Original Article

C-Terminal Part of Glutamate-Ammonia-Ligase Adenyltransferase Gene Identified by RAPD-HRM with 3H Primer for *E. Coli* Screening

(*E. coli* / random-amplified-polymorphic-DNA / 3H primer / high-resolution melt / sequence characterized amplified region)

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Abstract. A single random oligonucleotide 3H primer has been previously applied in random-amplified-polymorphic-DNA (RAPD)-PCR to distinguish stocked bacteria *E. coli* within a cocktail mixture also containing *Enterococcus faecalis*, *Bifidobacterium longum* and *Ruminococcus gnavus*. In this study, we demonstrate that a 702 base pair (bp) gene fragment can be amplified as a unique pattern by RAPD-PCR using a 3H primer in human faeces containing *E. coli*. This unique 702 bp amplicon contained a 687 bp gene fragment identified as the C-terminal region of the glutamate-ammonia-ligase adenyltransferase (*glnE*) gene of *E. coli*. By high-resolution melt (HRM) analysis, a mean melt-curve temperature of this 702 bp amplicon was determined to be approximately 88.1 ± 0.22 degrees Celsius (°C). A combination of RAPD with HRM in one single reaction based on this amplicon can achieve semi-quantitative detection of up to 10^2 CFU/ml of *E. coli*. To increase the signal intensity of HRM, a primer pair capable of screening *E. coli* directly from fresh human faeces was re-designed from the 687 bp gene segment, giving a mean peak melt-curve temperature at 88.35 ± 0.11 °C. Finally, single-nucleotide polymorphisms of this 687 bp gene segment were analysed for pathogenic *E. coli* strains, including UMN026, O83:H1, O104:H4, O157:H7 and O169:H41. We conclude that this 687 bp segment of the *glnE* gene has a high potential for screening of human faecal *E. coli*, including pathogenic strains, in contaminated food and water.

Introduction

The food hygiene and foodborne disease have attracted a great deal of attention in recent years. Foodborne illnesses are usually caused by opportunistic bacteria, viruses, parasites, or chemical substances getting through contaminated food or water to the body. In 2015, the World Health Organization (WHO) has estimated that 2.1 million deaths occur every year from contaminated food or drinking water (WHO and UNICEF, 2017). *Escherichia coli* is one of the most common infectious pathogens causing diarrheal diseases in 220 million children and 96,000 deaths from intestinal and extra-intestinal diseases every year (WHO, 2015). The transmission routes for diarrheal disease often include insufficiently processed animal products or manure, contaminated water, raw food, and human faecal materials (Rwego et al., 2008; FAO, 2011).

In the past, the diagnostics for foodborne microbes mostly relied on classical culture methods, using selective media to enrich the target organism hidden in the indigenous background flora of the food. Finally, presumptive typical colonies were verified by morphological, biochemical, genetic, and serological tests. Although advantages of the classical culture methods are ease of use in analytical laboratories, they are still labour-intensive, time-consuming and demanding for large amounts

Received March 26, 2019. Accepted April 23, 2019.

This study was supported by the Ministry of Science and Technology in Taiwan (grants MOST 106-2314-B-020-001, MOST 104-2314-B-020-001-MY3 and MOST 108-3017-F-020-001).

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Abbreviations: CFU – colony-forming units, ELFA – enzyme-linked fluorescent assays, ELISA – enzyme-linked immunosorbent assay, FISH – fluorescent *in situ* hybridization, *glnE* – glutamate-ammonia-ligase adenyltransferase gene, HRM – high-resolution melt, RAPD – random-amplified-polymorphic-DNA, PCR – polymerase chain reaction, PFGE – pulsed-field gel electrophoresis, qPCR – real-time PCR, SNP – single-nucleotide polymorphism.

of liquid and solid media (Vasavada, 1993; Jasson et al., 2010).

Numerous alternative methods for *E. coli* identification such as enzyme-linked immunosorbent assay (ELISA) (Segura-Alvarez et al., 2003), flow cytometry (Raybourne, 1999), enzyme-linked fluorescent assays (ELFA) (Stefen et al., 2007), fluorescent *in situ* hybridization (FISH) (Wu et al., 2010), pulsed-field gel electrophoresis (PFGE) (Kariuki et al., 1999), conventional polymerase chain reaction (PCR) (Holland et al., 2000; Segura-Alvarez et al., 2003; Hanna et al., 2005; Jasson et al., 2010), and real-time PCR (qPCR) (Heid et al., 1996; Ibekwe and Grieve, 2003; Stefen et al., 2007; Rodríguez-Lázaro et al., 2007) have been introduced to replace the conventional methods.

A technique modified from PCR called random-amplified-polymorphic-DNA (RAPD) has been successfully used to profile *E. coli* recently (Salehi et al., 2008; Packey et al., 2013; Marialouis and Santhanam, 2016). In contrast to traditional PCR, RAPD is a modification of PCR in which a single, short and random oligonucleotide primer can anneal and prime at multiple locations throughout the template DNA to produce a unique spectrum of amplification products. RAPD-PCR has found a wide range of applications in gene mapping, population genetics, molecular evolutionary genetics, and plant and animal breeding (Kumar and Gurusubramanian, 2011). In addition, Packey et al (2013) have developed an optimized RAPD-PCR using a 3H primer that can selectively screen colonized gnotobiotic rodents for contamination, especially of *E. coli* NC101, *E. coli* K12, *Enterococcus faecalis* OG1RF, *Bacteroides vulgatus*, and *Bifidobacterium longum* (Packey et al., 2013).

A fast and high-throughput method known as high-resolution melting (HRM) analysis has been developed for post-PCR diagnosis for many pathogens (Senapin et al, 2010; Cai et al., 2013; Gopaul et al, 2014; Elkins et al, 2016). Related to *E. coli* diagnosis, the HRM-qPCR method was able to identify swine-associated enterotoxigenic *E. coli* strains expressing five different types of fimbriae obtained from faecal samples of weaned piglets (Wang et al., 2017). Moreover, Harrison et al. (2017) also showed that the multiplex HRM-qPCR can be applied to distinguishing ST131 from non-ST131 *E. coli* without sequence analysis (Harrison et al., 2017). Elkins et al. (2016) used HRM-qPCR assays to identify multiple species present in a mixture of *Salmonella enterica* substrain *typhimurium*, *Escherichia coli*, and *Shigella flexneri.*

The RAPD-PCR combined with the high-resolution melt-curve analysis (RAPD-HRM) was first established as a novel technique to identify *Leptospira* serovars. A total of eight serovars belonging to four species of pathogenic *Leptospires* were characterized by each own serovar's characteristic melt profile (Tulsiani et al. 2010). In this study, we successfully applied an optimized RAPD-HRM for identification of *E. coli* in human faeces and discuss its use in detection of food and water contamination with *E. coli*.

Material and Methods

Collection of faecal samples from humans

The fresh faecal samples were provided from healthy human donors. Among the donors, there were 40 % males and 60 % females, and the age and dietary habits of the donors were recorded. Briefly, approximately 0.02 g faeces provided by the donor was placed in a 1.5 ml micro-centrifuge tube using a sterile toothpick and homogenized manually with 1 ml sterile water. The sample mixture was centrifuged and the supernatant was then collected.

Screening and identification of E. coli

The screening test of *E. coli* from the faeces was initially performed using the Eosin Methylene Blue Agar (EMB Agar, Difco™, Heidelberg, Germany) test followed by a TSI slant test, Compact Dry EC medium plate test, and Gram stain. For the EMB agar test, a disposable inoculation ring was used to pick up an adequate amount of the supernatants and performing quadrant streaking on EMB Agar (Difco). After incubation at 37 °C for 12 h, a greenish metallic single colony was picked from EMB for the TSI slant test (Difco), Compact Dry EC medium plate test (R-Biopharm AG, Darmstadt, Germany), and Gram staining (BASO®, Taipei, Taiwan) according to manufacturer's instructions.

E. coli DNA purification from faecal samples

The genomic DNA of *E. coli* from human faeces was purified by a QIAamp DNA Mini kit (QIAGEN, Hilden, Germany). According to the manufacturer's instructions, 1 ml of bacterial suspension was centrifuged and the supernatant was removed. To the pellet was added QIAamp DNA Mini kit buffer and rapid centrifugation proceeded. The QIAamp Mini spin column provided in the QIAamp DNA Mini kit was used for DNA isolation. The faecal DNA was quantified by Nanodrop (Maestrogen, Hsinchu City, Taiwan) and stored in –20 °C until further analysis.

Colony-formation unit assay

The assay was modified according to a previous study (Haase et al., 2017). *E. coli* was grown on EMB agar (Difco) as described for the screening test. The inoculation ring was used to pick the purified *E. coli* and seeded into LB broth (Neogen, Lansing, MI), and incubated at 37 °C for 12 h. Afterward, a series of 10-fold dilutions were prepared, of which 10 µl aliquots were placed on LB agar (MDBio, Inc, Xinbei, Taiwan) plates and incubated for 12 h at 37 °C. Colonies were counted for the serial dilutions at which distinguishable colonies were observable and used to calculate the number of viable bacteria in the original solution (Fig. 4).

RAPD-PCR assays

The single random oligonucleotide 3H primer (5'-AAGCTTGATTGCCC-3') was described in a pre-

vious study (Packey et al., 2013). Each RAPD-PCR reaction was performed in a final volume of 20 µl containing 90 ng of DNA template, 2 U of Taq Polymerase (JMR, London, UK), 3 µl $MgCl_2$ (25 mM), 2.5 µl 3H primer (20 µM) and 2 µl dNTPs (10 mM) (Promega, Madison, WI). The amplification of RAPD-PCR was performed in a thermal cycler (Biometra GmbH, Gottingen, Germany) by using the following conditions: 95 °C for 5 min, 36 cycles of 95 °C for 1 min, 36 °C for 1 min, and 72 °C for 2 min followed by one cycle of 72 °C for 7 min for completion of DNA extension. PCR products were stored at 4 °C. The *ETA* (Exotoxin A) gene expression of *Pseudomonas aeruginosa* served as a control. (*ETA* forward primer, 5'- TTCCGCTCCCCGCCAGCCTC- 3'; *ETA* reverse primer, 5'-AGTAGTGCAGCACGCCCTGG- 3'). The final products were examined in 1.7% agarose gel at 80 V for 35 min by electrophoresis.

DNA sequencing

The unique RAPD amplicon (702 bp) was excised from 1.7% agarose gel and extracted by a Qiaex II gel extraction kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Ligation of the purified DNA fragment to the yT & A vector (Yeastern Biotech Co., Ltd, Taipei, Taiwan) was performed in the reaction mixture containing 50 ng of DNA, 2 µl of 10x buffer A, 2 µl of 10× buffer B, 1 U of yT4 DNA ligase, 1 µl of yT & A vector (25 ng/µl), and 6 µl of sterilized water. The mixture was incubated for 24 h at 4 °C. Subsequently, the vector was transformed into *E. coli* DH5α competent cells after 24 h incubation at 4 °C. After growth of the colonies for at least 14 h, recombinant plasmid screening was performed by blue-white selection in agar plates containing 20–50 µg/ml ampicillin plus 0.1–0.5 mM IPTG and 40–60 µg/ml X-gal.

The white colonies were picked up for sequencing. DNA sequences were analysed by Cycle Sequencing Applied Biosystems 3730 DNA Analyser BigDyeR (TRI-I Biotech, Inc. Shanghai, China). The sequencing reactions were performed with a Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol. Briefly, the reaction was performed with M13 forward primer (5'-GGTTTTCCCAGTCACGAC-3') and M13 reverse primer (5'-CACACAGGAAACAGCTATGA-3') in GeneAmp PCR System 9700 (Applied Biosystems) using the following conditions: 96 °C for 10 s, 50 °C for 5 s and 60 °C for 4 min for 30 cycles. The DNA sequence was registered in GenBank under accession number MH361001.

RAPD-HRM assays

The RAPD/HRM reaction was modified according to a previous study (Tulsiani et al., 2010). Each 25-µl reaction mixture consisted of 12.5 µl HRM mix (QIAGEN), 0.5 µl of 25 mM $MgCl_2$, 1 µl of 20 µM 3H primer, 9 µl DNA template (90 ng) and 2 µl molecular-grade water. RAPD–HRM was performed in Rotor-Gene Q (QIAGEN) with green fluorescence emission. A 10-min hot start at 95 °C was followed by denaturation at 94 °C for 1 min, annealing at 40 °C for 1 min and extension at 72 °C for 2 min up to 40 cycles, then final extension at 72 °C for 7 min. During the RAPD-HRM, fluorescence data were acquired at the end of the annealing/extension cycle of the PCR. The temperature was ramped in increments of 0.1 °C between 75 °C and 95 °C for the HRM analysis. Melting curve data were statistically analysed from triplicate experiments using Microsoft Excel 2010 and presented as mean ± SD.

Semi-quantitation assay

The isolated DNA was processed in the RAPD-HRM reaction mixture containing 12.5 µl HRM mix (QIAGEN), 0.5 µl of 25 mM $MgCl_2$, 1 µl of a 3H primer (20 µM), 9 µl DNA template (90 ng, 180 ng, 270 ng, or 360 ng) and 2 µl molecular-grade water. The semi-quantitation analysis was performed with Graph Pad Prism version 7 (GraphPad Software, San Diego, CA). Data are presented as the mean ± SD in a linear regression graph, and the calculated values of the Spearman correlation coefficient (r) and two-tailed probability test. P values < 0.05 were considered statistically significant.

Sequence Characterized Amplified Region (SCAR) primer design and HRM-PCR amplification

The SCAR primers (forward primer, 5'-GATTGCCCAATGGATGCGAT-3'; reverse primer, 5'-TGCCCGCACCAGTTCA-3') were designed from the 5'- and 3'-end of 687 bp of the C-terminal part of the glutamate-ammonia-ligase adenylyltransferase (*glnE*) gene of *E. coli.* The specificity was assessed in an amplification reaction with a final reaction mixture volume of 25 µl, as follows: 12.5 µl HRM mix (QIAGEN), 1.75 µl of a primer mix (10µM), 2 µl DNA template and 8.75 µl molecular-grade water. Real-time PCR amplification was performed in Rotor-Gene Q (QIAGEN) according to the manufacturer's instructions (Type-it HRM PCR kit, QIAGEN) in the following running conditions: an initial denaturation step for 5 min at 95 °C, followed by 35 cycles of denaturation for 10 s at 95 °C, annealing for 30 s at 58 °C, and extension for 25 s at 72 °C, and a final step with HRM melting temperature increased from 65 °C–95 °C at the speed of 0.1 °C/step and held for 2 s at each step. A threshold value for the fluorescence of all samples was set manually.

Results

Screening, collection and identification of E. coli from human faeces

The human faecal samples were collected from healthy donors, 40 % males and 60 % females with an age range from 21 to 23 years (mean age 21.7 years). The faecal samples (approximately 0.02 g) collected from the do-

Table 1. Biochemical test for isolated faecal E. coli from human

	***E. coli* ATCC23815**	***E. coli* (Human faeces)**
Greenish metallic sheen colony (lactose fermentation)	+	+
Gas production	+	+
Sucrose / lactose metabolism	+	+
Facultative anaerobic	+	+
β-Glucuronidase	+	+
Gram staining/rod shape	–/+	–/+
H_2S production	–	–

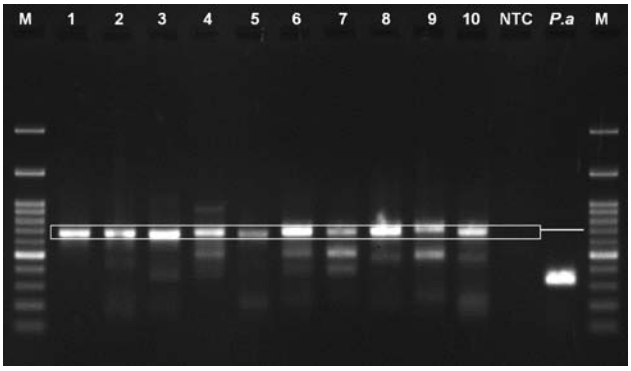


Fig. 1. RAPD-PCR fingerprints of *E. coli*
Agarose gel electrophoresis of RAPD-PCR amplicons of DNA isolated from healthy donors' faecal *E. coli* showing a consistent band at the size around 700 bp in contrast with that of *P. aeruginosa*. M: 100 bp DNA ladder, 1–10: donors' *E. coli*, NTC: non-template control, *P.a.*: Exotoxin A *(ETA)* gene expression of *Pseudomonas aeruginosa.*

nors were then diluted in 1 ml distilled water and immediately inoculated on Eosin Methylene Blue (EMB) Agar (BD Difco, Heidelberg, Germany) by the streaking method. After an incubation at 37 °C for 12 h, a single colony with greenish metallic sheen was picked up from EMB agar and expanded in 1 ml LB broth (BD Difco) for the next 12 h. A series of biochemical tests including Gram staining (BASO®, Taipei, Taiwan), Triple Sugar-Iron Agar Test (BD Difco) and Compact Dry EC (R-Biopharm AG, Darmstadt, Germany) were then performed to ensure the identities of *E. coli* from our selected colonies. *Pseudomonas aeruginosa* (ATCC 31156), *Escherichia coli* (ATCC 23815) and *DH5α* (ThemoFischer Scientific,Waltham, MA) were used as standard controls. All selected colonies were confirmed to be Gram-negative, rod-shape and coliform bacteria (Table 1). They manifested as anaerobic glucose-fermenting bacteria with gas production and without producing hydrogen sulphide in TSI slant agar (Table 1). In Compact Dry EC test, all selected colonies appeared blue due to β-glucuronidase activity produced by *E. coli* via cleavage of X-Gluc to produce colourless glucuronic acid and a strong blue-bromo-indigo precipitate (Table 1).

Detection of a unique amplicon of E. coli from human faeces using RAPD-PCR

In a previous study, a single random oligonucleotide 3H primer (5'-AAGCTTGA-TTGCCC-3') was used for screening of *E. coli* isolated from faecal samples of gnotobiotic rodents using RAPD-PCR assays (Packey et al., 2013). In this study, total genomic DNA was extracted from prepared human faecal *E. coli* by a QIAamp DNA mini kit (QIAGEN). After performing RAPD with the 3H primer, the pattern of PCR amplicons revealed a specific and reproducible DNA fragment at around 700 bp across all of our human faecal (100% accuracy) and stock *E. coli* samples (Fig. 1). We considered it as a unique DNA fingerprint representative of human faecal *E. coli* obtained by RAPD-PCR with the 3H primer. In contrast, this unique size of amplicon was not seen in *P. aeruginosa* after RAPD-PCR (data not shown).

Genetic identification of a 702 bp unique amplicon by DNA sequencing

The DNA fragment at molecular size of ≈ 700 bp was cut off from the gel and extracted with QIAEX® II Gel Extraction Kit (QIAGEN) (Fig. 2a). The extracted DNA was cloned into the yT & A vector (Yeastern Biotech, Taipei, Taiwan) and subjected to sequencing (TRI-I Biotech, New Taipei City, Taiwan). In the sequencing result (Fig. 2b), the 3H primer sequence was shown at both ends of a 702 bp DNA fragment. A 687 bp C-terminal part of the *glnE* gene fragment of *E. coli* partially overlapping with the 3H primer for eight nucleotides at the 5' end and five nucleotides at the 3' end was deposited to the NCBI GenBank database (GenBank accession number: MH361001). The *glnE* gene is ubiquitous, with very similar sequence throughout the *Escherichia* family. This 687 bp *glnE* gene fragment was confirmed to be a ubiquitous area presenting in over hundreds of *E. coli* strains according to the NCBI GenBank nr database (Fig. 2c).

RAPD in combination with high-resolution melt-curve (HRM) analysis of the 702 bp unique amplicon

The purified 702 bp DNA fragment was subjected to HRM analysis and the melting temperature was determined at 88.08 °C in the melting curve (Fig. 3a). Then, we carried out a RAPD-HRM analysis for all of our human faecal *E. coli* samples. Compared to the melting temperature of the purified 702 bp DNA fragment, we also obtained a dominant peak in *E. coli* samples (8 out of 10 samples) with the mean melting temperature at 88.1 ± 0.22 °C (Fig. 3b-c).

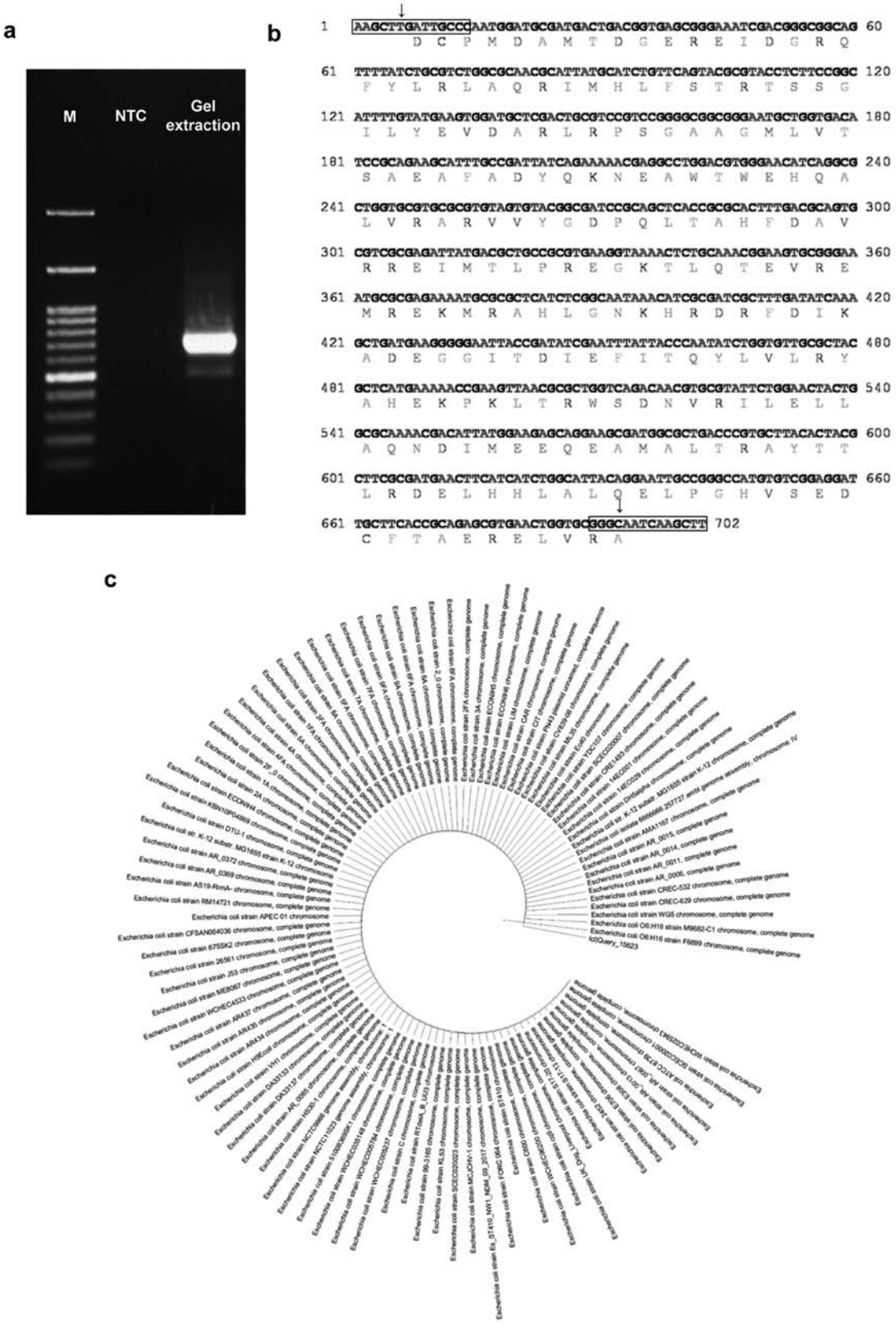


Fig. 2. DNA sequencing of the amplicon

(a) DNA amplicon at a size around 700 bp was purified by gel extraction after RAPD-PCR. **(b)** The amplicon was identified as a 702 bp nucleotide fragment including the 3H primer sequence (5'-AAGCTTGATTGCCC-3'). The 3H primer was marked in a frame (both 5'- and 3'-ends). The sequence between both of the arrows matches with the gene fragment of glnE, which is composed of 229 amino acids spanning the 687 base pairs shown by BioEdit software. M: 100 bp DNA ladder, NTC: non-template control. **(c)** According to the data from NCBI GenBank, this 687 bp amplicon was manifested as a gene ubiquitous for *E. coli* strains.

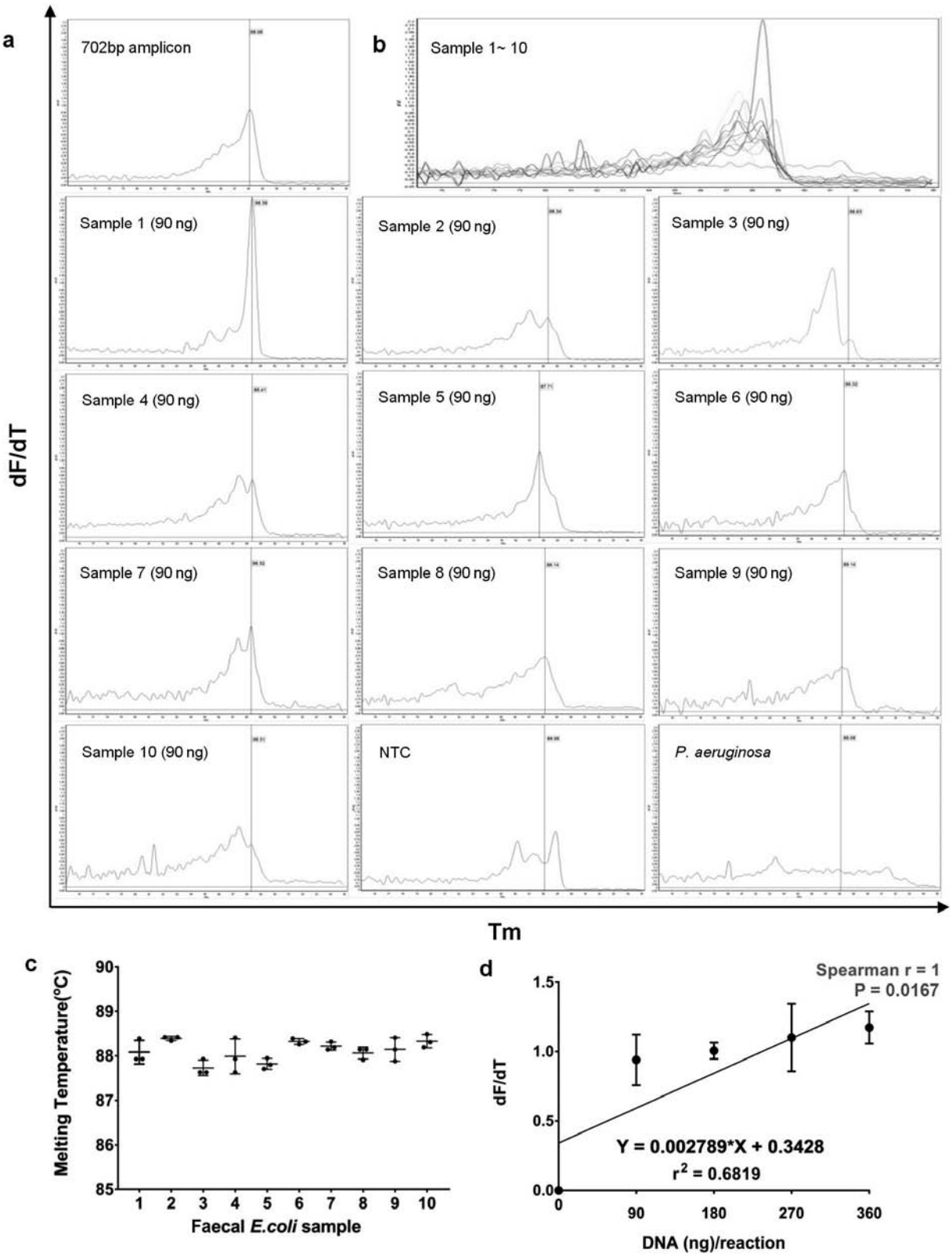
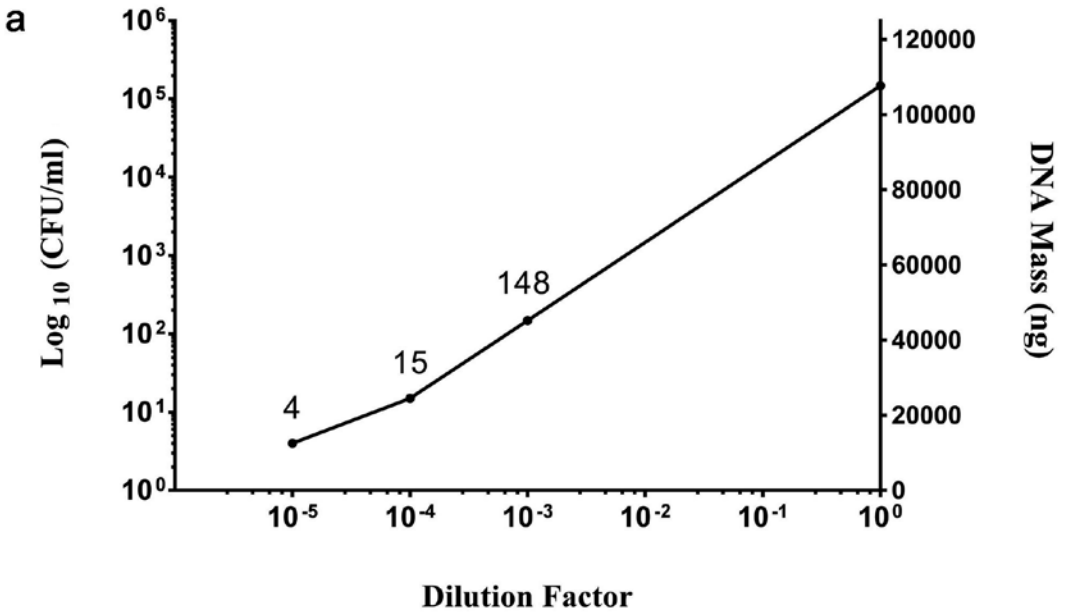


Fig. 3. RAPD-HRM assay of *E. coli* genomic DNA by using the 3H primer
(a) High resolution melt-curve (HRM) assay revealed that the melting temperature of the purified 702 bp amplicon made by RAPD-PCR was 88.08 °C. **(b-c)** RAPD-HRM of *E. coli* from groups 1–10 showing a representative melt-curve at 88.11 ± 0 .22 °C. NTC and *P. aeruginosa* were seen as a negative control. NTC: non-template control, samples 1–10: donors' *E .coli*. **(d)** Linear regression and correlations of the ratio of dF/dT with *E. coli* DNA concentration. Spearman correlation analysis showing positive correlation between fluorescence intensity dF/dT and the concentration of DNA undergoing RAPD-HRM. Calculated values of the Spearman correlation coefficient (r) and two-tailed probability test (P) are indicated, $P < 0.05$. $N = 6$.



$$\text{The sensitivity of RAPD} - \text{HRM} = \frac{E.coli \text{ concentration } \left(\frac{CFU}{ml}\right)}{\text{Total mass of extracted DNA } (ng)} \times \text{The mass of DNA applied in RAPD} - \text{HRM}$$

$$= \frac{1.48 \times 10^5 \left(\frac{CFU}{ml}\right)}{105652(ng)} \times 90(ng)$$

$$= 1.26 \times 10^2 \left(\frac{CFU}{ml}\right)$$

Fig. 4. Sensitivity of RAPD-HRM for *E. coli* detection
(a) CFU assay coordinated with DNA extract mass. **(b)** The sensitivity of RAPD-HRM for *E. coli* detection approaches 10^2 CFU/ml calculated by the formula.

Sensitivity of RAPD-HRM for E. coli detection

In this study, evaluation of colony-forming units (CFU) of *E. coli* was performed in order to calculate the extract of DNA concentration per human faecal *E. coli*. Hereby, we found that 1.48×10^5 CFU in 1 ml of *E. coli* contained 105,652 ng of DNA extract (Fig. 4a). Since each reaction of RAPD-HRM assay only required 90 ng of DNA extract, the sensitivity of our detection by RAPD-HRM can approach 1.26×10^2 CFU per ml of *E. coli* calculated by the formula (Fig. 4b).

Semi-quantitative E. coli detection by RAPD-HRM

In order to achieve quantitative determination of *E. coli* by RAPD-HRM, we tested whether the level of fluorescent signal (dF/dT) increases with the DNA content in *E. coli* samples. We tested different amounts of isolated *E. coli* DNA extract (90 ng, 180 ng, 270 ng, or 360 ng) in the RAPD-HRM reaction using the 3H primers. There was a significant increase in the level of fluorescent signal (dF/dT) with increasing *E. coli* DNA content in the samples. Spearman correlation analysis between the fluorescent signal (dF/dT) and *E. coli* DNA amount per reaction indicated a positive correlation (Spearman r = 1; P value = 0.0167) (Fig. 3d). This confirmed that RAPD-HRM can detect *E. coli* semi-quantitatively with the sensitivity starting at 10^2 CFU per ml, which is equivalent to 90 ng DNA per reaction.

SCAR primer pair targeting the C-terminal part of glnE (687 bp) for ubiquitous E. coli screening in fresh human faeces

We attempted to screen *E. coli* directly from 0.02 g fresh human faeces by RAPD-HRM using the 3H primer. The prior 702 bp amplicon as a control template in the reaction produced a melt curve temperature at 88.45 °C, and all five tested fresh human faeces samples manifested a tiny peak at 88.48 ± 0.13 °C after RAPD-HRM as well (Fig. 5a). In RAPD-HRM, samples 3, 4 and 5 had shown signal just above the noise at the indicated temperatures and the PCR product in these samples was barely to be seen in the gel (Fig. 5c). This result was clearly caused by the abundance of microbiota in fresh human faeces. Therefore, in order to increase the signal intensity of HRM, we re-designed the longer and specific primer pair (forward primer, 5'-GATTGCCCAATGGATGCGAT-3'; reverse primer, 5'-TGCCCGCACCAGT- TCA-3', sequences overlapped with 3H primer are underlined) targeting the 687 bp of *glnE*. This primer pair, originally derived from the product of RAPD-PCR, is referred to as the SCAR marker (Fig. 2a-b). We fur-

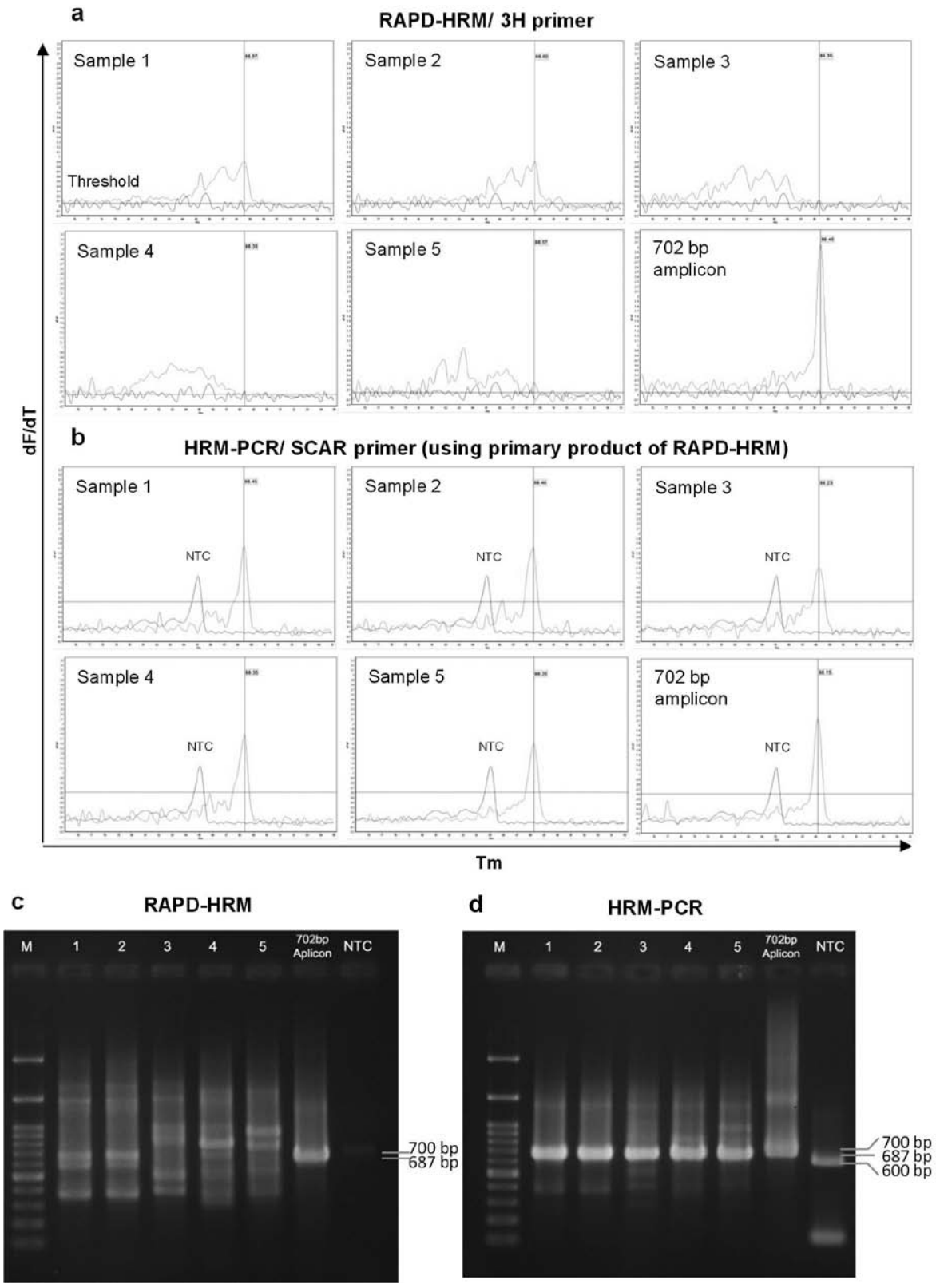


Fig. 5. Primer pair re-designed from SCAR specifically increases signal intensity of the C-terminal part of *glnE* of *E. coli* by HRM-PCR

(a) RAPD-HRM of five fresh human faeces samples that showed a weak representative melt-curve of *E. coli* at 88.48 ± 0.13 °C. **(b)** HRM-PCR using a specific primer pair re-designed from SCAR of 687 bp *glnE* to amplify the primary product of RAPD-HRM. The result showed a high-intensity representative melt-curve of *E. coli* at 88.35 ± 0.11 °C. **(c)** Agarose gel electrophoresis of faecal samples 1–5 using a specific 3H primer pair. **(d)** Agarose gel electrophoresis of faecal samples 1–5 using a specific SCAR primer pair; 687 bp of *glnE* was identified. In total, 17 fresh human faeces samples were tested and the results were 100 % reproduced. M: 100 bp DNA ladder marker, NTC: non-template control.

ther used the prior RAPD-PCR product as a DNA template for HRM-PCR using the SCAR primer. The result showed the expected dominant peak with a mean melting temperature at 88.35 ± 0.11 °C (Fig. 5b). After agarose gel electrophoresis, the amplicon of 687 bp of *glnE* was confirmed in the reaction using the SCAR primer in all five fresh human faecal samples (Fig. 5d). Although we also obtained an amplicon from non-template control (NTC) at around 600 bp, the melt curve temperature was 85.05 °C, easily being identified as a non-specific product of PCR by a 3 °C shifting (Fig. 5b). Finally, up to 17 fresh human faecal samples were tested and the results were 100 % reproduced.

The C-terminal part of glnE (687 bp) has potential for screening pathogenic E. coli

In the prior results, the 687 bp of the C-terminal part of *glnE* was shared among hundreds of *E. coli* strains (Fig. 2c). Next, we focused on the genetic variation in this 687 bp fragment of the pathogenic *E. coli* including the adherent and invasive *E. coli* (AIEC), enterotoxigenic *E. coli* (ETEC), extraintestinal pathogenic *E. coli* (ExPEC) and enterohaemorrhagic *E. coli* (EHEC). According to available data, single-nucleotide variations existed within the 687 bp gene fragment in *Escherichia coli* UMN026, O83:H1, O104:H4, O157:H7 and O169:H41 compared with that in *Escherichia coli* K-12 (Table 2, Fig. 6). On the other hand, *Escherichia coli* IAI39 and O15:H11 completely matched the regions of similarity with K-12 in the blast alignment by the NCBI GenBank nr and refseq representative genome database (Table 2). This valuable information could be used to develop new analytical approaches based on the single-nucleotide polymorphism (SNP) and applied to specific pathogenic *E. coli* screening.

Discussion

In a previous study, Packey et al. (2013) demonstrated that by using random amplification of polymorphic DNA (RAPD)-PCR with the 3H primer (5'-AAGCTT-GATTGCCC-3'), the SIHUMI cocktail mixed up with stock bacteria *E. coli* (LF82), *E. faecalis* (OG1RF), and *Bifidobacterium longum* (ATCC 15707) can be distinguished according to the unique pattern of amplicons shown in gel electrophoresis. However, the nucleotide sequence of amplicons and the potential of this method for identification of *E. coli* in human faeces remains unknown. In this study, we demonstrate a 702 bp amplicon representing a unique fingerprint of human faecal *E. coli* after RAPD-PCR using the 3H primer. Interestingly, Packey et al. (2013) showed a unique amplicon of *E. coli* from rodents' faeces with a molecular weight around 900 bp differing in size from the 702 bp amplicon of ours (*E. coli* from human faeces). These differences might be due to variable conditions of electrophoresis. By sequencing the 702 bp amplicon, we found that

Table 2. Genetic information of pathogenic E. coli within the 687 bp fragment

Match with K.12 strain's 687 bp	Type	Strain	Sequence ID	DNA strand (+/–)	Position (687 bp)		Variation in position (relative to 687 bp)	Nucleotide variation
					Start	End		
Complete	AIEC	IAI39	NC_011750.1	–	3701824	3701138	---	---
	ETEC	O15:H11	CP024239.1	+	3584784	3585470	---	---
Incomplete	ExPEC	UMN026	NC_011751.1	–	3652381	3651695	3652352(30) 3652331(51) 3652280(102) 3652246(136) 3652211(171) 3652208(174) 3652196(186) 3652088(294) 3652085(297) 3652082(300) 3652061(321) 3652055(327) 3652049(333) 3652040(342) 3652010(372) 3652007(375) 3651965(417) 3651956(426) 3651929(453) 3651863(519) 3651791(591) 3651785(597)	T -> C G -> A T -> A C -> A G -> T A -> C A -> G G ->A T -> C C -> G T -> C T -> C T -> C G -> A C -> T T -> C T -> C G -> C C -> T T -> C T -> G T -> G

	AIEC	O83:H1	NC_017634.1	–	3222518	3221832	3222489(30) 3222468(51) 3222438(81) 3222417(102) 3222384(135) 3222827(192) 3222228(291) 3222102(417) 3222093(426) 3221988(531) 3221961(558) 3221928(591) 3221922(597) 3221904(615) 3221873(646)	T -> C G -> A T -> C T -> A T -> A C -> T A -> G T -> C G -> C A -> G G -> A T ->G T -> G T -> C T -> C
	EHEC	O104:H4	NC_018658.1	+	756892	757578	756921(30) 756942(51) 757026(135) 757029(138) 757032(141) 757035(144) 757047(156) 757140(249) 757182(291) 757317(426) 757410(519) 757479(588) 757488(597) 757537(646)	T -> C G -> A T -> A A -> G G -> A T -> C G -> C T -> G A -> G G -> C T -> C C -> T T -> G T -> C
	EHEC	O157:H7	NC_002695.2	–	3937173	3936488	3937144(30) 3937126(48) 3937123(51) 3937087(87) 3937078(96) 3937072(102) 3937051(123) 3937039(135) 3937036(138) 3937030(144) 3937003(171) 3937000(174) 3936996(178) 3936949(225) 3936655(519) 3936528(646)	T ->C G ->A G ->A T ->C T ->C T ->A T ->C T ->G A ->G T ->C G ->T A ->C G ->A A ->G T ->C T ->C
	ETEC	O169:H41	CP024263.1	–	2728678	2727992	2728454(225) 2728160(519) 2728148(531) 2728139(540)	T -> C A -> G T -> C T -> A

Abbreviations: AIEC – adherent and invasive *E. coli*; ETEC – enterotoxigenic *E. coli*; ExPEC – extraintestinal pathogenic *E. coli*; EHEC – enterohaemorrhagic *E. coli*

this amplicon was composed of a 687 bp gene fragment encoding the C-terminal part of *glnE* of *E. coli* overlapping with a part of 3H primer sequences at both the 5'- and the 3'- end. The *glnE* gene encodes the adenylyltransferase responsible for reversible inactivation of glutamine synthetase by adenylylation (Muse and Bender, 1992). According to the GenBank database, this partial gene fragment of *glnE* appears as highly suitable for screening the contamination of food, water or soil with *E. coli* of human faeces origin.

The 702 bp amplicon extracted from gel electrophoresis had a melt-curve temperature at 88.08 °C in HRM analysis. The method combining RAPD and HRM (RAPD-HRM) was further applied to analysis of all of our human faecal *E. coli* samples. Expectedly, a dominant peak at a mean temperature of 88.11 ± 0.22 °C was reproduced in our samples. Compared to the RAPD-PCR analyses, the RAPD-HRM assays may provide a relatively rapid, cost-effective and less labour-consuming method for *E. coli* identification.

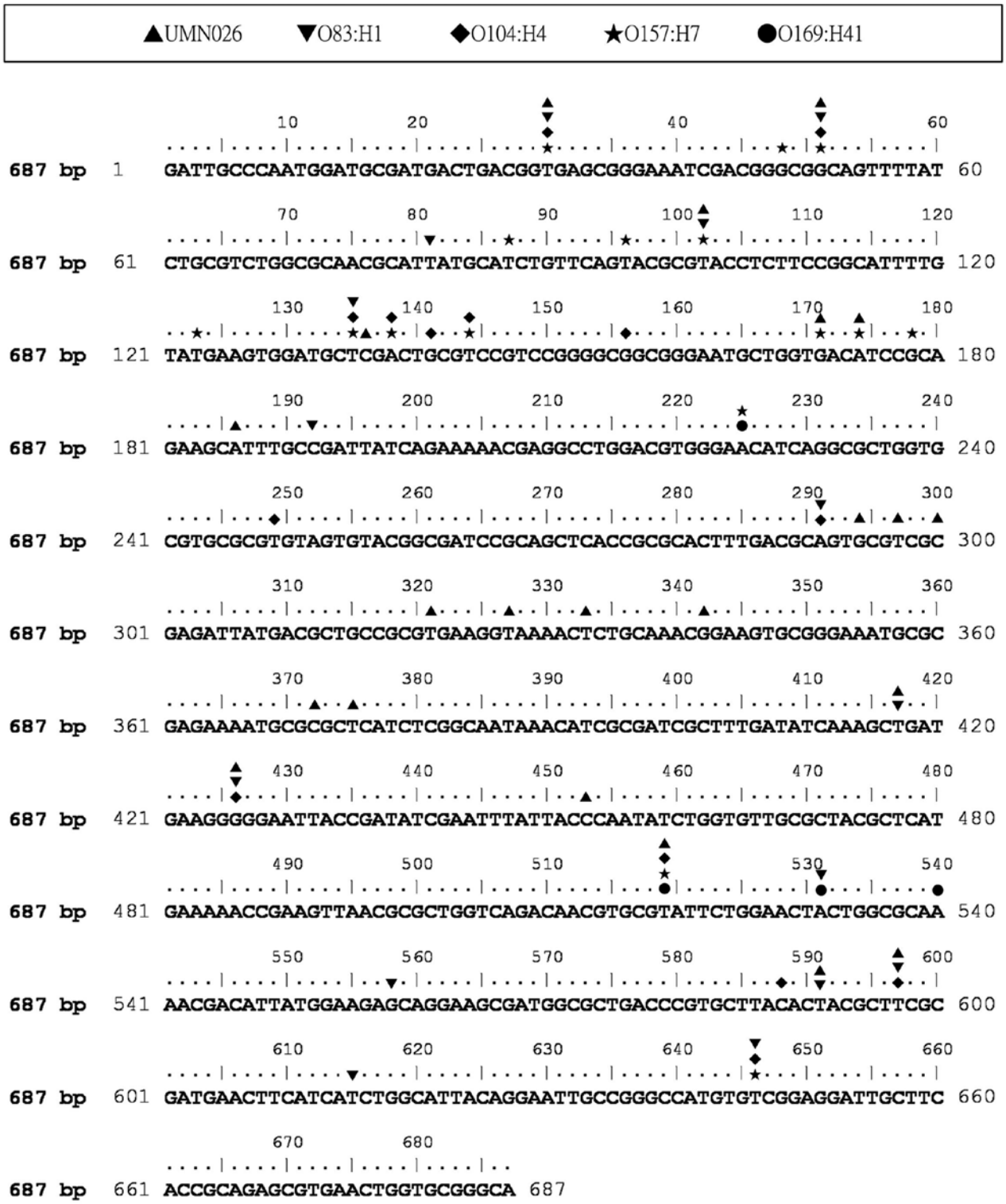


Fig. 6. Single-nucleotide polymorphism of 687 bp fragment for pathogenic *E. coli* strains
The sites of single-nucleotide polymorphism in 687 bp for *Escherichia coli* UMN026, O83:H1, O104:H4, O157:H7 and O169:H41 are shown by BioEdit software. The variations of the nucleotide are referred in Table 2.

In our work, we also demonstrated that the sensitivity of RAPD-HRM using the 3H primer for *E. coli* detection approached 10^2 CFU per ml. It is similar to other studies reporting the highest sensitivity for the enriched faecal samples and diarrhoeagenic *E. coli,* using conventional PCR assay, between 10^2 to 10^3 CFU (Dutta et al., 2001; Persson et al., 2007). Moreover, we have demonstrated a positive correlation between the ratio of the fluorescent signal (dF/dT) and the *E. coli* DNA concentration in the RAPD-HRM method using the 3H primer.

Hence, the RAPD-HRM method using the 3H primer can be used as a semi-quantitative tool for *E. coli* detection.

It is notable that we could directly detect the presence of *E. coli* in a very tiny amount of fresh human faeces (approximately 0.02 g) with only two consecutive steps: the first step is RAPD-HRM using the 3H primer, which is then followed by the second step consisting of HRM-PCR with the SCAR primer. The melt curve temperatures from both steps were very close, 88.48 ± 0.13 °C and 88.35 ± 0.11 °C, respectively. According to this data, we ruled out a possible shift in the melting temperature due to the distinct abundance of microbiota present in individual fresh human faeces (De Palma et al, 2017).

Finally, the 687 bp gene fragment encoding the C-terminal part of *glnE* of *E. coli* contains a single-nucleotide polymorphism in *Escherichia coli* UMN026, O83: H1, O104: H4, O157:H7 and O169:H41 strains when compared to the commensal *Escherichia coli* K-12. Therefore, this 687 bp gene segment is also a good candidate for the development of specific kits for detection of the pathogenic *E. coli* strains.

In conclusion, although RAPD has been replaced in a large part by whole-genome shotgun sequencing or next-generation sequencing for bacteria strain typing, RAPD costs less and has some other advantages. For example, a unique genetic region from unrelated bacterial strains can be easily found without expending vast work for gene comparison in the tremendous amount of data in Genbank. The combination of RAPD with HRM and SCAR techniques, developed in this study, provides a highly sensitive, semi-quantitative and speedy method for screening *Escherichia coli* in the food, water or soil contaminated by human faeces. Moreover, we identified a 687 bp fragment of the *glnE* gene of *E. coli* as a good target for the development of novel detection kits aimed at specific pathogenic *E. coli* strains.

Author contributions:

Co-first authorship: Y. C. C., Y. S. L., D. J. H. S. Conceived and designed experiments: K. T. C.; Performed experiments: Y. C. C., Y. S. L., D. J. H. S., Y. W. C., Z. R. C., Y. K. L., C. T. P. Analysed the data: Y. C. C., Y. S. L., D. J. H. S., K. T. C. Contributed reagents/materials: D. J. H. S., K. T. C. Contributed to the drafting and writing of the manuscript: Y. C. C., Y. S. L., D. J. H. S., K. T. C.

Conflicts of interest:

The authors declare no competing financial and non-financial interests.

Ethics approval:

This article contains human participants involved in the study. Volunteers gave informed consent for self-collection of non-identifiable faecal samples for an experimental teaching course and a sample for this study. In accordance with the Taiwan Ministry of Health Welfare (MOHW) Statement on the Ethical Conduct in Human Research (No. 1010265075), the research was considered as of a 'negligible risk' with no foreseeable risk of any harm or discomfort to the volunteers, and therefore deemed exempt from ethical review.

References

Cai, X. Q., Yu, H. Q., Ruan, Z. X., Yang, L. L. (2013) Rapid detection and simultaneous genotyping of *Cronobacter spp*. (formerly *Enterobacter sakazakii*) in powdered infant formula using real-time PCR and high resolution melting (HRM) analysis. *PLoS One* **8**, e67082.

De Palma, G., Lynch, M. D., Lu, J., Dang, V. T. (2017) Transplantation of fecal microbiota from patients with irritable bowel syndrome alters gut function and behavior in recipient mice. *Sci. Transl. Med*. **9**, eaaf6397.

Dutta, S., Chatterjee, A., Dutta, P., Rajendran, K. (2001) Sensitivity and performance characteristics of a direct PCR with stool samples in comparison to conventional techniques for diagnosis of *Shigella* and enteroinvasive *Escherichia coli* infection in children with acute diarrhoea in Calcutta. *India J. Med. Microbiol*. **50**, 667-674.

Elkins, K. M., Perez, A. C., Sweetin, K. C. (2016) Rapid and inexpensive species differentiation using a multiplex real-time polymerase chain reaction high-resolution melt assay. *Anal. Biochem.* **500**, 15-17.

FAO (2011) Preventing E. coli in Food. Available online: http://www.fao.org/fileadmin/user_upload/fcc/news/1_FAO_Preventing-E.Coli-inFood_FCC_2011.06.23.pdf (Accessed on 20 November 2017).

Gopaul, K. K., Sells, J., Lee, R., Beckstrom-Sternberg, S. M., Foster, J. T., Whatmore, A. M. (2014) Development and assessment of multiplex high resolution melting assay as a tool for rapid single-tube identification of five Brucella species. *BMC Res. Notes* **7**, 903.

Haase, H., Jordan, L., Keitel, L., Keil, C. (2017) Comparison of methods for determining the effectiveness of antibacterial functionalized textiles. *PloS One* **12**, e0188304.

Hanna, S. E., Connor, C. J., Wang, H. H. (2005) Real-time polymerase chain reaction for the food microbiologist: technologies, applications, and limitations. *J. Food Sci*. **70**, R49-R53.

Harrison, L. B., Hanson, N. D. (2017) High-resolution melting analysis for rapid detection of sequence type 131 *Escherichia coli. Antimicrob. Agents Chemother*. **61**, e00265.

Heid, C. A., Stevens, J., Livak, K. J., Williams, P. M. (1996) Real time quantitative PCR. *Genome Res*. **6**, 986-994.

Holland, J. L., Louie, L., Simor, A. E., Louie, M. (2000) PCR detection of *Escherichia coli* O157:H7 directly from stools: evaluation of commercial extraction methods for purifying fecal DNA. *J. Clin. Microbiol.* **38**, 4108-4113.

Ibekwe, A. M., Grieve, C. M. (2003) Detection and quantification of *Escherichia coli* O157:H7 in environmental samples by real-time PCR. *J. Appl. Microbiol.* **94**, 421-431.

Jasson, V., Jacxsens, L., Luning, P., Rajkovic, A. (2010) Alternative microbial methods: An overview and selection criteria. *Food Microbiol*. **27**, 710-730.

Kariuki, S., Gilks, C., Kimari, J., Obanda, A. (1999) Genotype analysis of *Escherichia coli* strains isolated from children

and chickens living in close contact. *Appl. Environ. Microbiol*. **65**, 472-476.

Kumar, N. S., Gurusubramanian, G. (2011) Random amplified polymorphic DNA (RAPD) markers and its applications. *Sci. Vis.* **11**, 116-124.

Marialouis, X. A., Santhanam, A. (2016) Antibiotic resistance, RAPD-PCR typing of multiple drug resistant strains of *Escherichia coli* from urinary tract infection (UTI). *J. Clin. Diagn. Res.* **10**, DC05.

Muse, W. B., Bender, R. A. (1992) Map position of the glnE gene from Escherichia coli. *J. Bacteriol*. **174**, 7876.

Packey, C. D., Shanahan, M. T., Manick, S., Bower, M. A. (2013) Molecular detection of bacterial contamination in gnotobiotic rodent units. *Gut Microbes* **4**, 361-370.

Persson, S., Olsen, K. E. P., Scheutz, F., Krogfelt, K. A. (2007) A method for fast and simple detection of major diarrhoeagenic *Escherichia coli* in the routine diagnostic laboratory. *Clin. Microbiol. Infect*. **13**, 516-524.

Raybourne, R. B. (1999) Flow cytometric detection of pathogenic E. coli in food. *Curr. Protoc. Cytom*. **8**, chapter 11, unit 11.6.

Rodríguez-Lázaro, D., Lombard, B., Smith, H., Rzezutka, A. (2007) Trends in analytical methodology in food safety and quality: monitoring microorganisms and genetically modified organisms. *Trends Food Sci. Technol*. **18**, 306-319.

Rwego, I. B., Gillespie, T. R., Isabirye-Basuta, G., Goldberg, T. L. (2008) High rates of *Escherichia coli* transmission between livestock and humans in rural Uganda. *J. Clin. Microbiol.* **46**, 3187-3191.

Salehi, T. Z., Madani, S. A., Karimi, V., Khazaeli, F. A. (2008) Molecular genetic differentiation of avian *Escherichia coli* by RAPD-PCR. *Braz. J. Microbiol.* **39**, 494-497.

Segura-Alvarez, M., Richter, H., Conraths, F. J., Geue, L. (2003) Evaluation of enzyme-linked immunosorbent assays and a PCR test for detection of Shiga toxins for Shiga toxin-producing *Escherichia coli* in cattle herds. *J. Clin. Microbiol*. **41**, 5760-5763.

Senapin, S., Molthathong, S., Phiwsaiya, K., Jaengsanong, C., Chuchird, N. (2010) Application of high resolution melt (HRM) analysis for duplex detection of *Macrobrachium rosenbergii* nodavirus (MrNV) and extra small virus (XSV) in shrimp. *Mol. Cell Probes*. **24**, 291-297.

Stefen, A., Scaramagli, S., Bergami, R., Mazzini, C. (2007) Real-time PCR and enzyme-linked fluorescent assay methods for detecting Shiga-toxin-producing *Escherichia coli* in mincemeat samples. *Can. J. Microbiol.* **53**, 337-342.

Tulsiani, S. M., Craig, S. B., Graham, G. C., Cobbold, R. C. (2010) High-resolution melt-curve analysis of random-amplified polymorphic DNA markers, for the characterisation of pathogenic *Leptospira*. *Ann. Trop. Med. Parasitol*. **104**, 151-161.

Vasavada, P. C. (1993). Rapid methods and automation in food microbiology: beyond Delphi forecast. *J. Rapid Methods Autom. Microbiol.* **2**, 1-7.

Wang, W., Zijlstra, R. T., Gänzle, M. G. (2017) Identification and quantification of virulence factors of enterotoxigenic *Escherichia coli* by high-resolution melting curve quantitative PCR. *BMC Microbiol*. **17**, 114.

WHO (2015) WHO's first ever global estimates of foodborne diseases find children under 5 account for almost one third of deaths. News release (media center), Geneva, Switzerland. Available online: http://www.who.int/mediacentre/news/releases/2015/foodborne-disease-estimates/en/ (Accessed on 20 November 2017).

WHO, UNICEF (2017) Progress on drinking water, sanitation and hygiene: 2017 update and SDG baselines. World Health Organization, New York, 2017.

Wu, S. M., Tian, Z. Q., Zhang, Z. L., Huang, B. H. (2010) Direct fluorescence in situ hybridization (FISH) in *Escherichia coli* with a target-specific quantum dot-based molecular beacon. *Biosens. Bioelectron*. **26**, 491-496.