



# Evaluation of the GeneFields® EHEC/SS PCR dipstick DNA chromatography kit for the detection of enteric bacterial pathogens in stool specimens of healthy humans

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## ABSTRACT

We developed a new GeneFields® EHEC/SS PCR dipstick DNA chromatography kit for the simultaneously detection of *invA*, *ipaH*, and *stx* genes in *Salmonella enterica* (56 strains), *Shigella* spp. (44), and enterohemorrhagic *Escherichia coli* (EHEC) (28), respectively, and evaluated the sensitivity and specificity with other bacteria (57) by this kit. The sensitivity and specificity were 100%, respectively. The detection limit of various methods was determined using 5% (w/v) stool suspensions spiked with each bacterium. The detection limit of the GeneFields® EHEC/SS kit ranged from approximately 10<sup>2</sup>–10<sup>3</sup> CFU/g. Additionally, the relative sensitivities and specificities of the GeneFields® EHEC/SS kit vs two commercially available real-time PCR kits were > 85.0% and > 90.0%, respectively. These results indicate that the GeneFields® EHEC/SS kit can be used for genetic screening of *S. enterica*, *Shigella* spp., and EHEC in human stool specimens with sensitivities and specificities similar to those of the commercially available real-time PCR kits.

## 1. Introduction

Approximately 1,000 outbreaks of foodborne illnesses, involving 20,000 patients, occur annually in Japan (Ministry of Health, Labour and Welfare of Japan, 2017a). To prevent foodborne illnesses, food handlers in Japan must submit a stool sample at least once a month as required by the Hygiene Management Manual for Large-scale Food Preparation Facilities (Ministry of Health, Labour and Welfare of Japan, 2017b). The test protocol detects the presence of *Salmonella enterica*, *Shigella* spp., and/or enterohemorrhagic *Escherichia coli* (EHEC).

Culture methods are presently the conventional gold standard for detecting pathogenic bacteria (Guerrant et al., 2001; Harrington et al., 2015). Although culture methods can readily detect and isolate possible pathogenic bacteria (O'Leary et al., 2009), they are time-consuming, labor-intensive, and require multiple reagents (Kawase et al., 2016).

Alternatively, the available genetic methods overcome the disadvantages of culture methods (Liu et al., 2012).

Recently, a rapid and simple laboratory PCR test was introduced that detects *S. enterica*, *Shigella* spp., and EHEC in a fecal sample mixed with 50 stool specimens (Baba et al., 2014; Nishimura et al., 2012). Further, multiplex real-time PCR kits, which detect these pathogens, are marketed by diagnostic companies and used in clinical laboratories. However, real-time PCR requires sophisticated equipment and considerable technical proficiency.

A recently introduced PCR dipstick DNA chromatography assay is now widely used for diagnostics, as only a PCR thermal cycler and a block incubator are required (Hayashi et al., 2013; Monden et al., 2014; Tian et al., 2014). These two components are commonly available in many diagnostic laboratories because of their lower costs compared to a real-time PCR system. Therefore, a PCR dipstick DNA chromatography

**Abbreviations:** EHEC, enterohemorrhagic *Escherichia coli*; UNG, uracil DNA glycosylase; DHL, deoxycholate-hydrogen sulfide-lactose; CFU, colony forming unit; FPE, food pathogen-enrichment; EMA, ethidium bromide monoazide; PMA, propidium monoazide

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assay is considered optimal for performing genetic screening by small-to-medium size diagnostic companies, which are unable to acquire real-time PCR equipment. However, to our knowledge, there is no commercially available kit that employs a nucleic acid chromatography component capable of detecting genes specific in *S. enterica*, *Shigella* spp., and EHEC in stool specimens. To address this deficiency in our clinical toolkit, we developed a genetic testing kit called “GeneFields® EHEC/SS,” which employs PCR dipstick DNA chromatography to detect pathogen-specific genes in stool samples.

In this study, we evaluated the kit's ability to sensitively and specifically detect *S. enterica*, *Shigella* spp., and EHEC. For this purpose, we tested the GeneFields® EHEC/SS kit, standard culture method, and commercially available real-time PCR kits using spiked and untreated stool specimens acquired from healthy people. Here, we demonstrate that the performance of the GeneFields® EHEC/SS kit is comparable to those of the selected real-time PCR kits.

## 2. Materials and methods

### 2.1. Bacterial strains

Bacterial strains were acquired from the culture collection of the Laboratory of International Prevention of Epidemics, Osaka Prefecture University, Osaka, Japan (Table 1). All strains, except for *Yersinia enterocolitica*, were aerobically cultured in LB Broth Miller (BD Difco, Detroit, MI, USA) at 37 °C for 12–18 h. *Y. enterocolitica* was aerobically cultured at 30 °C in LB broth Miller for 24 h. A fresh culture was used for each experiment.

**Table 1**  
Bacterial reference strains.

Species	Serotype	Number of strains	GF EHEC/SS <sup>a</sup>	Species	Serotype	Number of strains	GF EHEC/SS	
<i>Salmonella enterica</i>	Agona	2	Positive ( <i>invA</i> )	<i>Escherichia coli</i> : EHEC	O26	2	Positive ( <i>stx1</i> , <i>stx2</i> )	
	Anatum	1	Positive ( <i>invA</i> )				3	Positive ( <i>stx1</i> )
	Brunei	1	Positive ( <i>invA</i> )		O103	3	Positive ( <i>stx1</i> )	
	Derby	3	Positive ( <i>invA</i> )		O104	1	Positive ( <i>stx2</i> )	
	Eastbourne	1	Positive ( <i>invA</i> )		O111	2	Positive ( <i>stx1</i> , <i>stx2</i> )	
	Enteritidis	11	Positive ( <i>invA</i> )				2	Positive ( <i>stx1</i> )
	Huittingfoss	1	Positive ( <i>invA</i> )				1	Positive ( <i>stx2</i> )
	Infantis	1	Positive ( <i>invA</i> )		O121	1	Positive ( <i>stx1</i> , <i>stx2</i> )	
	i,8, 20:y-	1	Positive ( <i>invA</i> )				2	Positive ( <i>stx2</i> )
	Krefeld	2	Positive ( <i>invA</i> )		O145	3	Positive ( <i>stx2</i> )	
	Muenchen	1	Positive ( <i>invA</i> )		O157	3	Positive ( <i>stx1</i> , <i>stx2</i> )	
	Newport	1	Positive ( <i>invA</i> )				4	Positive ( <i>stx2</i> )
	Poona	1	Positive ( <i>invA</i> )		O165	1	Positive ( <i>stx2</i> )	
	Saintpaul	1	Positive ( <i>invA</i> )		Total		28	
	Senftenberg	1	Positive ( <i>invA</i> )		<i>Acinetobacter baumannii</i>		2	Negative
	Senovar Hadar	1	Positive ( <i>invA</i> )		<i>Citrobacter freundii</i>		2	Negative
	Stanley	5	Positive ( <i>invA</i> )		<i>C. koseri</i>		1	Negative
	Typhimurium	1	Positive ( <i>invA</i> )		<i>Enterobacter cloacae</i>		1	Negative
	Virchow	1	Positive ( <i>invA</i> )		Enteropathogenic <i>E. coli</i>		4	Negative
	Welikade	1	Positive ( <i>invA</i> )		<i>Escherichia coli</i>		16	Negative
Weltevreden	5	Positive ( <i>invA</i> )	<i>E. vulneris</i>		1	Negative		
O4	4	Positive ( <i>invA</i> )	<i>Klebsiella oxytoca</i>		2	Negative		
O7	4	Positive ( <i>invA</i> )	<i>Morganella morganii</i>		7	Negative		
O8	4	Positive ( <i>invA</i> )	<i>Pseudomonas aeruginosa</i>		1	Negative		
O9	1	Positive ( <i>invA</i> )	<i>Proteus mirabilis</i>		12	Negative		
Total	56		<i>P. penneri</i>		2	Negative		
<i>Shigella boydii</i>	ND	6	Positive ( <i>ipaH</i> )	<i>P. vulgaris</i>		3	Negative	
<i>S. dysenteriae</i>	ND	9	Positive ( <i>ipaH</i> )	<i>Yersinia enterocolitica</i>		3	Negative	
<i>S. flexneri</i>	ND	14	Positive ( <i>ipaH</i> )	Total		57		
<i>S. sonnei</i>	ND	15	Positive ( <i>ipaH</i> )					
Total		44						

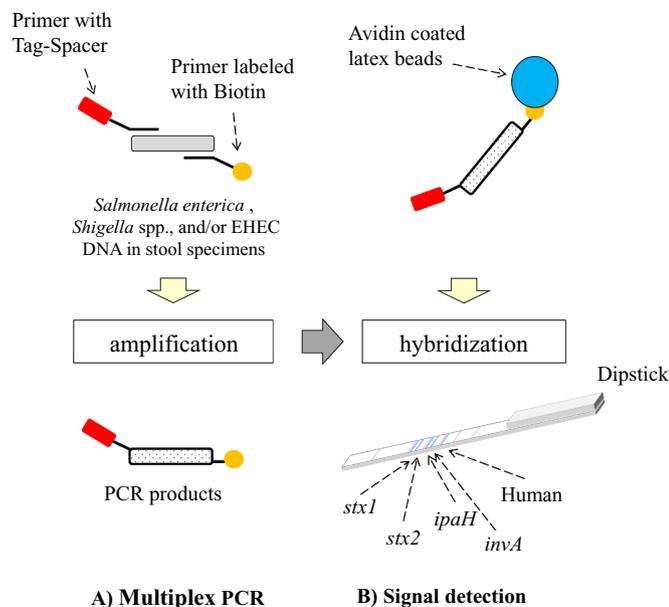
<sup>a</sup> GF EHEC/SS: GeneFields® EHEC/SS.

### 2.2. GeneFields® EHEC/SS

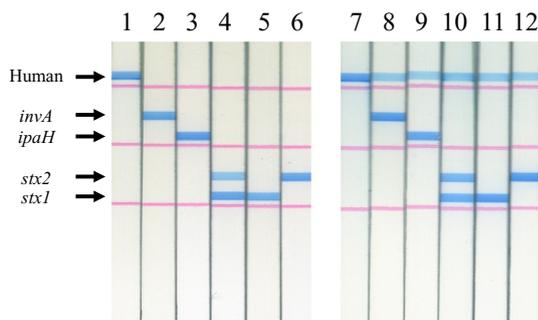
We applied the GeneFields® EHEC/SS kit according to the manufacturer's instructions (Cat# 551-41960-3; Kyokuto Pharmaceutical Industrial Co., Ltd., Tokyo, Japan). Briefly, 2.6 mg of a stool sample was suspended in 50 µl of Lysis Buffer B. The suspended samples were incubated at 100 °C for 10 min, and then centrifuged at 13,000g for 3 min. After centrifugation, the supernatant was collected and used as a template DNA for a multiplex PCR assay that amplifies the *invA*, *ipaH*, and *stx* (*stx1* and *stx2*) of *S. enterica*, *Shigella* spp., and EHEC, respectively. The gene encoding mammalian mitochondrial-derived 16S rRNA, which is present in human stools, served as internal positive control.

As shown in Fig. 1, GeneFields® EHEC/SS kit used 2 types of primers; one primer was labeled with biotin while the other primer was with a tag-spacer sequence. After amplification, the 5 terminus of the cocktail PCR products were labeled with biotin, and the 3 terminus was labeled with five different tags. Streptavidin-coated blue latex bound the biotinylated 5 terminal PCR products and the tagged 3 terminus was bound on the antitag lines printed on the dipstick strip.

Reaction mixtures included 5.0 µl of 2 × QuantiTect Multiplex PCR NoROX Master Mix (Qiagen, CA, USA), 2.9 µl of PCR Oligo Mix, 0.1 µl of uracil DNA glycosylase (UNG) (Takara Bio Inc., Shiga, Japan), and 2.0 µl of template DNA. The PCR assay was initiated by incubation with UNG, and incubated at 25 °C for 10 min, which was followed by denaturation at 95 °C for 9 min, 33 cycles of denaturation at 94 °C for 30 s, annealing at 68 °C for 30 s, and extension at 72 °C for 15 s. The PCR product was mixed with 30 µl of the visualization buffer. The dipstick was introduced into the mixture at 50 °C and after 10–15 min the results were interpreted according to the appearance of a blue line (Fig. 2). The



**Fig. 1.** Schematic representation of GeneFields® EHEC/SS. PCR amplification using 2 types of primers; one primer with a tag-spacer sequence and the other primer labeled with biotin. Hybridization between the tag sequence of the PCR products and an oligonucleotide complementary to the tag sequence on the Dipstick (blue line). Avidin immobilized on the blue latex beads binds to biotin on the primer of the PCR product. Red lines on the Dipstick indicate position marker. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)



**Fig. 2.** Detection of *invA*, *ipaH*, *stx1*, and *stx2* using the GeneFields® EHEC/SS PCR dipstick DNA chromatography kit. DNA samples extracted from reference strains were analyzed using PCR and the GeneFields® EHEC/SS kit (Lanes 1–6). Lane 1, Human genomic DNA; lane 2, *S. Enteritidis* (*invA*); lane 3, *S. sonnei* (*ipaH*); lane 4, EHEC O157 (*stx1*, *stx2*); lane 5, EHEC O103 (*stx1*); lane 6, EHEC O145 (*stx2*). Stool samples (5% percent [w/v]) were spiked with reference strains and analyzed using PCR and the GeneFields® EHEC/SS kit (Lanes 7–12). Lane 7, stool specimen only; lane 8, *S. Enteritidis* (*invA*); lane 9, *S. sonnei* (*ipaH*); lane 10, EHEC O157 (*stx1*, *stx2*); lane 11, EHEC O103 (*stx1*); lane 12, EHEC O145 (*stx2*).

reaction results began to appear after 10 min of reaction, and all reactions were completed after 40 min.

### 2.3. Multiplex real-time PCR analysis

Real-time PCR assays were performed using the following five commercially available kits: kit A, Intestinal pathogen gene detection kit ver.2 (Cat# 241-09600-91; Shimadzu Corporation, Kyoto, Japan); kit B, Intestinal bacterial gene detection kit -fluorescence detection- (Cat# FIK-301; TOYOBO Co., LTD, Osaka, Japan); kit C, TaKaRa qPCR intestinal pathogenic bacteria detection kit (Cat# RRL139A; Takara Bio, Shiga, Japan); kit D, Intestinal bacterial gene detection kit -fast

fluorescence detection- (Cat# FIK-311; TOYOBO, Osaka, Japan); and kit E, Intestinal pathogen gene detection kit ver.3 (Cat# 241-09450-91; Shimadzu, Kyoto, Japan), according to the manufacturers' instructions. Stool specimens suspended in sterile saline were incubated at 95 °C for 5 min and then centrifuged at 13,000 g for 3 min. After centrifugation, the supernatant was collected and used as the DNA template for each real-time PCR analysis.

### 2.4. Microcapillary electrophoresis

PCR products were visualized using high-resolution microcapillary electrophoresis with an Agilent DNA 1000 Kit and an Agilent 2100 Bioanalyzer (Agilent Technologies Inc., Santa Clara, CA, USA). The Agilent DNA 1000 Kit is designed to analyze DNA fragments from 25 to 1000 bp. Data were automatically analyzed using Agilent 2100 Bioanalyzer software.

### 2.5. Detection limit of the GeneFields® EHEC/SS kit

Each pathogenic bacterium was inoculated into LB Miller broth and aerobically cultured at 37 °C for 4–8 h. After cultivation, the cultures were serially diluted 10 times with sterile saline ( $10^1$ – $10^7$  CFU/ml). The viable cell count was determined using LB agar plates, except for *S. enterica*, which was measured using deoxycholate-hydrogen sulfide-lactose (DHL) agar (BD Biosciences, Franklin Lakes, NJ, USA). Stool samples of healthy volunteers were collected, diluted 20-times in sterile saline, and the suspension was thoroughly mixed to prepare a 5% (w/v) stool suspension.

Each diluted bacterial solution (0.1 ml) was mixed thoroughly with 0.9 ml of a 5% stool suspension for analysis (concentrations of the bacteria in the diluted 5% stool suspensions were approximately 1, 10,  $10^2$ ,  $10^3$ ,  $10^4$ ,  $10^5$ , and  $10^6$  CFU/g). Lysis Buffer B (22  $\mu$ l, 10 $\times$  stock) (for GeneFields® EHEC/SS) or sterile saline (for the culture method and the real-time PCR kit) was added to 200  $\mu$ l of each spiked stool suspension. These samples were used for the detection of *S. enterica*, *Shigella* spp., and EHEC employing the GeneFields® EHEC/SS kit, the culture methods, and real-time PCR kits, respectively, as well as to determine detection limits. DHL agar (BD Biosciences, Franklin Lakes, NJ), SS agar (Kyokuto Pharmaceutical Industrial Co., Ltd), CHROMagar™STEC (CHROMagar Microbiology, Paris, France) CT-SMAC, and CIX agar (Kyokuto Pharmaceutical Industrial Co., Ltd) were used for culture methods to isolate *S. enterica*; *S. enterica* and *Shigella* spp., EHEC O26, O103, O111, O121, O145 and O157, O157, and O26, O111 and O157, respectively.

### 2.6. Sensitivity and specificity of GeneFields® EHEC/SS for detecting bacterial pathogens in stool samples

We divided 5,000 stool specimens into 100 groups (50 specimens per group). Each group (about 0.13 g) was diluted with 2.5 ml of sterile distilled water. The concentrations of pooled stool samples were adjusted to approximately 5% (w/v). The stool samples used in this study were evaluated in accordance with the ethical standards of the facility where the experiment was conducted (TOHO Biological Laboratories and Japan Institute of Foods Ecology). The sample information was handled with caution, and we made the samples anonymous and completely separated the results and the donors personal information. Since this study did not involve collection of any additional information, there was no other inconvenience to the donor. Only the results of the stool cultures were reported to the donors (Rintala et al., 2016).

Next, 22  $\mu$ l of Lysis Buffer B or sterile distilled water was added to 200  $\mu$ l of each pooled stool sample. Samples suspended in Lysis Buffer B or sterile distilled water were analyzed using the GeneFields® EHEC/SS kit or real-time PCR kits D and E, respectively. Stool samples positive for *S. enterica*, *Shigella* spp., or EHEC in either or both GeneFields® EHEC/SS kit and real-time PCR kits D and E assays were then cultured

at 35 °C to 37 °C for 18–24 h using VitalMedia Twin-plate 39 (SS-NEO/CSIE) agar (Kyokuto Pharmaceutical Co., LTD), and S-Type Agar/X-EHEC II Agar “Nissui” (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan). Suspected colonies were identified as *S. enterica*, *Shigella* spp., or EHEC using conventional biochemical tests with TSI, LIM, SC, and SIM media (Eiken Chemical Co., Ltd., Tokyo, Japan). The kappa statistic was used to evaluate the extent of agreement among the data the tests.

### 3. Results

#### 3.1. Sensitivity and specificity of the GeneFields® EHEC/SS kit

The GeneFields® EHEC/SS kit is designed to detect *invA*, *ipaH*, and *stx* (*stx1* and *stx2*) encoded by the genomes of *S. enterica*, *Shigella* spp., and EHEC, respectively. Further, this kit is designed to detect the positive-control gene encoding mammalian mitochondrial-derived 16S rRNA, which is present in human stool specimens. Multiplex PCR was performed using control strains, such as *S. Enteritidis* (*invA*<sup>+</sup>), *S. sonnei* (*ipaH*<sup>+</sup>), *E. coli* O157:H7 (*stx1*<sup>+</sup>, *stx2*<sup>+</sup>), *E. coli* O103 (*stx1*<sup>+</sup>), *E. coli* O145 (*stx2*<sup>+</sup>), and human genomic DNA. The PCR products were detected using the dipstick DNA chromatography method of the GeneFields® EHEC/SS (Fig. 1). The kit detected a specific band corresponding to each gene product of *S. Enteritidis*; *S. sonnei*; and *stx1* or *stx2*, or both of *E. coli* (EHEC), as well as the positive control (Fig. 2). PCR products with expected sizes were confirmed using electrophoresis, while nonspecific products were considered undetectable (Fig. S1).

We next determined the sensitivity of the kit using 56, 44, and 28 isolates of *S. enterica*, *Shigella* spp., and EHEC, respectively. The sensitivity of detection of each bacterium was 100% (Table 1). The specificity of the kit was evaluated using 57 other bacterial strains, representing 9 genera and 13 species. The specificity of the kit for these strains was 100% (Table 1).

#### 3.2. Detection limit of the GeneFields® EHEC/SS kit

We next assessed the detection limits of the GeneFields® EHEC/SS kit versus those of the culture method. For this purpose, we used a 5% (w/v) stool suspension spiked with 2, 4, and 7 strains of *S. enterica*, *Shigella* spp., and EHEC, respectively (Table 2). The GeneFields® EHEC/SS kit detected up to 10<sup>3</sup> CFU/g each of *S. Enteritidis* and *S. Stanley*, which was the same result as that achieved using the culture methods.

The detection limits of the GeneFields® EHEC/SS kit for *S. sonnei*, *S. dysenteriae*, and *S. boydii* were each approximately 10<sup>2</sup> CFU/g, while that for *S. flexneri* was approximately 10<sup>3</sup> CFU/g. In contrast, the detection limits of the culture method ranged from approximately 10<sup>3</sup>–10<sup>5</sup> CFU/g (Table 2).

The detection limits of the GeneFields® EHEC/SS kit for EHEC strains ranged from approximately 10<sup>2</sup>–10<sup>3</sup> CFU/g versus approximately 10<sup>2</sup>–10<sup>4</sup> CFU/g achieved using the culture method.

#### 3.3. Comparison of the sensitivities of the GeneFields® EHEC/SS kit and real-time PCR kits

We used *S. enterica*, *S. sonnei*, *S. dysenteriae*, *S. flexneri*, and *E. coli* O157 (EHEC) to compare the detection limits of the GeneFields® EHEC/SS kit and three commercially available real-time PCR kits (Table 3). The detection limits of GeneFields® EHEC/SS for *S. Enteritidis* and EHEC O157 were approximately 10<sup>3</sup> CFU/g, which was equal to those of real-time PCR kits A and C and lower when compared with that of real-time PCR kit B (Table 3). The detection limits of the GeneFields® EHEC/SS kit for *Shigella* spp. ranged from approximately 10<sup>2</sup>–10<sup>3</sup> CFU/g; however, those of the real-time PCR assays depended on the nature of the kit or the bacterial species (Table 3). For example, the detection limits of real-time PCR kits A, B, and C for *Shigella* spp. ranged from 10<sup>4</sup> to 10<sup>5</sup>, 10<sup>1</sup> to 10<sup>5</sup>, and 10<sup>2</sup> to 10<sup>5</sup>, or > 10<sup>6</sup> CFU/g, respectively. These data suggest that the detection limit of the GeneFields® EHEC/SS kit is

nearly comparable to that of the real-time PCR kits.

#### 3.4. Evaluation of the GeneFields® EHEC/SS kit for routine testing

In the spike experiment, the GeneFields® EHEC/SS kit showed almost equivalent results to the real-time PCR kits A to C for the detection of *S. enterica*, *Shigella* spp., and EHEC. Therefore, the GeneFields® EHEC/SS kit and real-time PCR kit D were employed to detect these target bacteria in a routine surveillance test of 5,000 stool specimens collected from healthy adults. Of 100 samples, 4 and 90 were positive or negative, respectively, for *invA*. In contrast, the GeneFields® EHEC/SS kit detected *invA* and *stx2* in 5 and 1 samples, respectively, which were not detected using real-time PCR kit D (Table 4).

The relative sensitivity and specificity of the GeneFields® EHEC/SS kit were 100% and 95.7% compared with real-time PCR kit D. The percentage of observed agreement was 96.0%, while the kappa statistic was 0.73 (Table 4). We further found that 10 stool samples positive in either or both tests using the GeneFields® EHEC/SS kit and the real-time PCR kit D, were also positive using the culture method (Fig. 3). For example, 4 samples positive in the GeneFields® EHEC/SS assay and in real-time PCR kit D assays were positive using the culture methods. Among 6 samples positive in the GeneFields® EHEC/SS assay but not the real-time PCR kit D assay, 3 were positive using the culture method.

Further, the utility of the GeneFields® EHEC/SS kit was compared with that of real-time PCR kit E in a routine screen of another 5,000 stool specimens acquired from healthy adults. Of 100 samples, 7 were positive in the GeneFields® EHEC/SS kit and real-time PCR kit E assays, while 85 samples were negative in both. However, the results of 8 samples were discordant between the two assays (Fig. 4). For example, 7 samples were positive for *stx2* (3), *ipaH* (2), *invA* (1) and *stx1* (1) only in the GeneFields® EHEC/SS assay, while 1 sample was positive for *stx1/stx2* genes only in the real-time PCR kit E assay. The relative sensitivity and specificity of the GeneFields® EHEC/SS kit were 87.5%, and 92.4%, respectively, compared with those of real-time PCR kit E. The percentage of observed agreement was 92.0%, while the kappa statistic was 0.60 (Table 5). Among 15 stool samples positive for *invA* (*S. enterica*), *ipaH* (*Shigella* spp.), and *stx* (EHEC), only 5 *S. enterica* (*invA*<sup>+</sup>) isolates were detected using the culture-dependent method (Fig. 4).

### 4. Discussion

It is well known that food handler who is a healthy carrier with *Salmonella*, *Shigella* and EHEC may contaminate the foods and as a result, it may cause huge outbreak of food-poisoning (Hancock-Allen et al., 2016; Marineli et al., 2013; Murata et al., 1993). Although culture methods are widely used for detecting and identifying pathogenic bacteria, they are time-consuming and laborious, particularly when analyzing large numbers of samples (Law et al., 2014). To identify healthy carriers of bacterial pathogens in food handlers, the detection of *S. enterica*, *Shigella* spp., and EHEC in stool samples of food handlers is regularly performed as required by the Law Concerning the Prevention of Infectious Diseases in Japan. Genetic screening using a real-time PCR assay was recently introduced for this purpose. Compared with the culture method, the real-time PCR assay is faster, easier to perform, and reduces labor costs. Furthermore, a sampling system that analyzes 50 stool specimens in one PCR assay tube was introduced to perform regular examinations of healthy food handlers in Japan. However, real-time PCR remains costly due to the initial investment of acquiring a real-time PCR system. Therefore, it is important to provide small to medium-scale clinical testing companies with more economical and faster genetic screening methods to help protect the public from foodborne enteric bacterial diseases.

Here we show that the GeneFields® EHEC/SS kit specifically detected *S. enterica*, *Shigella* spp., and EHEC (Table 1). Using this kit is very simple, because the detection steps include amplification of target

**Table 2**  
Detection limits of the GeneFields® EHEC/SS kit and the culture method for 4 target genes in spiked experiment.

Species (target gene)	Serotype/serogroup	Strain	Assay method	N	N × 10 <sup>x</sup> CFU/g							
					0	X = 1	2	3	4	5	6	
<i>Salmonella enterica</i> ( <i>invA</i> )	Enteritidis	9	GF EHEC/SS <sup>a</sup> Culture <sup>b</sup>	3.0	0 <sup>c</sup> /3 <sup>d</sup>	0/3	0/3	3/3	3/3	3/3	3/3	3/3
	Stanley	Thai-104	GF EHEC/SS Culture	7.4	0/3	0/3	0/3	3/3	3/3	3/3	3/3	3/3
<i>Shigella sonnei</i> ( <i>ipaH</i> )		SS-2	GF EHEC/SS Culture	5.8	0/3	0/3	2/3	3/3	3/3	3/3	3/3	3/3
<i>Shigella dysenteriae</i> ( <i>ipaH</i> )		AQ7017	GF EHEC/SS Culture	3.0	0/3	0/3	2/3	3/3	3/3	3/3	3/3	3/3
<i>Shigella flexneri</i> ( <i>ipaH</i> )		SF-1	GF EHEC/SS Culture	1.9	0/3	0/3	0/3	2/3	3/3	3/3	3/3	3/3
<i>Shigella boydii</i> ( <i>ipaH</i> )		SB-2	GF EHEC/SS Culture	4.4	0/3	0/3	2/3	3/3	3/3	3/3	3/3	3/3
<i>E. coli</i> : EHEC ( <i>stx1</i> , <i>stx2</i> )	O157	Sakai	GF EHEC/SS Culture	4.5	0/3	0/3	0/3	3/3	3/3	3/3	3/3	3/3
	O26	KB0176	GF EHEC/SS Culture	3.7	0/3	0/3	3/3	3/3	3/3	3/3	3/3	3/3
	O111	E535–5	GF EHEC/SS Culture	3.2	0/3	0/3	0/3	3/3	3/3	3/3	3/3	3/3
	O121	KB0732	GF EHEC/SS Culture	1.6	0/3	0/3	1/3	3/3	3/3	3/3	3/3	3/3
<i>E. coli</i> : EHEC ( <i>stx1</i> )	O103	KB1750	GF EHEC/SS Culture	2.5	0/3	0/3	0/3	3/3	3/3	3/3	3/3	3/3
<i>E. coli</i> : EHEC ( <i>stx2</i> )	O104	LB226692	GF EHEC/SS Culture	1.3	0/3	0/3	3/3	3/3	3/3	3/3	3/3	3/3
	O145	KB0748	GF EHEC/SS Culture	2.3	0/3	0/3	2/3	3/3	3/3	3/3	3/3	3/3

<sup>a</sup> GF EHEC/SS: GeneFields® EHEC/SS.  
<sup>b</sup> Culture: Culture method.  
<sup>c</sup> The number of sample showing positive result.  
<sup>d</sup> The total number of sample analyzed.

genes using conventional PCR, nor is it as costly as real-time PCR. For example, dipstick DNA chromatography can be performed much faster than agarose electrophoresis. Generally, we could obtain sufficient results by incubating the PCR products for 10–15 min. Therefore, the GeneFields® EHEC/SS kit combined with a standard PCR assay will provide a DNA detection system that can be performed in the time it

takes to run real-time PCR assays or even earlier.

Furthermore, the sensitivity of the GeneFields® EHEC/SS kit when applied to the analysis of 5% (w/v) stool samples was higher compared with that of the culture method and equal to that of 3 commercially available real-time PCR kits (Tables 2 and 3). The detection limits for *S. enterica*, *Shigella* spp., and EHEC achieved by the GeneFields® EHEC/SS

**Table 3**  
Detection limits of the GeneFields® EHEC/SS kit and real-time PCR kits A, B, and C for 4 target genes.

Species (target gene)	Serotype	Strain	Assay method	N	N × 10 <sup>x</sup> CFU/g							
					0	X = 1	2	3	4	5	6	
<i>Salmonella enterica</i> ( <i>invA</i> )	Enteritidis	9	GF EHEC/SS <sup>a</sup>	3.0	0 <sup>c</sup> /3 <sup>d</sup>	0/3	0/3	3/3	3/3	3/3	3/3	3/3
			rtPCR kit A <sup>b</sup>									
			rtPCR kit B									
			rtPCR kit C									
<i>Shigella sonnei</i> ( <i>ipaH</i> )		SS-2	GF EHEC/SS	5.8	0/3	0/3	2/3	3/3	3/3	3/3	3/3	3/3
			rtPCR kit A									
			rtPCR kit B									
<i>Shigella dysenteriae</i> ( <i>ipaH</i> )		AQ7017	GF EHEC/SS	3.0	0/3	0/3	2/3	3/3	3/3	3/3	3/3	3/3
			rtPCR kit A									
			rtPCR kit B									
<i>Shigella flexneri</i> ( <i>ipaH</i> )		SF-1	GF EHEC/SS	1.9	0/3	0/3	0/3	2/3	3/3	3/3	3/3	3/3
			rtPCR kit A									
			rtPCR kit B									
<i>E. coli</i> : EHEC ( <i>stx1</i> , <i>stx2</i> )	O157:H7	Sakai	GF EHEC/SS	5.8	0/3	0/3	0/3	3/3	3/3	3/3	3/3	3/3
			rtPCR kit A									
			rtPCR kit B									
			rtPCR kit C									

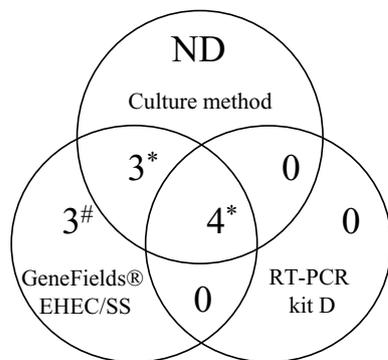
<sup>a</sup> GF EHEC/SS: GeneFields® EHEC/SS.  
<sup>b</sup> rtPCR kit: real-time PCR kit.  
<sup>c</sup> The number of sample showing positive result.  
<sup>d</sup> The total number of sample analyzed.

**Table 4**  
Comparative results using the GeneFields® EHEC/SS kit and the real-time PCR kit D to screen 5,000 stool specimens.

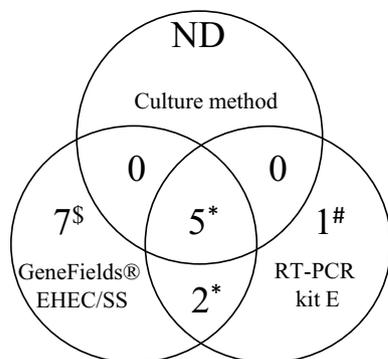
GeneFields®EHEC/SS	Real-time PCR kit D				Total positive	Culture positive
	<i>invA</i> +	<i>ipaH</i> +	<i>stx1/stx2</i> +	Negative		
<i>Salmonella</i> ( <i>invA</i> +)	4	0	0	5	9	7
<i>Shigella</i> ( <i>ipaH</i> +)	0	0	0	0	0	0
EHEC ( <i>stx1</i> +)	0	0	0	0	0	0
EHEC ( <i>stx2</i> +)	0	0	0	1	1	0
Negative	0	0	0	[90 <sup>a</sup> ]	0	3
Total positive	4	0	0	6	10	7
Total	100 (96)	100 (100)	100 (100)	100 (94)	100 (90)	10

Fifty stool samples were collected and analyzed as one sample. Namely, 100 stool mixtures consisting of 5000 specimens.

<sup>a</sup> The number showing negative result by both kits but not being counted for total number.



**Fig. 3.** Comparison of the GeneFields® EHEC/SS kit, the real-time PCR kit D, and the culture method for detecting enteric pathogens in stool specimens collected from food handlers. ND: not done. \*All were positive for *invA* (*Salmonella*). #Two were positive for *invA* (*Salmonella*), and one was positive for *stx2* (EHEC).



**Fig. 4.** Comparison of the GeneFields® EHEC/SS kit, the real-time PCR kit E, and the culture method for detecting bacterial pathogens in stool specimens of food handlers. ND: not done. \*All were positive for *invA* (*Salmonella*). #One was positive for *stx1/stx2* (EHEC). \$Three were positive for *stx2* (EHEC), two were positive for *ipaH* (*Shigella*), and one each was positive for *stx1* (EHEC) and *invA* (*Salmonella*).

kit ranged from  $10^1$  to  $10^3$  CFU/g, which is nearly equal to those of real-time PCR assays (Table 3). These results suggest that the GeneFields® EHEC/SS kit is useful for genetic screening of *S. enterica*, *Shigella* spp., and EHEC in stool samples. To the best of our knowledge, the present report is the first to show the detection of *S. enterica*, *Shigella* spp., and EHEC in stool samples using PCR dipstick DNA chromatography, although there are several similar commercially available kits.

An available PCR dipstick DNA chromatography assay detects *invA*, *ipaH*, and *stx1/stx2* in food pathogen-enrichment (FPE) broth (Hayashi et al., 2013). In this system, DNA is extracted using a physical

disruption method after 6-h enrichment culture in FPE broth. However, this DNA detection system was not designed or evaluated for detecting DNA directly from prepared stool samples that may contain proteins and enzymes that inhibit PCR assays. Therefore, to prepare DNA directly from a stool sample, we developed a new DNA extraction method that removes PCR inhibitors and avoids using a physical disruption method.

Specifically, we improved detection sensitivity by denaturing proteins, including inactivating enzymes, in stool samples using Lysis Buffer B in the DNA extraction step (Table S1). We expect that this DNA extraction method will detect numerous, diverse bacterial species present in stool samples. Moreover, we believe it is reasonable to predict that this DNA extraction method will be widely applicable to other samples, such as foods that contain an abundance of proteins and fats.

We compared the GeneFields® EHEC/SS and real-time PCR kits D and E using 5,000 stool specimens. As shown in Figs. 3 and 4, the GeneFields® EHEC/SS kit, real-time PCR kits (D or E), and the culture method detected *S. enterica* in 9 samples (Figs. 3 and 4). The GeneFields® EHEC/SS kit detected *stx1/stx2* in 5 samples, while only one sample was positive using real-time PCR kit E. However, the culture method did not detect EHEC or *Shigella* strains (Tables 4 and 5). Only the GeneFields® EHEC/SS kit detected 2 *ipaH*-positive samples (Table 5). Consequently, we sequenced the PCR products of *ipaH* and found that the sequence was 100% identical to those of *ipaH* of *S. sonnei* and *S. flexneri* (data not shown). Therefore, the sensitivity of GeneFields® EHEC/SS for detecting *Shigella* spp. was higher when compared with that of the culture method. These results indicate that the sensitivities of the GeneFields® EHEC/SS kit and the real-time PCR kit E for detecting *S. enterica*, *Shigella* spp., and EHEC were higher compared with that of the culture method. However, real-time PCR kit D was less sensitive than the GeneFields® EHEC/SS kit and the culture method. This may be explained by the medium used for isolating bacteria. For example, O157 is the most important O serogroup of EHEC strains (Verhaegen et al., 2015). Therefore, CT-SMAC was used, however, non-O157 strains may not grow on this medium (Perry and Freydiere, 2007). Another possibility is the detection of dead bacteria by genetic methods. To solve this problem, ethidium bromide monoazide (EMA) or propidium monoazide (PMA) can differentiate dead from live bacteria (Nocker et al., 2007; Soejima et al., 2008). However, the membrane permeabilities of EMA and PMA are species-specific (Nocker et al., 2006). Therefore, these reagents must be tested for detecting the serovars of *S. enterica*, *Shigella* spp. and EHEC in stool samples.

The relative sensitivity of the GeneFields® EHEC/SS kit compared with those of real-time PCR kits D and E was > 85.0%. The relative specificity of the GeneFields® EHEC/SS kit compared with those of real-time PCR kits D and E was > 90.0%; the percentage of observed agreement was > 90.0%, and the kappa statistic was > 0.60 (Tables 4 and 5). These results indicate that the GeneFields® EHEC/SS kit can be used instead of real-time PCR kits as a genetic screening assay for *S. enterica*, *Shigella* spp., and EHEC in stool specimens.

**Table 5**  
Comparative results using the GeneFields® EHEC/SS kit and real-time PCR kit E to screen 5,000 stool specimens.

GeneFields®EHEC/SS	Real-time PCR kit E			Total positive	Culture positive
	<i>invA/ipaH</i> +	<i>stx1/stx2</i> +	Negative		
<i>Salmonella</i> ( <i>invA</i> +)	7	0	1	8	5
<i>Shigella</i> ( <i>ipaH</i> +)	0	0	2	2	0
EHEC ( <i>stx1</i> +)	0	0	1	1	0
EHEC ( <i>stx2</i> +)	0	0	3	3	0
Negative	0	1	[85 <sup>a</sup> ]	1	5
Total positive	7	1	7	15	5
Total (negative)	100 (93)	100 (99)	100 (93)	100 (85)	10

Fifty stool samples were collected and analyzed as one sample. Namely, 100 stool mixtures consisting of 5000 specimens.

<sup>a</sup> The number showing negative result by both kits but not being counted for total number.

## 5. Conclusions

We demonstrated that the GeneFields® EHEC/SS kit detected *S. enterica*, *Shigella* spp., and EHEC in stool specimens with specificities and sensitivities almost equivalent to those of commercially available real-time PCR kits. Importantly, the GeneFields® EHEC/SS kit is an economical, simple, and rapid genetic screening method for detecting these enteric pathogens. Further comparative studies using more clinical samples are required, together with conventional culture and genetic methods, to establish the GeneFields® EHEC/SS kit as a rapid, economical, and reliable method for identifying *S. enterica*, *Shigella* spp., and EHEC in stool specimens of healthy people, as well as patients with diarrhea.

## Conflict of interest

None to declare.

## Ethical statement

None to declare.

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