-1	tofu	−	−	+ + +	+	−	−	−	+
Hafnia alvei	b14-2	sliced beef	−	−	−	+ + + +	−	−	+	+
Hafnia alvei	b15-3	sliced beef	−	−	+	+ + + +	+	−	−	+
Hafnia alvei	b17-2	sliced beef	+	+ + +	+ +	+ + + +	+ + + +	−	+	+
Klebsiella sp.	b6-2	ground beef	+ + +	+ +	+ + + +	+ + + +	+ + + +	+	+	+
Klebsiella sp.	b15-2	sliced beef	+	+ + +	+ +	+ + +	+ + + +	+	+	+
Klebsiella sp.	b16-1	sliced beef	+ +	+ +	+ + + +	+ + +	+ +	+ +	+ + +	+
Klebsiella sp.	b16-2	sliced beef	+ +	+ +	+ + + +	+ + + +	+ + +	+	+ +	+
Klebsiella sp.	p18-3	sliced pork	+ + +	+ + +	+ + + +	+ + + +	+ + + +	−	+ +	+
Klebsiella sp.	x4-1	ready-to-eat	+ + + +	+ + +	+	+ + +	+ +	−	+	+
Klebsiella sp.	x5-1	ready-to-eat	+ +	+ +	+	+ + +	+	−	−	+
Klebsiella sp.	s3-1	tofu	−	−	−	−	+	−	−	+
Raoultella sp.	c2-3	chicken liver	−	+	+	−	+ + +	−	+	+
Raoultella sp.	c3-3	chicken liver	−	+ +	+	+ + +	+ + + +	+	+	+
Raoultella sp.	p13-4	pork liver	+	−	+	+ +	+ + + +	+	+	+
Pantoea sp.	c8-2	minced chicken	−	+ + +	+ +	−	−	+ + + +	+ +	+
Pantoea sp.	p18-1	sliced pork	+	+ + +	+ + + +	+ +	+	+ + + +	+ +	+
Serratia sp.	b14-1	sliced beef	−	+	+	+	−	+	+ +	+
Serratia sp.	c2-1	chicken liver	+	−	+	+	+	+	+ + +	+
Serratia sp.	c5-1	chicken thigh	−	−	−	−	+	−	+ +	+
Serratia sp.	c8-1	minced chicken	−	−	−	−	−	−	+ + + +	+
Serratia sp.	c9-1	minced chicken	−	−	−	+	+ + +	−	+ +	+
Serratia sp.	c10-2	minced chicken	−	+	+	+	+ +	+	+ + +	+
Serratia sp.	c10-3	minced chicken	−	−	+	−	+ +	+	+ + + +	+
Serratia sp.	p11-1	pork giblets	−	−	+ + +	+ +	−	+	+ +	+
Serratia sp.	p12-2	pork gut	−	−	+	−	+	−	+ + +	+
Serratia sp.	xk1-1	ready-to-eat	−	−	−	−	−	−	+ +	+
Serratia sp.	xk2-1	ready-to-eat	+ +	+ +	−	+ +	−	−	+ +	+
Serratia sp.	xk6-1	ready-to-eat	−	−	+	−	−	−	+ + +	+
Average[b]			1465	1913*	2044*	1844	1772	1219	1450	/
SD[b]			865	1064	1296	1263	1353	1051	905	/
Coverage[c] (%)			53	58	76	71	67	38	69	100

[a] The presence of coliform bacteria was defined as "+" when any of the 7 LFTS assays was positive.
[b] Averages and standard deviations are based on assays that were positive.
[c] Percentage of assays that were positive.
* Average value significantly greater than that of S9-LFTS ($p < 0.05$).

bound to antibodies (Abs) labeled with a metal or latex colloid. Then, the cells migrate on the test strip by capillary force, and other Abs immobilized on the strip capture them. Finally, colloid-derived colored spots or lines are generated, indicating the presence of target bacteria. This analysis can be performed in approximately 10 min and requires no special equipment.

Here, an LFTS assay was developed for the detection of coliform bacteria. Seven polyclonal antibodies (pAbs) against the genera *Aeromonas, Citrobacter, Enterobacter, Hafnia, Klebsiella/Raoultella, Pantoea* and *Serratia* were prepared and used on LFTSs. The detection rates of coliform bacteria in food isolates and culture collection strains containing clinical isolates were investigated using the seven LFTS assays. A feasibility study was performed on meat samples to investigate whether coliform bacteria could be detected by an LFTS assay at

incubation times of 0, 8 and 24 h.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Tables 1 and 3 list the strains used in this study. LB medium (Becton, Dickinson and Company, NJ) was used for culturing, and shaking cultivation was performed at 37 °C for 16 to 20 h. If necessary, 2% (w/v) agar was added to the medium for the determination of bacterial counts.

2.2. Production of pAbs

The strains used as antigens were isolated from foods. Five strains of *Aeromonas* were used to prepare pAb A5. Four strains of *Citrobacter* were used to prepare pAb C4, 8 strains of *Enterobacter* were used to prepare pAb E8, 3 strains of *Hafnia alvei* were used to prepare pAb H3, 5 strains of *Klebsiella* and 3 strains of *Raoultella* were used to prepare pAb KR8, 2 strains of *Pantoea* were used to prepare pAb P2, and 9 strains of *Serratia* were used to prepare pAb S9. All strains used for immunization were grown for 18 h in 3 mL of LB medium at 37 °C. Cultures were evenly mixed, and the total liquid volume was 12 mL in a 50 mL tube. Then, the mixture was centrifuged at 5,000 ×g for 10 min at 4 °C, and the pellet was washed 3 times in 12 mL of 0.85% NaCl with centrifugation at 5,000 ×g for 10 min at 4 °C. Finally, 1×10^{10} cells were suspended in 1 mL of 0.85% NaCl with formaldehyde (0.74%, v/v). The cells were stored at 4 °C for 24 h.

Immunization and pAb preparation was performed at Kawaguchi Chemical (Saitama, Japan). Rabbits received intravenous injections of 5×10^8 cells per dose without adjuvant. Each animal received 11 injections every 3 to 4 days. Preparation of IgG fractions from collected sera was performed using immobilized protein A. After binding of sera diluted in binding buffer (1.5 M glycine + 3.0 M NaCl, pH 8.9) followed by washing of the column, the IgG fraction was eluted using a buffer solution (0.1 M citrate acid + 0.5 M NaCl, pH 4.0). The absorbance was measured at 280 nm, and the IgG molar extinction coefficient ($2.1 \times 10^{-5}\,M^{-1}\,cm^{-1}$) and molecular weight (150 kDa) were used to calculate the concentration.

2.3. Development of LFTSs

LFTSs were constructed as reported previously (Tominaga, 2018). Briefly, purified pAbs (0.2 μg/test) were immobilized onto the nitrocellulose membrane (FF80HP PLUS, GE Healthcare, IL). Identical pAbs were labeled with colloidal nanoparticles (0.25 μg/test) and used as detection pAbs. Bacteria were collected from the culture solution by centrifugation, washed in 0.85% NaCl, and heated for 15 min at 100 °C. Then, the cells were mixed with detection pAbs and applied to the membrane. For simplicity, all treatments are identified using abbreviations (*e.g.*, "A5-LFTS" for "pAb A5-based LFTS"). An image of the test paper was acquired with a scanner (MX 893, Canon, Tokyo, Japan) at a resolution of 600 dpi, and the density of each spot was quantified as "++++" (> 3,001 arbitrary unit: a.u.), "+++" (2,001–3,000 a.u.), "++" (1,001–2,000 a.u.), "+" (401–1,000 a.u.), or "–" (< 400 a.u.).

2.4. Sensitivity test

The bacterial solutions were serially diluted 10 times, and a sample with 3 to 8 $\log_{10}$ (cfu) of bacteria was applied to the LFTS. The signal intensity was evaluated as described above.

2.5. Food sample test

Twelve types of meat were purchased from retail stores, stored overnight at 4 °C, and tested on the following day. A 100 g sample of each meat was placed on a styrofoam dish, covered with cling film, and incubated at 37 °C. After 0, 8, and 24 h of incubation, 10 g of meat was sampled and homogenized in 90 mL of 0.85% NaCl using a Masticator (IUL, Barcelona, Spain) for 30 s. Then, 1.25 mL of the suspension was centrifuged at 380 ×g for 2 min, and 1 mL of the supernatant was further centrifuged at 9,600 ×g for 1 min. The pellet was washed in 0.85% NaCl, heated at 100 °C for 15 min, and then subjected to the LFTS assay.

For determination of bacterial counts, the homogenized foods were diluted in 0.85% NaCl, applied to XM-G agar medium (Nissui Pharmaceutical, Tokyo, Japan), cultured at 37 °C for 20 h, and the purple colonies were counted.

2.6. Statistical analysis

Student's *t*-test was used to determine statistical significance using Excel 2013 (Microsoft Corporation, WA).

3. Results

3.1. Detection of bacteria in food isolates

LFTS assays were conducted against 55 food isolates (Table 1). All Abs recognized the original antigens and had signal intensities higher than "++". The Abs also showed cross-reactions in that they recognized strains other than the original genus. Thus, the C4-LFTS assay detected *Enterobacter*, *Hafnia*, *Klebsiella*, *Pantoea* and *Citrobacter*. Similarly, the H3-LFTS assay detected *Citrobacter* and *Klebsiella* in addition to *Hafnia*. For the individual LFTS assays, the detection rate of coliform bacteria was 38% to 76%. However, the coordinated use of all 7 LFTS assays led to a detection rate of 100%.

3.2. Sensitivity test

The sensitivity of the LFTS assay was examined (Table 2). Positive signals were produced at ≥6 $\log_{10}$ (cfu/test) in the A5-LFTS assay, ≥4 $\log_{10}$ (cfu/test) in the S9-LFTS assay and ≥5 $\log_{10}$ (cfu/test) in the other assays.

Table 2
Sensitivity of the LFTS assays.

Tested strain	pAb name used for LFTS	log (cfu/test)					
		8	7	6	5	4	3
Aeromonas sp. c2-2	A5	++	++	++	−	−	−
Citrobacter sp. c4-2	C4	++++	+++	+++	+	−	−
Enterobacter sp. p7-2	E8	++++	++++	+++	++	−	−
Hafnia alvei b14-2	H3	++++	+++	+++	++	−	−
Raoultella sp. p13-4	KR8	++++	++	++	++	−	−
Pantoea sp. c8-2	P2	++++	++	++	+	−	−
Serratia sp. c8-1	S9	++++	++++	++++	++	+	−

Table 3
LFTS assays of culture collection strains.

	Origin	pAb name used for LFTS							Coliform bacteria[a]
		A5	C4	E8	H3	KR8	P2	S9	
Aeromonas hydrophila subsp. *hydrophila* NBRC 3820		−	−	−	+ + +	−	−	−	+
Aeromonas hydrophila subsp. *hydrophila* NBRC 12658		+	−	−	−	−	−	−	+
Aeromonas hydrophila subsp. *hydrophila* NBRC 12978		+	−	−	+	+	−	+	+
Aeromonas hydrophila subsp. *hydrophila* NBRC 12981		−	−	−	−	−	−	−	−
Aeromonas hydrophila subsp. *hydrophila* NBRC 13286	juvenile silver salmon intestine	+	−	−	−	+	−	+	+
Citrobacter amalonaticus JCM 1661^T	stool	−	−	−	−	+	−	−	+
Citrobacter freundii IAM 12471^T		−	+ +	−	+	−	+	−	+
Citrobacter freundii NBRC 13539	milk set for cottage cheese	−	+	−	−	−	−	+	+
Citrobacter freundii NBRC 13546		−	+	−	+	+	−	+	+
Citrobacter freundii NBRC 16624		−	+ +	+	+	+ +	−	+ +	+
Citrobacter rodentium NBRC 105723^T	hamster	−	−	−	+	+	−	+	+
Cronobacter sakazakii JCM 1233^T	child's throat	+	+	+	+ +	+	−	+ +	+
Enterobacter aerogenes IAM 12348^T	sputum	+ + +	−	−	+	+ + + +	−	+ +	+
Enterobacter aerogenes NBRC 12010	feces	−	−	−	−	−	−	+	+
Enterobacter cloacae subsp. *cloacae* IAM 12349^T	spinal fluid	−	−	+ + + +	+	−	+	+	+
Enterobacter cloacae subsp. *cloacae* NBRC 13536		−	−	+ + + +	−	+	−	+	+
Escherichia coli NBRC 102203^T	urine	−	−	−	−	−	−	−	−
Hafnia alvei JCM 1666^T		−	−	−	−	−	−	−	−
Hafnia alvei NBRC 3731		−	−	−	+	+	−	+	+
Klebsiella oxytoca ATCC 43086		+ + +	+	+ + + +	+ + +	+ + +	−	+ +	+
Klebsiella oxytoca ATCC 43165	clinical isolate, California	−	+ + + +	+ + + +	+ + + +	+ + + +	+	+ + +	+
Klebsiella oxytoca ATCC 8724		+ + + +	+ + + +	+ + + +	+ + + +	+ + +	−	+	+
Klebsiella oxytoca NBRC 102593^T	pharyngeal tonsil	+ +	+ + +	+ +	+	+	+ +	+	+
Klebsiella pneumoniae IAM 1063		+ +	+ + +	+	+	+ +	−	+	+
Klebsiella pneumoniae NBRC 3318		−	+ + +	−	+	+	+ +	+ +	+
Klebsiella pneumoniae NBRC 3319		+ +	+ + + +	+	+ + +	+ + + +	+ + + +	+ + +	+
Klebsiella pneumoniae subsp. *pneumoniae* NBRC 14940^T	−	−	−	+	+	−	+	+	
Kluyvera ascorbata IAM 14203^T	human sputum	−	−	+	+	−	−	+	+
Leclercia adecarboxylata JCM 1667^T	drinking water	−	−	+	+	−	−	+	+
Pantoea agglomerans NBRC 12686		+	+	+	+	+	+	+ +	+
Raoultella ornithinolytica JCM 6096^T	urine	−	−	−	+	+	−	+	+
Raoultella planticola JCM 7251^T	radish root	−	−	−	−	+ + + +	−	+	+
Raoultella terrigena JCM 1687^T	drinking water	−	−	+	−	+ + + +	−	+	+
Serratia ficaria NBRC 102596^T	fig	−	−	+ +	−	−	−	+ + +	+
Serratia grimesii NBRC 13537^T		−	−	−	−	−	−	+ +	+
Serratia fonticola JCM 1242^T	spring water	−	−	−	+ + + +	+	−	+	+
Serratia liquefaciens JCM 1245^T		−	−	−	+	−	−	−	+
Serratia odorifera NBRC 102598^T	sputum	−	−	−	−	−	−	−	−
Average[b]		1507	1930*	1734*	1315	1650	1277	1095	/
SD[b]		1111	1322	1362	1189	1460	920	651	/
Coverage[c] (%)		29	34	39	63	61	18	76	89

[a] The presence of coliform bacteria was defined as "+" when any of the 7 LFTS assays was positive.
[b] Averages and standard deviations are based on assays that were positive.
[c] Percentage of assays that were positive.
* Average value significantly greater than that of S9-LFTS ($p < 0.05$).

3.3. Detection of bacteria in culture collections

LFTS assays were conducted on against 38 culture collection strains (Table 3). The S9-LFTS assay had significantly lower signal intensity than the C4-LFTS and E8-LFTS assays, but it tested positive for more strains. The detection rate of coliform bacteria was 18% to 76% for the individual LFTS assays but was 89% for the combined use of all 7 LFTS assays.

3.4. Feasibility study

The detection of coliform bacteria in food samples was examined in tests on 20 types of meat (Table 4). Without incubation (0 h), the LFTS assay was positive for 3 types of meat (4.9 $\log_{10}$ [cfu/g] ~ 6.6 $\log_{10}$ [cfu/g]). For other meats, coliform bacteria could not be detected by the LFTS assays (2.0 $\log_{10}$ [cfu/g] ~ 4.3 $\log_{10}$ [cfu/g]). After 8 h of incubation, the LFTS assay was positive for all tested meats, and the number of coliform bacteria was 5.4 to 7.4 $\log_{10}$ (cfu/g). After 24 h of incubation, the LFTS assay was also positive for all tested meats, and the number of coliform bacteria was 7.6 to 9.8 $\log_{10}$ (cfu/g).

Fig. 1 shows the number of LFTS assays that had positive signals and the maximum signal intensities for 8 h and 24 h incubation times. These results indicate that an increased incubation time led to more positive signals (1.8 at 8 h and 4.6 at 24 h) and an increased signal intensity (1366 at 8 h and 2678 at 24 h) ($p < 0.01$).

4. Discussion

This paper describes the development and feasibility testing of a rapid method for the detection of coliform bacteria using an LFTS assay.

Table 4
LFTS assays of meats from a retail store that were incubated for 0, 8 and 24 h.

Foodstuff	Incubation time (h)	log(cfu/g)	pAb name used for LFTS							Coliform bacteria[a]
			A5	C4	E8	H3	KR8	P2	S9	
Ground pork	0	3.3	−	−	−	−	−	−	−	−
	8	6.1	−	−	−	−	+	−	−	+
	24	9.5	−	+ +	+ +	+	+	+ +	+ +	+
Ground pork	0	2.0	−	−	−	−	−	−	−	−
	8	5.4	−	−	+	−	−	−	−	+
	24	7.6	−	+	+	+	+ +	−	−	+
Ground pork	0	3.7	−	−	−	−	−	−	−	−
	8	6.2	−	−	−	+	+	−	−	+
	24	8.6	−	+ + + +	+	+ + + +	+	+ + +	+ + +	+
Ground pork	0	3.0	−	−	−	−	−	−	−	−
	8	6.3	−	−	+	−	−	−	+ + +	+
	24	8.7	−	+	+ + +	+ + +	−	+ +	+ + +	+
Ground pork	0	2.9	−	−	−	−	−	−	−	−
	8	6.5	−	−	+ +	−	−	−	+	+
	24	9.2	−	−	+ + +	−	+	−	+	+
Ground pork	0	4.9	−	−	−	−	−	−	+	+
	8	7.4	−	−	+ +	−	−	−	+	+
	24	8.6	−	−	+ +	+	−	−	+ +	+
Ground pork	0	3.9	−	−	−	−	−	−	−	−
	8	5.8	−	−	−	−	−	−	+	+
	24	8.4	−	−	+ + +	+ +	−	+	+ +	+
Ground beef	0	2.7	−	−	−	−	−	−	−	−
	8	6.0	−	−	+	−	+	−	+ +	+
	24	8.8	−	+ + + +	+ + + +	+ +	+ + +	+ + +	+ + + +	+
Minced chicken	0	3.3	−	−	−	−	−	−	−	−
	8	6.1	−	−	+	−	−	−	+	+
	24	7.8	−	+ +	+ + +	−	+	−	+ + +	+
Minced chicken	0	4.0	−	−	−	−	−	−	−	−
	8	6.9	−	−	+	−	−	−	−	+
	24	7.8	−	+	+ +	+	−	+	+	+
Minced chicken	0	4.3	−	−	−	−	−	−	−	−
	8	6.9	−	−	+	−	−	−	−	+
	24	8.6	−	−	+	−	−	−	+ +	+
Minced chicken	0	4.3	−	−	−	−	−	−	−	−
	8	6.8	−	−	+	−	−	−	−	+
	24	9.2	−	+	+ + +	+	−	−	+	+
Sliced pork	0	3.0	−	−	−	−	−	−	−	−
	8	6.0	−	−	−	−	−	−	+	+
	24	8.3	−	−	+ +	−	−	−	+ + +	+
Sliced pork	0	6.6	+ + + +	−	−	−	−	−	+ +	+
	8	6.8	+ + + +	−	−	−	−	−	+ +	+
	24	7.9	+ + + +	−	−	−	−	−	+ +	+
Sliced pork	0	3.4	−	−	−	−	−	−	−	−
	8	6.7	−	−	+ + +	−	−	−	+ + +	+
	24	9.8	−	+	+ + + +	+ +	−	−	+ + +	+
Sliced pork	0	2.9	−	−	−	−	−	−	−	−
	8	7.0	−	+	+ + + +	−	−	−	+	+
	24	8.4	+ +	+ +	+ + + +	+	+	+	+ +	+
Sliced pork	0	5.0	−	−	−	−	−	−	+	+
	8	5.7	−	−	+	+	−	−	+ +	+
	24	8.3	+	+ +	+ + +	+ + +	+	+ +	+ +	+
Sliced beef	0	2.7	−	−	−	−	−	−	−	−
	8	6.2	−	−	+ +	−	−	−	−	+
	24	7.9	+	−	+ + +	+ +	+ + +	−	+ +	+
Sliced beef	0	2.5	−	−	−	−	−	−	−	−
	8	6.4	−	−	−	+ + +	−	−	−	+
	24	9.2	−	+	+	+ + + +	−	+ +	+ +	+
Sliced beef	0	2.3	−	−	−	−	−	−	−	−
	8	5.9	−	−	+ +	−	+ +	+ +	+ +	+
	24	9.1	+ +	+ + +	+ +	+ + +	+ +	+ +	+ + + +	+

[a] The presence of coliform bacteria was defined as "+" when any of the 7 LFTS assays was positive.

4.1. Why were coliform bacteria targeted?

Foods are exposed to the risk of contamination by various pathogens, such as *E. coli* O157, *Listeria monocytogenes, Y. enterocolitica.* In the past, the presence of coliform bacteria was thought to indicate potential pathogen contamination, so these bacteria were regarded as an indicator of food safety. However, it seems that the detection of coliform bacteria from food is not always associated with pathogen contamination (Trmčić et al., 2016). The implication that coliforms are a marker for food safety is diminishing. Nevertheless, foods for which coliforms are detected in excess of the specified amount may suggest insufficient heating or secondary contamination after the sterilization process. Then, coliform bacteria are considered to be sanitary indicators. Thus, to ascertain food safety, it may be preferable to combine an LFTS assay that detects individual pathogens (Tominaga, 2018) with an LFTS assay that detects coliforms.

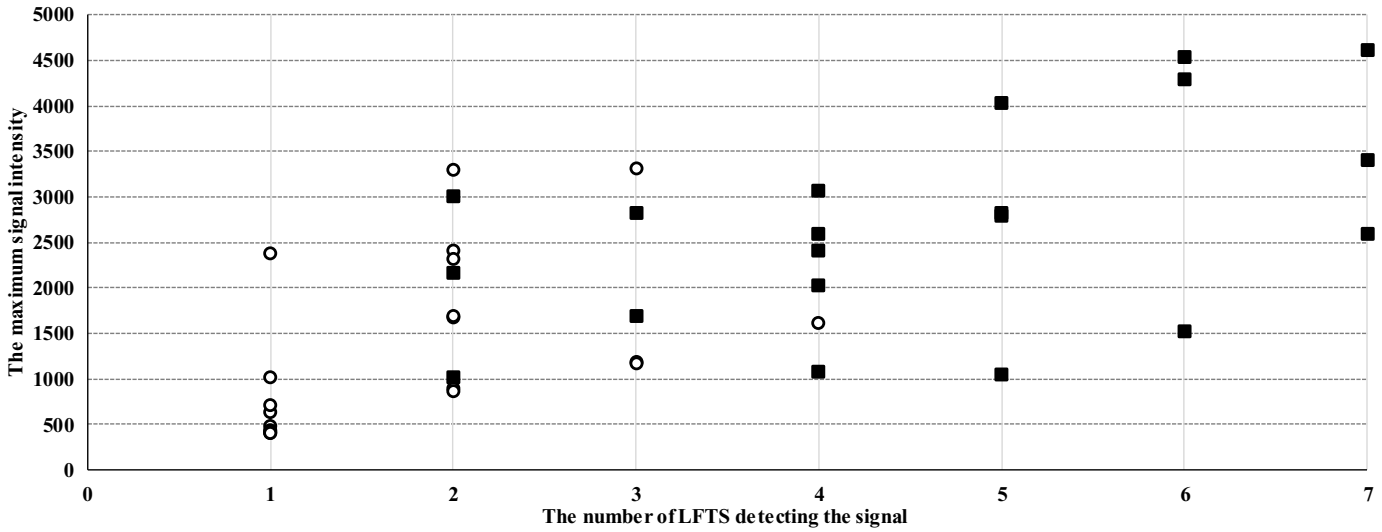

Fig. 1. Effect of incubation time on LFTS assay positivity.
Abscissa: number of assays with a positive signal; ordinate: maximum signal intensity of each assay. ○: 8 h incubation, ■: 24 h incubation.

Coliform bacteria are detected in a wide variety of foods, such as meats, pastries and ready-to-eat food. The growth rate of coliforms is fast in a wide temperature range from low to medium temperatures (Yoshida et al., 2015). Once the number of coliforms contaminating food reaches a certain level, they produce off-flavors and reduce the shelf life (Martín et al., 2010). Therefore, coliform bacteria are considered to be indicators of food quality in this context. It is also assumed that the LFTS assay developed here will be used as a freshness marker.

In starting the present study, a preliminary test for isolating coliforms from food using XM-G medium was carried out, and the genus to be the target of antibody production was selected. Since this medium contains β-galactosidase and β-glucuronidase substrates as ingredients, an *E. coli* colony becomes blue-violet in color, whereas other coliform bacteria develop a reddish-purple color, enabling the number of both to be counted separately. In the preliminary test, although *E. coli* was occasionally detected in meat, it was not targeted in this study because the isolation number was too small. In the future, if LFTSs for *E. coli* are prepared and added to the present LFTSs set, improvement in detection sensitivity can be expected.

4.2. Detection of coliform bacteria using the LFTS assay

The LFTS assay detects target bacteria utilizing Abs that recognize specific bacterial antigens, surface polysaccharides and proteins in particular (Van Regenmortel, 2014). An Ab usually only binds to an antigen on a specific strain of bacteria. For example, there are more than 180 O-antigens in *E. coli* and 9 O-antigens in *Klebsiella pneumoniae* (Joensen et al., 2015; Clarke et al., 2018), and Abs used for O-typing have very high specificity. Therefore, the use of an LFTS assay for the detection of coliform bacteria, which includes many genera, requires an Ab preparation with reduced specificity.

Some Abs can cross-react with multiple species. For example, an Ab against *Moraxella catarrhalis* lipooligosaccharide cross-reacts with *Haemophilus influenzae* and *Haemophilus parainfluenzae* (Gergova et al., 2007), and an Ab against *Klebsiella* sp. cross-reacts with *Serratia* sp. (Tominaga, 2018). Cross-reactivity can occur when similar surface polysaccharides occur in different species (Shashkov et al., 2015). In addition, because an Ab only recognizes a 2 to 5 amino acid epitope, cross-reactivity can occur when there are structurally related antigens (Van Regenmortel, 2014). The advantage of cross-reactive Abs is that a small set of different Abs can be used to detect multiple types of bacteria.

The polysaccharides of the outer cell wall of bacteria include an O-antigen, which withstands heating at 100 °C for 2 h (Yoshida et al., 2015). Therefore, to obtain Abs against the O-antigen, cells are heated at 100 °C for 1 to 2 h to eliminate other components prior to immunization (Gaston et al., 1983; Hochel and Škvor, 2009; Xu et al., 2014). In this study, cells were fixed not by heating but by formaldehyde treatment, so a part of the sugar chains and proteins other than O-antigen might have remained on the cell surface. The purpose of this fixation method was to provide a wide variety of antigenic substances for immunization so that more diverse Abs could be obtained, some of which should be cross-reactive. Moreover, Abs against multiple strains were obtained by mixing cells of 3 to 9 strains and immunizing with the mixture. The results indicated that even when cells of 9 strains were mixed, all strains used as antigens were recognized. The prepared Abs could recognize food and clinical isolates such as *Cronobacter* and *Kluyvera* because of cross-reactivity. The individual LFTS assays were only 38% to 76% positive against food isolates and 18% to 76% positive against culture collection strains; however, the 7 LFTS assays in combination were 100% positive against food isolates and 89% positive against culture collection strains.

In contrast, because the assay failed to detect some strains of coliform bacteria, it may be necessary to collect these unreacted strains and obtain Abs against them. Alternatively, development of an LFTS assay based on an Ab that recognizes a commonly expressed surface antigen, such as enterobacterial common antigen (Liu et al., 2015), poly-(β-1,6)-*N*-acetyl glucosamine (Skurnik et al., 2016), or MrkA, a major protein in the type III fimbriae complex (Wang et al., 2016), that can be used in conjunction with the LFTS assay described here may allow detection of all coliform bacteria.

4.3. Significance of monitoring coliform bacteria in meat

Meat is rich in nutrients (De Filippis et al., 2013), and microorganisms attached to raw meat during the processing and preservation processes are likely to propagate (Nychas et al., 2008; Casaburi et al., 2015). *Enterobacteriaceae, Pseudomonas*, lactic acid bacteria, and *Brochothrix thermosphacta* are the major causative agents of meat decay (Doulgeraki et al., 2012; De Filippis et al., 2013; Stellato et al., 2016). When these bacteria proliferate, fresh meat spoils, leading to gross discoloration, strong off-odors, and the development of surface “slime” (bacterial pellicle) (Nychas et al., 2008; Dave and Ghaly, 2011; De Filippis et al., 2013; Casaburi et al., 2015). It is important for consumers to avoid the consumption of spoiled meat, which is unhealthy (Gram et al., 2002), even when it is cooked. It is also important for retailers to

predict the shelf life of their products (Nychas et al., 2008). This is why a method for evaluating and monitoring meat freshness is needed.

The freshness of meat can be simply evaluated based on the presence of odor and surface liquids (Chabela et al., 1999), although this assessment is generally subjective (Nychas et al., 2008). Although there are more objective testing methods based on sensory evaluation by experts and microbial analysis (Nychas et al., 2008), the former requires expert groups in a restricted testing site, and the latter often requires excessive amounts of time. An objective and rapid method of evaluation has yet to be developed. Thus, an LFTS assay that overcomes the above limitations was developed in the present work.

All the genera examined in this study are common in raw meat (Doulgeraki et al., 2012; Casaburi et al., 2015). After an 8 or 24 h incubation period, the LFTS assay successfully detected coliform bacteria in all tested raw meats. The freshness of the meat can be estimated from the incubation time required for signal detection, the number of positive LFTS assays, and the maximum signal intensity. The time required for the assay (including pretreatment) is only approximately 30 min. Moreover, the assay is objective and does not require specialized equipment or personnel. Therefore, the LFTS assay described here is suitable for use in food production plants, as well as supermarkets and retail stores.

4.4. Future prospects

The presence of coliform bacteria indicates poor hygiene in milk and cheese (Martin et al., 2016; Trmčić et al., 2016), and the LFTS assay described here can be used as a "kit" to evaluate the freshness of such foods. The LFTS assay appears to be a promising tool that has the potential to secure the safety of many types of foods.

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