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A cocktail PCR and DNA strip method for quick confirmation of multiple pathogenic factors in BSL3 stock cultures

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Repositories of culture collections of Biosafety Level 3 (BSL3) pathogens are expected to provide information on the pathogenic factors present in stock bacteria. However, it is difficult to maintain valid pathogenic information on each of the different BLS3 pathogens because quality control of the stock strains generally has to be performed by a few staff members.

We developed a common platform to differentiate six different PCR amplicons on a DNA strip. The six amplicons can be differentiated with the help of three position markers printed on the strip. With this method, the pathogenic factors in BSL3 pathogens can be determined within 40 min after cultured bacteria are picked up from a single colony.

INTRODUCTION

The provision of quality information on specific pathogens associated with Japanese Infectious Diseases (SPJID) through the activities of the National Bioresource Project of the Japanese Ministry of Education, Culture, Sports, Science and Technology, is especially important for both the collection of cultures and their use. The pathogenic factors of Biosafety Level 3 (BSL3) pathogens and SPJID are often encoded on plasmids or insertion sequences on the bacterial chromosomes. These factors are, therefore, unstable and are often "cured" (i.e. eliminated) from the host. The important pathogenic factor *invE* (virB) of *Shigella* spp. and enteroinvasive *Escherichia coli* is required for the invasiveness of these bacteria (Mitobe *et al*., 2009). This factor is encoded on a plasmid and is easily cured from the host when stock bacteria are cultured. Once the factor has been eliminated from the host, it is difficult to differentiate these pathogens from non-pathogenic *E. coli*. The Vi antigen of *Salmonella enterica* serovar Typhi is the most important virulence factor used for differentiating this serovar from the 2000 other serovars of *S. enterica*, but the Vi synthesis gene clusters are naturally deleted from the host chromosome (Hashimoto *et al*., 1996, Hashimoto & Khan, 1997).

Capsular antigen fraction 1 (caf1) (Galyov, 1990) and plasminogen activator surface protease (pla) are located on plasmids of *Yersinia pestis* (Guinet & Carniel, 2012). Once the plasmids are cured from the host, it becomes very difficult to differentiate *Y. pestis* from the closely related *Yersinia pseudotuberculosis*.

Type strains and strains used for serotyping of SPJID are especially important for maintaining quality information on the pathogenic factors in these bacteria. Simple and rapid confirmation methods are required so that these strains can be checked before they are distributed. To fulfill this need, we developed a tagged-cocktail PCR method (Hayashi, 2013) and established a protocol to differentiate the PCR amplicons by using a DNA strip containing six printed anti-tags and three position markers. We applied this technology to confirm the presence of pathogenic factors of BSL3 and SPJID strains. The PCR amplification method was also optimized for this with the use of a low-profile PCR tube and a quick amplification PCR machine. We established a protocol to confirm the presence of pathogenic factors of BSL3 and SPJID strains within 40 min from the time bacteria were collected from a single colony on a culture plate.

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MATERIALS AND METHODS

Cell cultures

Strains from the Gifu Type Culture Collection (GTC), and the Japan National Collection of Bacterial Pathogens (JNBP), along with wild *Y. pestis* strains stocked at the Mongolian National Center for Zoonotic Diseases (Ulaanbaatar, Mongolia) were used. The tagged primers used are listed in Table 1. Upstream primers were labeled with a common polyA sequence and downstream primers were labeled with six different tags. These primers were synthesized by Sigma-Aldrich Japan (Tokyo, Japan) and Tohoku Bio-Array Corporation (Sendai, Japan). The DNA strips printed with six different anti-tags and three position markers were kindly provided by Tohoku Bio-Array Corporation (Fig. 1).

Examination methods

Bacteria from a single colony plated the day before and incubated overnight were suspended in 500 μl of distilled water, and the solution was boiled to disrupt the cells. A volume of 2.5 μl of the solution was used for cocktail PCR analysis. The concentrations of the single-colony suspensions ranged from 10^7 to 10^8 cfu/ml. To determine the detection limit, cell suspensions of 10^1 to 10^8 cfu/ml were also prepared. For rapid PCR, a low-profile PCR tube was selected and a total of 10 μl of the reaction mixtures was used for analysis. The reaction mixtures contained 2.5 μl of 4× primer mixture (0.8 μM), 2.5 μl of crude DNA suspension, and 5 μl of 2× premixed Taq polymerase (TakaraBio, Kyoto, Japan). After denaturation at 97℃ for 2 min, samples were amplified under the following two-step PCR conditions: 60℃ for 10 s and 97℃ for 5 s. Thirty PCR cycles were performed. The total amplification time was within 30 min.

After PCR amplification, 10 μl of a 0.5% blue latex suspension labeled with polyT was added to the PCR tube, and a DNA strip was inserted into the PCR tube for chromatography. Ten minutes later, the blue latex had been completely absorbed by the DNA strip and the target line that captured the PCR amplicon on the DNA strip had turned blue.

Sensitivity of the DNA strip

Two-fold serial dilutions of the PCR products of *E. coli* shiga2-positive strain 157:H7 (GTC 14509) were prepared. PCR products in the diluted solutions were detected with a standard latex solution to determine the endpoint of detection. Ten-fold serial dilutions of a *Salmonella enterica* GTC 3P0637 cell suspension from 10^8 to 10^1 cfu/ml were prepared, and the *invA* amplicon was detected by using SYBR green (Takara Bio, Kyoto, Japan) and a Step One Plus PCR machine (Applied Bio, Tokyo, Japan). After amplification, the amplicon was also detected by using the DNA-strip method, and the sensitivities of the two methods were compared.

Screening for pathogenic factors

Bacteria from a single colony plated the day before and incubated overnight were suspended in 500 μl of distilled water to prepare a 10^8 cfu/ml cell suspension. The solution was boiled at 100℃ for 3 min. Next, 2.5 μl of the boiled cell suspension was mixed with 2.5 μl of 4× cocktail primers (0.8 μM) and 5 μl of 2× SYBR Premix EX Taq polymerase (Takara Bio). All cocktail primers were denatured at 97℃ for 2 min. The following two-step PCR cycle was applied 30 times for all primers: 60℃ for 10 s and 97℃ for 5 s.

All JNBP strains of genus *Shigella* listed in Table 2 were analyzed for *invE, stx1* (Shiga 1) and *stx2* (Shiga 2). Reference serotyping strains, including 13 *Shigella dysenteriae*, 28 *Shigella flexneri*, 26 *Shigella boydii* and 10 *Shigella sonnei*, were analyzed. *dnaJ* primer to detect both *Shigella* species and *E. coli* was also included in the cocktail primers.

Salmonella enterica serovar Typhi and serovar Paratyphi A are SPJID pathogens. The *invA* gene is shared among all 2000 *Salmonella* serovars. To differentiate these two serovars from other *Salmonella* serovars, Vi antigen regulator (*vipR*) and flagellar antigen gene (fliC:a) were prepared. Vi antigen is specific for serovar Typhi, but it is often deleted from the chromosome (Hashimoto *et al*., 1996, Hashimoto & Khan, 1997). We therefore selected another differential factor, CRISPR2, which is a recently reported serovar Typhi-specific factor that was found by complete genome analysis (Fabre *et al*., 2014). CRISPR1 and FliC:a were selected for differentiating serovar Paratyphi A. FliC:a is a stable gene, but several other salmonella serovars belonging to group A flagellar groups cross-react with FliC:a primers. CRISPR-1 has thus far been detected only in serovar Paratyphi A (Fabre *et al*., 2014).

CT toxin of *Vibrio cholerae*

A cholera toxin (CT) and DnaJ chaperone gene

Table 1 Pathogenic factors and cocktail primers

Species	Tag position	Protein (gene)	Positive species	Forward	Reverse	Source
Escherichia-Shigella group	Tag_5	DnaJ (dnaJ)	*E. coli, E. albertii, E. fegusonii, Shigella* spp.	PolyA-TGACAGAATCCTCAGTTTTTCA	Tag_5-AGATAAGACGGCTGGTACTGA	This study
	Tag_4	InvE (virB)	*E. coli* EIEC, *Shigella* spp.	PolyA-GATACAGAGAGAATTTCGTC	Tag_4-GCCAGTTATCTGACATTCTG	Hayashi, 2013
	Tag_2	Shiga2 (stx2)	*E. coli* EHEC	PolyA-GATACAGAGAGAATTTCGTC	Tag_2-TCTGTATTTGCCGAAAACGT	Hayashi, 2013
	Tag_1	Shiga1 (stx1)	*E. coli* EHEC, *S. dysenteriae* O1	PolyA-ACAGGATTTGTTAACAGGAC	Tag_1-GCCAGTTATCTGACATTCTG	Hayashi, 2013
Salmonella enterica serovar Typhi group	Tag_5	InvA (invA)	*Salmonella* spp	PolyA-TGACAGAATCCTCAGTTTTTCA	Tag_5-AGATAAGACGGCTGGTACTGA	Hayashi, 2013
	Tag_4	FliC	*S. enterica* serovar Paratyphi A	PolyA-GAACGTGCAGAAAGCGTATGA	Tag_4-CGCATCATCAAGGCCTGAGGCA	This study
	Tag_3	CRISPR1	*S. enterica* serovar Paratyphi A	PolyA-ACGGGTTGCTGTAATGATGC	Tag_3-GCATCATCGCGCATAGTGTC	Fabre, 2014
	Tag_2	VipR (vipR)	*S. enterica* serovar Typhi	PolyA-TCGGGGTTGGAGCTGCTGGCATTA	Tag_2-CAGGGCATTTAACAGGCTGTAGCGA	This study
	Tag_1	CRISPR2	*S. enterica* serovar Typhi	PolyA-ACGTAGACTCATCCTCGACC	Tag_1-GCGTGTAGCAGTATTCACCA	Fabre, 2014
Vibrio cholerae	Tag_5	DnaJ (dnaJ)	*V. cholerae*	PolyA-ACCTGATCCATCGCAAGCGTCA	Tag_5-GAAGAAGCGGTTCGCGGCTGC	This study
	Tag_1	CT (ctxA)	*V. cholerae* O1, O139	PolyA-GGTCTTATGCCAAGAGGACAGAG	Tag_1-TCCCGTCTGAGTTCCTCTTGC	This study
Francisella tularensis group	Tag_4	Noncoding4	*F. tularensis* subsp. *tularensis I*	PolyA-AGCTTATGCATCGAGTTGAGGTA	Tag_4-AAAGCTGGCGATCCAAGG	Gunnell, 2012
	Tag_3	Noncoding3	*F. tularensis* subsp. *tularensis II*	PolyA-CGAGATTTTGTCCACGCTTC	Tag_3-TTTGCGCCAAGACCAGAG	Gunnell, 2012
	Tag_2	Noncoding2	*F. tularensis* subsp. *holarctica*	PolyA-TTGCCTATCCAATACTCCGAGTTAG	Tag_2-CAAGCGCCTGGCTTTGATAA	Gunnell, 2012
	Tag_1	Noncoding1	*F. tularensis* subsp. *novicida*	PolyA-TCAATGTTGCTAAAGTCTCTGGAG	Tag_1-AATGATGGTAATAAAAGAAGTGGAGC	Gunnell, 2012
Yersinia pestis group	Tag_4	DnaJ (dnaJ)	*Y. pestis, Y. pseudotuberculosis*	PolyA-GATCCGAATCCCAACGCTGG	Tag_4-TGGGCAAGTGACTGGTGAACTC	This study
	Tag_3	F1 (caf1)	*Yersinia pestis*	PolyA-GTTGGTACGCTTACTCTTGGCG	Tag_3-TGGGATCACCCGCGGCATCTG	This study
	Tag_1	Pla (pla)	*Yersinia pestis*	PolyA-ATACTGTGACGGCGGGTCTGCA	Tag_1-ACCTCCTTTCCACAGACATCC	This study
Burkholderia mallei group	Tag_3	Phage transposase	*B. mallei, B. pseudomallei*	PolyA-CCGATTGGCGCTTCGTGCGC	Tag_3-AGCGGGTAGTCGAAGCTGCA	Janse, 2013
	Tag_1	Phage integrase	*B. mallei*	PolyA-GTGGGCGAAAGAACGCGAAC	Tag_1-GCGTTCCACGATCAACTC	Janse, 2013
Actin	Tag_7	Actin	Internal control	PolyA-CGGGAAATCGTGCGTGAC	Tag_7-ATGGTGATGACCTGGCCG	This study

cocktail was prepared to detect both CT-positive and -negative strains of *V. cholerae*. Strains belonging to the O1 and O139 serotypes are generally both *ctx*- and *dnaJ*-positive. The other 140 serotypes were expected to be CT-negative and *dnaJ*-positive.

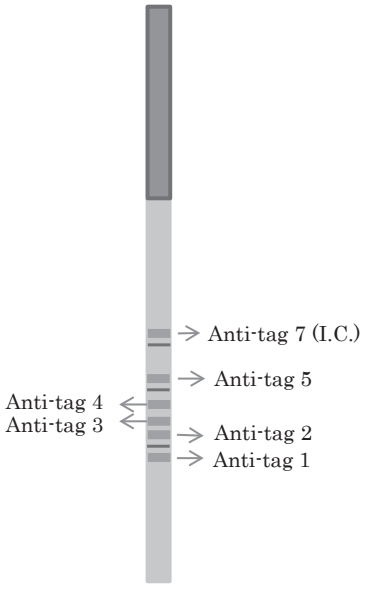

Fig. 1 A DNA strip for differentiating six cocktail PCR amplicons. Six anti-tags and three marker lines are printed on a nitrocellulose membrane to differentiate six PCR amplicons. Anti-Tag 7 is internal control (I.C.)

pla and *dnaJ* of *Y. pestis*

Both the plasmid-encoded plasminogen activator gene *pla* (Guinet & Carniel, 2012) and the chromosomal chaperone gene *dnaJ* of *Y. pestis* were prepared. *pla* was recently reported in a wild strain of *Escherichia coli* and in *Citrobacter koseri* (Hänsch *et al*., 2015). Pathogenic wild-type strains of *Y. pestis* were expected to be both *pla*- and *dnaJ*-positive, but plasmid-cured strains were expected to be only *dnaJ*-positive. Because another plasmid-encoded specific F1 antigen gene, *caf1* (Galyov *et al*., 1990; Tsui *et al*., 2015), produced nonspecific amplicons, *calf1* was not mixed with *pla,* but was instead amplified independently from the *pla* and *dnaJ* cocktail. After PCR, the two amplicons were combined and detected with a single DNA strip. The closely related *Y. pseudotuberculosis* has an identical *dnaJ* sequence, and the *dnaJ* PCR was expected to be positive. Once the plasmids are cured from *Y. pestis,* it cannot be differentiated from *Y. pseudotuberculosis* by using this cocktail primer (Table 3).

Subspecies and pathovar differentiation of *Francisella tularensis*

Genome analyses have revealed sequences for differentiating subspecies of *Francisella tularensis* (Gunnell *et al*., 2012). We added tags to these four primers and made a cocktail to differentiate two subspecies, namely *F. tularensis* subsp. *holarctica and F. tularensis* subsp. *novicida,* from *F. tularensis*

Table 2 Pathogenic factors and percentage of positive results

Species	Virulent factor	% of positive results among stock strains
Shigella dysenteriae	*stx1*	8% (1/13)
Shigella dysenteriae	*InvE*	15% (2/13)
Shigella flexneri	*InvE*	25% (7/28)
Shigella boydii	*InvE*	31% (8/26)
Shigella sonnei	*InvE*	20% (2/10)
Salmonella enterica serovar Typhi	*vipR*	85% (17/20)
Salmonella enterica serovar Typhi	CRISPR2*	100% (3/3)
Salmonella enterica serovar Paratyphi	CRISPR2*	0% (0/3)
Salmonella enterica serovar Typhi	CRISPR1*	0% (0/5)
Salmonella enterica serovar Paratyphi	CRISPR1*	100% (3/3)
Vibrio cholerae O1/O139	*ctxa*	87% (13/15)
Vibrio cholerae other serotypes	*ctxa*	0% (0/140)
Yersinia pestis	*caf1*	60% (3/5)
Yersinia pestis	*pla*	60% (3/5)
Yersinia pseudotuberculosis	*pla*	0% (0/5)

*CRISPAR1, CRISPAR2, are not pathogenic factors but cited in this table.

Table 3 Differentiation of closely related subspecies and serovars

Francisella tularensis group	Tag 1	Tag 2	Tag 3	Tag 4	Tag 5
	Noncoding1	Noncoding2	Noncoding3	Noncoding4	*dnaJ*
subsp. tularensis GTC 3P0421	−	−	−	+	+
subsp. holarctica GTC 3P0422	−	+	−	−	+
subsp. novicida GTC 3P0425T	+	−	−	−	+
Salmonella enterica subsp. *enterica* group	Tag 1	Tag 2	Tag 3	Tag 4	Tag 5
	CRISPR2	*vipR*	CRISPR1	FliC:a	*invA*
serovar Typhi GTC 3P0637 Vi positive	+	+	−	−	+
serovar Typhi GTC 3P0612 Vi negative	+	−	−	−	−
serovar Paratyphi A GTC 3P0433	−	−	+	+	+
serovar Dublin GTC 02558 Vi positive	−	−	−	−	+
Citrobacter freundii GTC 14916 Vi positive	−	−	−	−	−
Bukholderia mallei group	Tag 1	Tag 2	Tag 3	Tag 4	Tag 5
	Phage integrase		Phage transposase		
B. pseudomallei GTC 3P0028	−		+		
B. pseudomallei 10 wild-type strains	(0/10)		(10/10)		
B. mallei GTC 3P0003T	+		+		
B. mallei 4 wild-type strains	(4/4)		(4/4)		
B. thailandensis GTC 3P0407T	−		−		
B. vietnamiensis GTC 3P0436T	−		−		
Yersinia pestis group	Tag 1	Tag 2	Tag 3	Tag 4	Tag 5
	pla		*caf1*	*dnaJ*	
P. pestis GTC 3P0417T	+		−	+	
P. pestis Russian vaccine strain	+		not tested	+	
P. pestis 3 Mongolian wild-type strains	(2/3)		not tested	(3/3)	
P. pseudotuberculosis GTC 03445	−		−	+	

subsp. *tularensis*.

Differentiation and identification of *Burkholderia mallei* and *Burkholderia pseudomallei*

Burkholderia mallei and *Burkholderia pseudomallei* are genetically similar organisms (Yabuuchi *et al*., 1992). Housekeeping genes that are useful for differentiating two such closely related species are rare. Several multiplex PCR methods have been reported (Rattanathongkom *et al*., 1997; Wongratanacheewin *et al*., 2000), but closely related species, such as *Burkholderia thailandensis* and environmental isolates of *B. pseudomallei* are not clearly differentiable. However, a recent whole-genome analysis has revealed several sequences that are specific to each species (Janse, 2013). Using information on these noncoding sequences, we prepared tagged primers to make cocktail PCR mixtures to differentiate the two species. Phage transposase, which is common to both species, and phage integrase, which is found only in *B. mallei* (Table 1), were prepared for differentiating the two closely related species.

RESULTS AND DISCUSSION

The standard PCR method employs a high-profile PCR tube to run 20 to 50 μl of PCR reaction mixtures, and 96 specimens can usually be amplified with a standard PCR machine.

To reduce the amplification time, we designed a four-well PCR machine (Quick Mobile), and employed low-profile PCR tubes to run experiments with a 10 μl reaction volume. The DNA extraction protocol was also simplified. Bacteria from a single colony were suspended in 500 μl of distilled water (to a concentration of around 10^7 to 10^8 cfu/m*l*) and boiled for 3 min. With this simple sample preparation, the entire experiment could be completed within 40 min from the time bacteria were collected from a single colony. Gram-positive *Mycobacterium tuberculosis* and *Clostridium botulinum,* which have

tough cell walls, were successfully analyzed by using this simple protocol, (Data not shown).

Sensitivity of the DNA strip

The PCR amplicon of the *E. coli* Shiga2-positive strain O157:H7 (GTC 14509) was diluted by two-fold serial dilution. Up to 64-fold diluted amplicon was visualized (Fig. 2). PCR amplicon in the same diluted solutions was also detected by electrophoresis and ethidium bromide staining; the amplicon could be detected at dilutions up to eight-fold (Data not shown.)

The PCR product from cell suspensions of *S. enterica* serovar Typhi GTC 3P0637 at 10^8 to 10^3 cfu/m*l* could be clearly visualized by using the DNA-strip method (Fig. 3A). However, the real-time PCR results for the solutions at 10^4 to 10^2 cfu/m*l* could not be differentiated from that for the negative con-

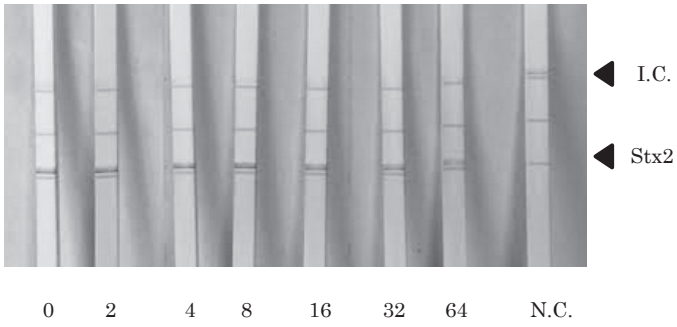

Fig. 2 Sensitivity of the DNA strip
Serially diluted PCR amplicons of *Escherichia coli* Shiga2-positive strain O157:H7 (GTC 14509) were detected with a standard latex solution. The DNA-strip method could detect the amplicon in all dilutions up to the 64-fold dilution.

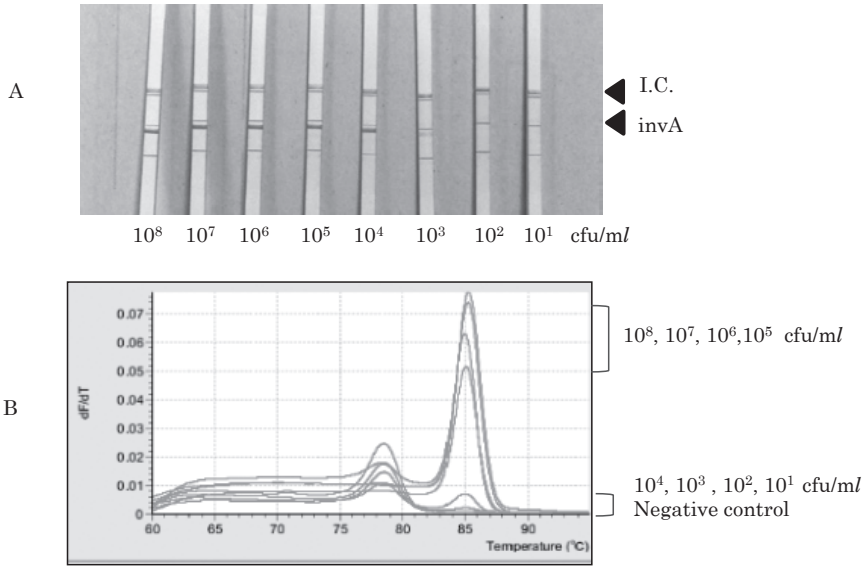

Fig. 3 Real-time PCR and cocktail PCR DNA strips
A. The PCR products from the cell suspensions of *Salmonella enterica* GTC 3P0637 at 10^8 cfu/m*l* to 10^3 cfu/m*l* could be clearly visualized using the DNA-strip method.
B. Real-time PCR using the cell suspensions at 10^8 cfu/m*l* to 10^5 cfu/m*l* could detect amplicon, but the method could not differentiate the cell suspensions at 10^4 cfu/m*l* to 10^2 cfu/m*l*. The Tm of the *invA* amplicon is 85.1℃.

trol (Fig. 3B).

Screening for pathogenic factors

Freshly isolated *Shigella* species usually carry the *invE* (*virB*) plasmid. Among 76 stock strains of *Shigella* species, only 25% were *invE*-positive, and only the *Shigella dysenteriae* O1 GTC 00786 strain was *stx1*-positive (Table 1). Data for *S. dysenteriae* O1 GTC 00786 and other representative stock strains are shown in Figure 4.

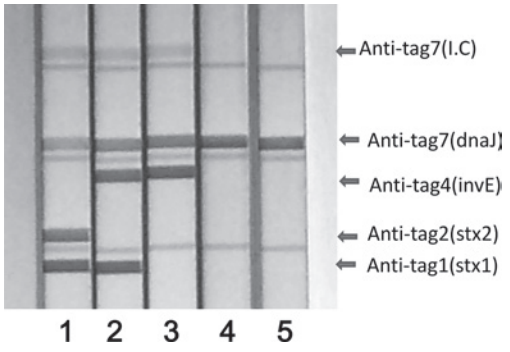

Fig. 4 Cocktail to amplify *Shigella invE* and *dnaJ*
Freshly isolated *Shigella* species usually carry the *invE* plasmid. Among the 76 stock strains of *Shigella* species analyzed, only 25% of the strains were *invE*-positive. *Shigella dysenteriae* O1 GTC 00786 was the only strain that was *stx1*-positive. Data for *S. dysenteriae* O1 GTC 00786 and the following representative stock strains are shown.
1. *Escherichia coli* (O157:H-) GTC 14508 (Shiga1+Shiga2+ dnaJ),
2. *Shigella dysenteriae* (O1) GTC 00786 (Shiga1+invE+ dnaJ)
3. *Shigella flexneri* GTC 01924 (invE+dnaJ)
4. *Shigella flexneri* GTC 01927 (invE negative+dnaJ)
5. *Shigella boydii* GTC 14835 (invE negative+dnaJ)

The Vi antigen is generally believed to be specific to *S. enterica* serovar Typhi. However, some strains of *S. enterica* serovar Dublin and *Citrobacter freundii* also carry Vi genes. The *vipR* primer that we prepared did not amplify the DNA from Vi gene-positive *S. enterica* serovar Dublin GTC 02558 or *C. freundii* GTC 14916 (Fig. 5). *Salmonella enterica* serovar Typhi GTC 10007-1 was originally Vi-positive, but the strain has lost its Vi gene (GTC 10007-3) (Hashimoto *et al*., 1996). Among the 20 stock strains analyzed, three had lost the Vi antigen (Table 2). The recently described Crispr-2 and Crispr-1 were specific to each serovar (Table 3).

The CT toxin of *V. cholerae* is reportedly present only in serotypes O1 and 139 (Shi *et al*., 1998). The CT gene is reported to be stable, but two strains among 15 stock cultures of O1 and O139 were CT-negative (Table 2). The non O1-O139 *V. cholerae* were all CT-negative among the 140 strains analyzed (Table 2).

The Fl antigen (*caf1*) and *pla* genes of *Y. pestis* are located on plasmids, and these genes are often cured from the host. The type strain GTC 3P0417 of *Y. pestis* had already lost the *caf1*. Among four

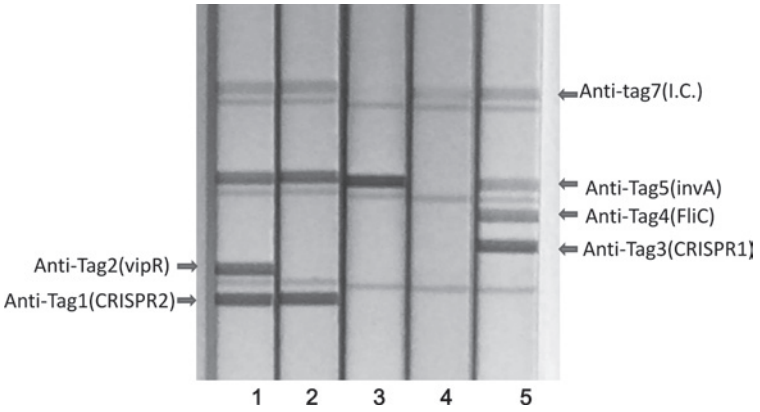

Fig. 5 Cocktail to differentiate closely related *Salmonella* serovars and *Citrobacter freundii*
1. *S. enterica* serovar Typhi GTC 10007-1 (Vi+ invA)
2. *S. enterica* serovar Typhi GTC 10007-3 (del Vi, invA)
3. *S. enterica* serovar Dublin GTC 02558 (Vi positive)
4. *C. freundii* GTC 14916 (Vi positive)
5. *S. enterica* serovar Paratyphi A GTC 3P0433

strains isolated from animals in Mongolia, three were *pla*-positive, but one strain had lost *pla* (Table 3). The *dnaJ* primer amplified sequences in both *Y. pestis* and the closely related *Y. pseudotuberculosis*. Both F1 antigen-gene and *pla*-deleted strains were positive for *dnaJ*, and it was difficult to differentiate *Y. pestis* from *Y. pseudotuberculosis* by using PCR-based methods.

The three subspecies of *F. tularensis* need to be differentiated from each other, because only *F. tularensis* subspecies *tularensis* and subspecies *holarctica* are classified as SPJID pathogens. Housekeeping genes such as the 16S rRNA gene and dnaJ gene are almost identical among species (Siddaramappa *et al*., 2012). Sequences specific to subspecies have been found by complete genome analysis (Gunnell *et al*., 2012). Four cocktail primers designed from these genomic sequences could be used to differentiate each of the three subspecies and the wild-type strains (Table 3).

Burkholderia pseudomallei and *B. mallei* are genetically similar species (Yabuuchi *et al*., 1992) and the 16S rRNA gene and housekeeping genes cannot be used to differentiate them. *Burkholderia pseudomallei* is a motile species, whereas *B. mallei* is highly adapted to its host animals and has lost its motility. Complete genome analysis, has revealed many phage-derived genes in their chromosomes (Janse *et al*., 2013). Phage-derived sequences and insertion sequences were selected to differentiate the two species. Primers for amplifying the phage integrase in *B. mallei* did not amplify any sequences in *B. pseudomallei* (Table 2). However, primers for the integrase in *B. pseudomallei* could amplify sequences in all clinical strains. These two integrase primer sets did not amplify any sequences in the closely related *B. thailandensis*.

CONCLUSIONS

The cocktail PCR and DNA-strip method for differentiating six different amplicons is simple to perform. The same device can be used to detect pathogenic factors in BLS3 and SPJID pathogens. Culture collection involves the handling of a broad range of different pathogens. Such collections are generally maintained by only a few staff members, who are expected to offer pathogenic information on all items in collections. This quick and simple method of detecting pathogenic factors in BLS3 and SPJID strains in less than an hour is expected to be very useful in the handling of culture collections.

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BSL 3の保存細菌株の病原因子を迅速に確認するカクテルPCR-DNAクロマト法

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BSL3の細菌の分譲にあたって分譲株が不安定な病原性因子を保有しているかどうかの情報が要求される．多種類にわたる菌種の病原因子を確認し，分譲することは少数のスタッフで運営されている分譲業務には大きな負担になる．我々は最大6種類のプライマーにTagをつけ，一本のPCRチューブ内で増幅するカクテルプライマー増幅法を作成し，増幅産物をDNA-Strip上にプリントしたAnti-Tagに結合させ，3本の位置マーカー情報と対比させ，増幅産物を目視判定する方法を作成した．この方法で単一集落からPCR産物の目視判定まで40分以内でBSL3の病原体の病原因子の有無を判定する方法を確立した．