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(54) **Method for detecting Escherichia coli**

Detektionsverfahren für Escherichia coli

Méthode pour détecter Escherichia coli

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(Cont. next page)

- CHEN J ET AL: "PCR DIFFERENTIATION OF ESCHERICHIA COLI FROM OTHER GRAM NEGATIVE BACTERIA USING PRIMERS DERIVED FROM THE NUCLEOTIDE SEQUENCES FLANKING THE GENE ENCODING THE UNIVERSAL STRESS PROTEIN" LETTERS IN APPLIED MICROBIOLOGY, OXFORD, GB, vol. 27, no. 6, December 1998 (1998-12), pages 369-371, XP008005257

Description

[0001] The present invention pertains to a method for detecting *Escherichia coli* in a sample, which comprises amplifying the nucleic acid contained in a sample therein with a set of nucleic acids as described herein and detecting an amplification product, wherein the formation of an amplification product is indicative of the presence of *Escherichia coli* in said sample. Moreover, the present invention also relates to the set of nucleic acids useful in the selective amplification of *E. coli* DNA.

[0002] Traditional methods of detecting microorganisms in a sample rely on time-consuming growth in culture media, followed by isolation and biochemical or serological identification. The entire process usually takes 24-48 hours. Many methods for rapid detection of microorganisms have recently been developed, including miniaturized biochemical analyses, antibody- and DNA-based tests, and modified conventional assays. However, one of the shortcomings of the methods presently available resides in that most of them are not capable to detect a variety of different *E. coli* strains, but are focused on specific ones only.

[0003] Detection of the microorganism *Escherichia coli* in water and food has been considered as an indicator of the possible presence of enteric pathogens. Indeed, certain *E. coli* strains are pathogenic themselves. Rapid and accurate identification of *E. coli* is therefore important for public health.

[0004] In consequence an object of the present invention resides in providing a rapid and reliable method for detecting the presence of *E. coli* in a sample obviating the prior art shortcomings.

[0005] This objective has been achieved by providing a specific nucleic acid sequences for detecting *Escherichia coli.* In consequence, according to one aspect, the present invention features a set of nucleic acids including a first nucleic acid that contains SEQ ID NO: 1 or 3 and a second nucleic acid that contains SEQ ID NO:2 or 4, each nucleic acid being 18-40 nucleotides in length. These nucleic acids can be used as PCR primers for detecting *E. coli.*

[0006] According to a preferred embodiment the first nucleic acid contains SEQ ID NO:1, the second nucleic acid contains SEQ ID NO:2, and each nucleic acid is 18-40 (e.g., 18-30) nucleotides in length. According to another preferred embodiment, the first nucleic acid contains SEQ ID NO:3, the second nucleic acid contains SEQ ID NO:4, and each nucleic acid is 24-40 (e.g., 24-32) nucleotides in length.

[0007] Yet, according to another preferred embodiment the nucleic acids are as identified by SEQ. ID. Nos: 1 to 4.

[0008] In another aspect, this invention features a nucleic acid obtained from amplification of an *Escherichia coli* nucleic acid template with an upstream primer containing SEQ ID NO:1 or 3 and a downstream primer containing SEQ ID NO:2 or 4, each primer being 18-40 nucleotides in length. The amplification product can be used as a hybridization probe for *E. coli* detection. According to an embodiment, the upstream primer contains or is SEQ ID NO:1, the downstream primer contains or is SEQ ID NO:2, and each primer is 18-40, preferably 18-30 nucleotides in length. According to another embodiment, the upstream primer contains or is SEQ ID NO:3, the downstream primer contains or is SEQ ID NO:4, and each primer is 24-40, preferably 24-32 nucleotides in length.

[0009] In yet another aspect, this invention features a nucleic acid that is 26-1000, preferably 26-500, 26-200, or 26-50 nucleotides in length containing any of SEQ ID NO:5, 6, 7, or 8, or its complementary sequences. The nucleic acid can simply be SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, or SEQ ID NO:8, or its complementary sequences. These nucleic acids can be used as hybridization probes for detecting *E. coli.*

[0010] Also, the present invention provides a method of detecting *Escherichia coli* using two or more of the nucleic acids described above. The method includes (1) providing a sample, e.g. a biological sample having a nucleic acid from an unknown microorganism; (2) amplifying the nucleic acid with an upstream primer containing SEQ ID NO: 1 or 3 and a downstream primer containing SEQ ID NO:2 or 4, each primer being 18-40 nucleotides in length; and (3) detecting an amplification product. Detection of an expected amplification product indicates the presence of *Escherichia coli.*

[0011] According to an embodiment the detecting step includes hybridizing the amplification product to a nucleic acid probe that is 26-1000 (e.g., 26-500, 26-200, or 26-50) nucleotides in length and contains SEQ ID NO:5, 6, 7, or 8, or its complementary sequence.

[0012] Further within the scope of this invention is a kit for detecting *E. coli.* The kit contains one or more of the nucleic acids described above. It may include other components such as a DNA polymerase, a PCR buffer, or a solid support on which one or more of the above-described probes are immobilized.

[0013] The present invention provides a fast, accurate, and sensitive method for *E. coli* detection. Specifically, a nucleic acid template from a sample suspected of containing *E. coli* is amplified with a pair of *E. coli*-specific primers. Non-limiting examples for such samples are food material or water or from a patient. The amplification product, if any, is detected by either gel electrophoresis and staining, e.g. by ethidium bromide, or by probe hybridization. Detection of an expected amplification product indicates the presence of *E. coli* in the sample.

[0014] The nucleic acid template can be DNA (e.g., a genomic fragment or a restriction fragment) or RNA, in a purified or unpurified form.

[0015] According to the present invention *E. coli*-pecific primers are provided selected from the nucleotide sequence between 81889 and 83238 of *E. coli* genome (GenBank Accession No. AP002562). This region contains three open reading frames (ORFs), i.e, ECs3458, ECs3459 and ECs3460, encoding three hypothetical proteins with unknown

functions. The present inventors have now found that the DNA sequence in this region is conserved in both pathogenic and non-pathogenic *E. coli* groups, which should render them a suitable means for detecting *E. coli* in general, i.e. comprising a variety of different strains.

[0016] In one of the selected primer pairs, the forward primer is a 24 oligonucleotide N1 (5'-TGAATGCGCAAGCT-GAAAAAGTAG-3'; SEQ ID NO:3; corresponding to nucleotides 82568-82591 of GenBank Accession No. AP002562), and the reverse primer is another 24 oligonucleotide N2 (5'-ACGCCGTTAGGTGTATTGATTGTG-3', SEQ ID NO:4, corresponding to a complementary sequence of nucleotides 83075-83052 of GenBank Accession No. AP002562).

[0017] In another selected primer pair, the forward primer (SEQ ID NO:1) is the 18 oligo-nucleotide located at the 3'-end of N1, and likewise, the reverse primer (SEQ ID NO:2) is the 18 oligo-nucleotide located at the 3'-end of N2. A search against GenBank indicates that these two primers are *E. coli*-specific.

[0018] In two additional examples, SEQ ID NO:1 is paired with SEQ ID NO:4, and SEQ ID NO:2 is paired with SEQ ID NO:3.

[0019] Typically, a primer is 14-40 nucleotides in length (PCR Application Manual, Boehringer Mannheim, 1995, page 37). In this invention, primers that contain SEQ ID NO:1, 2, 3, or 4, and have 18-40 (e.g., 18-32) nucleotides in length can be used to amplify an *E. coli* template. *E. coli* sequences can be added to either the 5'-end or the 3'-end of SEQ ID NO:1, 2, 3, or 4; non-*E. coli* sequences can be added to the 5'-end of SEQ ID NO:1, 2, 3, or 4. An example of a non-*E. coli* sequence is a sequence containing a restriction site, which can be used to facilitate cloning of the amplification product.

[0020] The present invention also features four *E. coli*-specific probes chosen from the region amplified with primers N1 and N2 described above.

(a) From the sense strand of the amplified nucleic acid sequence:

N1-1: 5'-AATACATAACAGAAACCTGAAACACAA-3' (SEQ ID NO:5), corresponding to nucleotides 82618-82644 of GenBank Accession No. AP002562; and
N1-2: 5'-AAAACACCTCTTCCTGCGATTTCTCAC-3' (SEQ ID NO:6), corresponding to nucleotides 82758-82784 of GenBank Accession No. AP002562.

(b) From the antisense strand of the amplified nucleic acid sequence:

N2-1: 5'-ATTTTACCTCTTGTCTTCCCGTCTTGG-3' (SEQ ID NO:7), which is a complementary sequence of nucleotides 82894-82868 of GenBank Accession No. AP002562; and
N2-2: 5'-GTTATGTATTGCTGCTGTTTGCGGCG-3' (SEQ ID NO:8), which is a complementary sequence of nucleotides 82626-82602 of GenBank Accession No. AP002562.

[0021] N1-1, N1-2, N2-1, N2-2, and longer probes that contain N1-1, N1-2, N2-1, or N2-2 and have 26-1000 (e.g., 10-500, 10-200, and 10-50) nucleotides in length can each be used for detecting *E. coli* by hybridizing to an non-amplified *E. coli* nucleic acid or an *E. coli* nucleic acid amplified with the above-described primer pairs. For instance, the amplification product described above is one of such probes. A search against GanBank indicates that the nucleic acid sequence amplified with primers N1 and N2 is *E. coli*-specific.

[0022] The probes can be immobilized on the surface of a solid support, such as a membrane (a nylon-membrane or a nitrocellulose membrane), a glass, or a plastic polymer. Immobilization of probes to a membrane can be achieved by conventional means, such as baking at 80°C or UV cross-linking. The probes can also be covalently linked to a material (e.g., poly-lysine) coated on the surface of a glass. In addition, a novel method of immobilizing probes on a plastic polymer has recently been developed. See US Application Serial No. 09/906,207. Alternatively, the probes can be synthesized *de novo* at precise positions on a solid substrate. See Schena *et al.*, 1995, *Science* 270: 467; Kozal *et al.*, 1996, *Nature Medicine* 2(7): 753; Cheng *et al.*, 1996, *Nucleic Acids Res.* 24(2): 380; Lipshutz *et al.*, 1995, *BioTechniques* 19(3): 442; Pease *et al.*, 1994, *Proc. Natl. Acad. Sci. USA* 91: 5022; Fodor *et al.*, 1993, *Nature* 364: 555; and Fodor *et al.*, WO 92/10092.

[0023] A target amplification product described above can be detected by binding it to an immobilized probe. To facilitate detection, a labeled amplification product can be generated with a labeled amplification primer. Alternatively, the labeling can be done, chemically or enzymatically, after amplification. Examples of labeling reagents include, but are not limited to, a fluorescent molecule (e.g., fluorescein and rhodamine), a radioactive isotope (e.g., ^{32}P and ^{125}I), a colorimetric reagent, and a chemiluminescent reagent. Biotin and digoxgenin are frequently used for colorimetric detection on a membrane or a plastic polymer. Fluorescent labels, such as Cy3 and Cy5, are widely used for detection on a glass. In addition, artificial tagging tails (e.g., a protein or its antibody) can be conjugated to the 5'-end of the primers or either end of the probes. See Stetsenko and Gait, 2000, *J. Org. Chem.* 65(16): 4900.

[0024] The specificity of the *E. coli* detection method of this invention is unexpectedly high. Only *E. coli* templates are amplified with the selected primers, and there is no amplification of nucleic acid templates prepared from other bacteria

such as *Salmonella spp., Shigella spp., Enterobacter aerogenes, Citrobacter freundii, Klebsiella pneumoniae, Staphylococcus aureus, Listeria monocytogenes, Vibrio parahaemolyticus, Bacillus cereus,* and *Streptococcus aglactiae* (see Example 1 below). Most unexpected is the ability of the selected primers to discriminate an *E. coli* template from a *Shigella spp*. template, which has not been previously achieved in the art (cf. Hellyer *et al.*, US-P-6,207,818). Moreover, amplification of an *E. coli* template is not affected by the presence of nucleic acid templates prepared from other bacteria (see Example 3 below) or contaminants in a crude sample (see Example 4 below).

[0025] The sensitivity of the *E. coli* detection method of this invention is also unexpectedly high. More specifically, 1 ng and even 1 fg of *E. coli* genomic DNA can be detected on an agarose gel after 20 and 30 cycles of amplification, respectively (see Example 2 below).

[0026] Hybridization, following PCR amplification, further increases the sensitivity by 5-50 folds (see Example 5 below). Indeed, according to the teaching of the present invention *E. coli* genomic DNA equivalent to an extract from 1 ml of a 1 cfu/ml culture may be detcted (see Examples 2 and 5 below).

[0027] Also within the scope of this invention is the use of *E. coli*-specific sequences described above in combination with other species-specific nucleic acid sequences for simultaneously identification of multiple microorganisms.

[0028] Furthermore, at positions where single nucleotide polymorphisms occur, nucleotide variations are allowed in primers and probes described in this invention. As single nucleotide polymorphisms may be associated with a particular genotype or phenotype, these primers and probes can be used to distinguish and categorize different *E. coli* strains.

[0029] The specific examples below are to be construed as merely illustrative, and not limitative of the remainder of the disclosure in any way whatsoever. Without further elaboration, it is believed that one skilled in the art can, based on the description herein, utilize the present invention to its fullest extent.

Example 1:

Amplification and detection of *Escherichia coli* using specific oligo-nucleotide primers

(1) Bacteria strains

[0030] All bacterial strains used in this example are listed in Table 1 below. *Escherichia coli* strains used include non-pathogenic and pathogenic subtypes, such as ETEC, EIEC, EPEC, EAggEC and EHEC strains. Non-*E. coli* bacteria tested include some coliform bacteria and common food-borne pathogen bacteria. These bacterial strains were obtained from different sources, such as Culture Collection and Research Center (CCRC), Hsin-Chu, Taiwan; National Laboratory for Food and Drugs (NLFD), Taipei, Taiwan; American Type Culture Collection (ATCC), Rockville, MD, USA; United States Department of Agriculture (USDA), Washington, DC, USA; Center of Vaccine Development (CVD), University of Maryland, Baltimore, MD, USA; and Pingtung University of Technology (PT), Pingtung, Taiwan.

[0031] One loop of test strains were plated on Luria-Bertani agar (LB; 0.5% yeast extract, 1% trypton, 0.5% NaCl, 1.5-2% agar) and incubated for overnight (14 hr) at 37°C. A single colony was picked and inoculated into 10 ml sterilized LB broth. The culture was incubated for overnight at 37°C. Colony forming unit (cfu) was calculated after serial dilutions, and bacterial genomic DNA was prepared as described below.

(2) Preparation of bacterial genomic DNA

[0032] Genomic DNA was prepared from 1 ml of bacterial overnight culture. Cells were harvested by centrifugation at 6,000g for 5 minutes, and were resuspended in 50 ml STET buffer (0.1 M NaCl; 10 mM Tris-HCl, pH 8.0; 1 mM EDTA; 0.05 mg lysozyme; and 5% Triton X-100). Cells were subsequently lysed after incubation for 15 minutes at 37°C followed by boiling for 10 minutes. DNA-containing supernatant was roughly separated from cell debris after 5 minutes of centrifugation. Genomic DNA was further purified by treatment with phenol/chloroform (1:1), and centrifugation at 10,000g for 10 minutes. The upper layer of the extraction mixture, ca. 40 ml, was transferred into a new Eppendorf tube and was ready to be amplified.

(3) Amplification and detection of bacterial genomic DNA with specific oligonucleotide primers

[0033] 50 μl of amplification reaction mixture contains 5 ml 10X Taq DNA polymerase buffer, 5 ml 25 mM $MgCl_2$, 4 ml 2.5 mM dNTPs (Promega, Madison, WI, USA),

Table 1

Bacterial strains		
Number of Strains	Strain	Strain No. and Source
8	Non-pathogenic *E.coli*	FP2-27 (CCRC11509), FP3-27, FP3-28 (ATCC25922), FP3-29 (ATCC11775), FP3-30, FP3-31, FP3-32, FP3-33
5	Enteroaggregative *E. coli* (EAggEC)	FP2-32 (TVGH), FP2-33 (CVD), FP2-34 (CVD, O86: H2), FP2-35 (TVGH2), FP2-36 (TVGH)
2	Enteroinvasive *E. coli* (EIEC)	*E. coli* O124:NM (CCRC15375), *E. coli* O164:H (CVD)
37	Enterohemoehagic *E. coli* (EHEC)	FP1-37 (CCRC14825), FP1-40 (CCRC15373), FP1-41 (CCRC14824), FP1-42 (CCRC13089), FP3-9 (CCRC14815), FP3-10 (CCRC13084), FP3-11 (CCRC13086), FP3-12 (CCRC35150), FP3-13 (CCRC43890), FP3-14 (CCRC13093), FP3-15 (CCRC13094), FP3-16 (CCRC13095), FP3-17 (CCRC13096), FP3-18 (CCRC13097), FP3-19 (CCRC13098), FP3-20 (CCRC13099), FP2-45 (NLFD I20), FP2-46 (NLFD I61), FP2-47 (NQS317), FP2-48 (USDA014-90), FP2-49 (USDA115-93), FP2-50 (USDA028-00), FP3-1 (USDA177-93), FP3-2 (USDA042-91), FP3-3 (USDA037-90), FP3-4 (USDA45750), FP3-5 (USDA45753), FP3-6 (USDA45756), FP3-7 (USDA54a-7), FP3-8 (USDAAMF1847), FP3-9 (USDA008-90), FP3-26 (USDA012-89), FP3-21, FP3-22, FP3-23, FP3-24, FP3-25 (USDA008-90)
5	Enteropathogenic *E. coli* (EPEC)	FP1-35 (CCRC15530), FP1-36 (CCRC15536), FP2-38 (CVD), FP2-39 (CVD), FP2-40 (NQS)
7	Enterotoxigenic *E. coli* (ETEC)	FP1-38 (CCRC15372), FP2-25 (CCRC15370), FP2-26 (CCRC15371), FP2-41 (CCRC41443), FP2-42 (WHO103), FP2-43 (WHO110), FP2-44 (WHO112)

Table continued

Bacterial strains		
Number of Strains	Strain	Strain No. and Source
56	*Salmonella sp.*	FP1-23 (*typhi* , CCRC14875), FP1-24 (*typhimurium* , CCRC10747), FP1-25 (*salamae* , CCRC15450), FP1-26 (*typhimurium* , CCRC10248), FP1-27 (*paratyphi* A, CCRC14878), FP1-28 (*typhimurium* , CCRC12947), FP1-29 (*california* , CCRC15454), FP1-30 (*enteritidis* ,), FP1-32 (*paratyphi* B, CCRC14897), FP1-33 *(etterbeele,* CCRC15455), FP1-34 (*postsdam* . CCRC15433), FP3-34 (*aberdeen* , US), FP3-35 (*adelaide* , US), FP3-36 (*albany* , USDA), FP3-37 (*amager* , US), FP3-38 (*anatum* , PT), FP3-39 (*bareilly* , USDA), FP3-40 (*berta* , US), FP3-41 (*california* , US), FP3-42 *(cerro,* USDA671D), FP3-43 (*cerro* , USDA), FP3-44 (*chester* , USDA), FP3-45 (*coleypark* , US), FP3-46 (*crossness* , US), FP3-47 (*cubana* , USDA), FP3-48 (*djakarta* , US), FP3-49 (*drypool* , USDA607E), FP3-50 *(dublin,* US), FP4-1 (*dugbe* , US), FP4-2 (*enteritidis* , ATCC13076), FP4-4 (*emek* , US), FP4-5 (*eppendorf* , PT633), FP4-6 (*florida* , US), FP4-7 (*hartford* , USDA), FP4-8 (*havana* , US), FP4-9 (*hvttingfoss* , USDA), FP4-10 (*hvttingfoss* , US), FP4-11 (*infantis* , US), FP4-12 (*java* , US), FP4-13 (*kentuky* , US), FP4-14 (*litchfield* , US), FP4-15 *(london,* PT1004), FP4-16 (*miami* , US), FP4-17 (*munster* , PT1014) FP4-18 (*newbrunswick* , US), FP4-19 *(newington,* USDA), FP4-20 *(newwington,* USDA), FP4-21 (*newport* , US), FP4-22 (*ohio* , PT1007), FP4-23 (*panama* , US), FP4-24 (*pomona* , USDA), FP4-25 (*Poona* , USDA), FP4-26 (*taksony* , USDA1121D), FP4-27 (*thomasville* , USDA1101E), FP1-31 (*typhi* , CCRC10746), FP4-3 *(enteritidis,* US)
1	*Staphylococcus aureus*	FP1-1 (CCRC10780)
4	*Shigella sp.*	FP2-18 (*dysenteria* , CCRC13983), FP2-19 (*boydii* , CCRC15961), FP2-20 (*flexneri* , CCRC10772), FP2-21 *(sonnei,* CCRC10773)
1	*Streptococcus agalactiae*	FP1-43 (CCRC10787)
		FP2-16 (CCRC11827)
1	*Vibrio parahaemolyticus*	FP2-22 (CCRC10806)
1	*Listeria monocytogenes*	FP2-24 (CCRC14930)
1	*Enterobacter aerogenes*	FP2-29 (CCRC10370)
1	Citrobacter freundii	FP2-27 (CCRC 12291)
1	Klebsiella pneumoniae	FP2-30 (CCRC 15627)

[0034] 1 μl 20 μM of oligonucleotide primer N1, 1 μl 20 μM of oligonucleotide primer N2, 1 μl DNA template, 0.1 U of Taq DNA polymerase (Promega, Madison, WI, U.S.A.), and sterilized dH_2O.

[0035] Amplification was carried out using Peltier-effect Thermal Cyclers (PTC-100, MJ Research Inc., MA, U.S.A.) as follows: 95°C for 2 minutes; 30 cycles of 95°C for 40 sec, 55 °C for 40 sec, 72 °C for 40 sec; and a final extension at 72 °C for 6 minutes.

[0036] All amplified products (50 μl) were analyzed by electrophoresis on a 2% agarose gel in TAE buffer (40 mM

Tris, 20 mM sodium acetate, 2 mM EDTA, pH adjusted with glacial acetic acid) and stained with ethidium bromide.

[0037] The experimental results, summarized in Table 2 below, show that the selected oligo-nucleotide primers are highly specific for detecting *E. coli.* The expected amplified product (molecular weight of 500 bp) could only be detected for all 64 *E. coli* species, including 8 non-pathogenic and 56 pathogenic subtypes or serotypes. No amplification product was observed for the other 68 bacterial strains. More specifically, there was no cross reaction between *E. coli* and the 4 *Shigella* spp. strains tested, which is unexpected from previously described PCR-gel-based methods.

Example 2:

Detection sensitivity of PCR-gel analysis

(1) Amount of bacterial genomic DNA required for 30 PCR thermal cycles

[0038] Detection sensitivity of the PCR-gel analysis method was determined by titrating the amount of genomic DNA required for amplification. *E. coli* DNA was extracted, purified using QIAamp DNA mini Kit (QIAGEN, Hilden, Germany). Quantification of the purified genomic DNA was performed by electrophoretic agarose gel method. DNA marker (Gene Mark, Taiwan, R.O.C.) loaded on the same agarose gel was used as a reference for quantification. All components except the DNA template in the amplification reaction mixture were the same as described in Example 1, section 3. The amount of genomic DNA tested was in the range of 100 ng to 0.1 fg. Unexpectedly, as low as 1 fg of *E. coli* genomic DNA was detected after amplification using the selected oligo-nucleotide primer pair.

(2) Amount of bacterial genomic DNA required for 20 PCR thermal cycles

[0039] Less cycle number for amplification reaction means less time needed for *E. coli* detection. Twenty amplification cycles could approximately save 30 minutes than thirty amplification cycles, and the detection sensitivity might be high enough for the purpose of

Table 2

***Escherichia coli* detection using specific oligonucleotide primers**			
Bacterial strains	Number of strains tested	Number of PCR positive strains	Number of PCR negative strains
Escherichia coli			
Non-pathogenic *E. coli*	8	8	0
Enteroaggregative *E. coli* (EAggEC)	5	5	0
Enterotoxigenic *E. coli* (ETEC)	7	7	0
Enterohemorrhagic *E. coli (EHEC)*	37	37	0
Enteropathogenic *E. coli* (EPEC)	5	5	0
Enteroinvasive *E. coli* (EIEC)	2	2	0
Shigella sp.			
Shigella dysenteria	1	0	1
Shigella boydii	1	0	1
Shigella flexneri	1	0	1
Shigella sonnei	1	0	1
Staphylococcus aureus	1	0	1

Table continued

***Escherichia coli* detection using specific oligonucleotide primers**			
Bacterial strains	Number of strains tested	Number of PCR positive strains	Number of PCR negative strains
Streptococcus agalactea	1	0	1
Bacillus cereus	1	0	1
Vibrio parahaemolyticus	1	0	1
Listeria monocytogenes	1	0	1
Citrobacter freundii	1	0	1
Klebsiella pneumoniae	1	0	1
Enterobacter aerogenes	1	0	1
Salmonella sp.			
Sal. typhi	2	0	2
Sal. typhimisum	3	0	3
Sal. salamae	1	0	1
Sal. paratyphi	2	0	2
Sal. California	2	0	2
Sal. enteritidis	3	0	3
Sal. etterbeele	1	0	1
Sal. postsdam	1	0	1
Sal. aberdeen	1	0	1
Sal. albany	1	0	1
Sal. amager	1	0	1
Sal. anatum	1	0	1
Sal. bareilly	1	0	1
Sal. berta	1	0	1
Sal. cerro	2	0	2
Sal. Chester	1	0	1
Sal. coleypark	1	0	1
Sal. crossness	1	0	1
Sal. cubana	1	0	1
Sal. djakarta	1	0	1
Sal. drypool	1	0	1

Table continued

***Escherichia coli* detection using specific oligonucleotide primers**			
Bacterial strains	Number of strains tested	Number of PCR positive strains	Number of PCR negative strains
Sal. dublin	1	0	1
Sal. dugbe	1	0	1
Sal. emek	1	0	1
Sal. eppendorf	1	0	1
Sal. florida	1	0	1
Sal. hartford	1	0	1
Sal. Havana	1	0	1
Sal. hvittingfoss	2	0	2
Sal. infantis	1	0	1
Sal. java	1	0	1
Sal. kentucky	1	0	1
Sal. litchfield	1	0	1
Sal. london	1	0	1
Sal. miami	1	0	1
Sal. munster	1	0	1
Sal. newbrunswicks	1	0	1
Sal. newington	2	0	2
Sal. newport	1	0	1
Sal. ohio	1	0	1
Sal. panama	1	0	1
Sal. pomona	1	0	1
Sal. poona	1	0	1
Sal. thomasville	1	0	1
Sal. adelaide	1	0	1
Sal. taksony	1	0	1

[0040] *E. coli* detection in food industry. Therefore, we tried to determine the detection limits of genomic DNA and

vital cells (cfu, see section 3 below) required for 20 amplification cycles.

[0041] Amplification was carried out as described in section 1 of this example, except that 20 amplification cycles were applied instead of 30 cycles. The amount of genomic DNA tested was in the range of 100 ng to 0.1 fg. Unexpectedly, as low as 1 ng of *E. coli* genomic DNA was detected.

(3) Number of bacterial cells required for 20 PCR thermal cycles

[0042] An overnight culture of pathogenic *E. coli* O157:H7 (H type, CCRC 14825) was grown in LB broth at 37°C, and the cell concentration, colony forming unit/ml (cfu/ml), was determined. Genomic DNA was extracted from 1 ml of a 10^9 cfu/ml culture, and was serially diluted to concentrations equivalent to extracts from 1 ml of 0-10^7 cfu/ml cultures. Amplification was carried out as described in section 2 of this example. Unexpectedly, *E. coli* DNA equivalent to an extract from 1 ml of a 1 cfu/ml culture was detected.

Example 3

Detection of Escherichia coli in a bacterial mixture

[0043] Bacterial strains used to make a bacterial mixture include: *Staphylococcus aureus* (CCRC10780), *Salmonella typhimurium* (CCRC10747), *Escherichia coli* (CCRC14825), *Streptococcus agalactea* (CCRC10787), *Bacillus cereus* (CCRC11827), *Shigella dysenteria* (CCRC13983), *Vibrio parahaemolytticus* (CCRC10806), and *Listeria monocytogenes* (CCRC14930). A single colony was picked from each strain, and was inoculated into 5 ml LB broth for overnight growth at 37°C with shaking at 100 rpm. Genomic DNA was prepared and amplified as described in Example 1. An expected amplification product (500 bp) was only detected on an agarose gel when *E. coli* was present in the mixture. DNA from other bacterial species did not affect the sensitivity and specificity of the selected amplification primer pair.

Example 4

Detection of Escherichia coli in a milk sample

[0044] Whole milk samples were purchased from a local supermarket and were pasteurized. The pathogenic *E. coli* O157:H7 (H type, CCRC 14825) culture was inoculated in a concentration of 10^6 cfu/ml, and centrifuged at 10,000g for 5 minutes. Genomic DNA was extracted and serially diluted to the concentrations equivalent to extracts from 1 ml of 0-10^6 cfu/ml cultures. Amplification was carried out as described in Example 2, section 2.

[0045] The expected amplification product (500 bp) was detected on an agarose gel. The sensitivity and specificity of the selected amplification primer pair were not affected by the simple DNA preparation method, or by the presence of other microorganisms and cattle somatic cells in milk.

Example 5

Detection of Escherichia coli by hybridization after amplification

(1) Probe design

[0046] Four types of oligo-nucleotide probes were designed for hybridization.

Type 1: Four oligonucleotides (i.e., N1-1, N1-2, N2-1, and N2-2) were chosen from the region amplified with *E. coli*-specific oligo-nucleotide primers N1 and N2.

Type 2: A DNA probe for positive control of hybridization.

Pco: 5'-$(T)_{25}$-GAGCGGGAAATCGTGCGCGACATCAAGGAG-3'

(SEQ ID NO:9)

Type 3: DNA probes for negative control of hybridization, which can be any non-complementary sequences against the target nucleic acid and have around 25 bases.

Nco1: 5'-$(T)_{25}$-ATGAAGCAYGTCAGGGCRTGGATACCTCG-3'

(SEQ ID NO:10)

Nco2: dH_2O

Type 4: An orientation probe, a short oligo-nucleotide with biotin labels at the 5'-end. The sequence is 5'-GTAATAC-GACTCACTATAGGGC-3' (SEQ NO:11).

(2) Hybridization

[0047] Each oligonucleotide probe was dissolved in a probe solution (Dr. Probsol, Dr.Chip Biotechnology Inc., Taiwan) to a final concentration of 10 μM, spotted, and immobilized on a solid substrate (Dr. Chip Biotechnology Inc., Taiwan). Amplification was carried out as described in Example 1, except that both primers were labeled with biotin at the 5'-end. The amplification reaction mixture was diluted with a hybridization buffer in a ratio of 1:(50-100). The diluted mixture was boiled for 5 minutes, chilled on ice, and applied to the solid support. Hybridization was performed at 50-55°C for 1-2 hours in an oven. The solid support was then washed with a wash buffer (0.5 ml) (DR. Wash from Dr.Chip Biotechnology Inc., Taiwan) for at least three times. Biotin-specific colorimetric detection was performed by incubating the solid substrate in a Blocking Reagent (Roche) containing alkaline phosphatase-conjugated streptavidin (Promega). The solid substrate was subsequently washed three times with the wash buffer, and incubated with NBT/BCIP solution (Roche) diluted with a detection buffer in a ratio recommended by the supplier for about 10 minutes in dark. Colored type 1 probe spots indicate the presence of an amplification product from *E. coli* genomic DNA. Type 2 probes were stained in all hybridization reactions. No signal was detected from type 3 probes.

(3) Detection specificity

[0048] Genomic DNA isolated from *E. coli,* 8 other bacterial strains, and a mixed bacterial culture containing *E. coli* were amplified and hybridized to the probes. Only samples containing *E. coli* DNA template resulted in colored spots on the solid support. Probe N2-1 showed the highest signal intensity of all type 1 probes under hybridization conditions described above. No signal was detected for the 8 other bacterial samples.

(4) Detection sensitivity

[0049] One-fifth (1/5) volume of the amplification mixture was sufficient for hybridization analysis. *E. coli* DNA templates equivalent to extracts from 1 ml of 10^0-10^7 cfu/ml cultures were amplified and hybridized to the probes. The amount of DNA that could be detected was equivalent to an extract from 1 ml of a 1 cfu/ml culture. Furthermore, 0.2 ng and 0.02 fg *E. coli* genomic DNA was detected after 20 and 30 cycles of amplification, respectively. Therefore, hybridization analysis is, unexpectedly, 5 - 50 times more sensitive than analysis on an ethidium bromide-stained agarose gel.

[0050] From the above description, one skilled in the art can make various changes and modifications of the invention to adapt it to various usages and conditions. Thus, other embodiments are also within the scope of the following claims.

SEQUENCE LISTING

[0051]

<110> DR. Chip Biotechnology Incorporation

<120> METHOD FOR DETECTING ESCHERICHIA COLI

<130> 51021

<150> US 10/025,137

<151> 2001-12-19

<160> 11

<170> PatentIn version 3.1

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<211> 22

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<213> Artificial Sequence

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<223> probe

<400> 11
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Claims

1. A set of nucleic acids comprising:

   a first nucleic acid containing a sequence selected from SEQ ID NO:1 or 3, and
   a second nucleic acid containing a sequence selected from SEQ ID NO:2 or 4,

   wherein each nucleic acid is 18-40 nucleotides in length.

2. The set of nucleic acids of claim 1, wherein the first nucleic acid contains SEQ ID NO: 1 and the second nucleic acid contains SEQ ID NO:2, or wherein the first nucleic acid contains SEQ ID NO:3 and the second nucleic acid contains SEQ ID NO:4.

3. The set of nucleic acids of claim 1 or 2, wherein each nucleic acid is 18-30 or 24-32 nucleotides in length.

4. A nucleic acid obtained from amplification of an *Escherichia coli* nucleic acid template with a set of nucleic acids according to any of the preceding claims or fragment thereof, wherein said nucleic acid or fragment thereof is 26-1000 nucleotides in length comprising a sequence selected from the group consisting of SEQ ID NOs:5-8, or a sequence complementary thereto.

5. The nucleic acid of claim 4, wherein said nucleic acid is 26-500 nucleotides, preferably 26-200 nucleotides, more preferably 26-50 nucleotides in length.

6. A method of detecting *Escherichia coli* in a sample, comprising:

   providing a sample;
   amplifying the nucleic acid contained therein with a set of nucleic acids according to any of the claims 1 to 3; and
   detecting an amplification product;

   whereby detection of an amplification product indicates the presence of *Escherichia coli.*

7. The method according to claim 6, wherein the detecting step includes hybridizing the amplification product to a nucleic acid probe that is 26-1000 nucleotides in length and contains a sequence selected from the group consisting of SEQ ID NOs:5-8, or a sequence complementary thereto.

8. The method of claim 7, wherein said nucleic acid probe is 26-50 nucleotides in length.

9. Use of a nucleic acid according to any of the claims 1 to 3 for the detection of *E. coli.*

10. A kit for detecting the presence of *E. coli* in a sample, which comprises a set of nucleic acids according to any of the claims 1 to 3; and buffers, enzymes or a support, suitable for the detection step.

Patentansprüche

1. Satz von Nukleinsäuren, welcher umfasst:

   eine erste Nukleinsäure, welche eine Sequenz enthält, die ausgewählt ist, aus SEQ ID NO: 1 oder 3, und
   eine zweite Nukleinsäure, welche eine Sequenz enthält, die ausgewählt ist, aus SEQ ID NO: 2 oder 4,

   wobei jede Nukleinsäure eine Länge von 18 - 40 Nukleotiden aufweist.

2. Satz von Nukleinsäuren nach Anspruch 1, worin die erste Nukleinsäure SEQ ID NO: 1 enthält und die zweite Nukleinsäure SEQ ID NO: 2 enthält, oder die erste Nukleinsäure SEQ ID NO: 3 enthält und die zweite Nukleinsäure SEQ ID NO: 4 enthält.

3. Satz von Nukleinsäuren nach Anspruch 1 oder 2, worin jede Nukleinsäure eine Länge von 18 - 30 oder 24-32 Nukleotiden aufweist.

4. Nukleinsäure, die erhalten wird, durch Amplifikation eines *Escherichia coli* Nukleinsäure-Templats mit einem Satz von Nukleinsäuren nach einem der vorstehenden Ansprüche, oder ein Fragment hiervon,
   worin die Nukleinsäure oder das Fragment hiervon eine Länge von 26 - 1000 Nukleotiden aufweist, umfassend eine Sequenz ausgewählt aus der Gruppe, bestehend aus SEQ ID NO: 5 - 8, oder eine hierzu komplementäre Sequenz.

5. Nukleinsäure nach Anspruch 4, worin die Nukleinsäure aufweist, eine Länge von 26 - 500 Nukleotiden, vorzugsweise von 26 - 200 Nukleotiden, bevorzugterweise von 26 - 50 Nukleotiden.

6. Verfahren zum Nachweis von *Escherichia coli* in einer Probe, welches umfasst:

   Bereitstellen einer Probe;
   Amplifizieren der darin enthaltenen Nukleinsäure mit einem Satz von Nukleinsäuren nach einem der Ansprüche 1 bis 3; und
   Nachweisen eines Amplifikationsprodukts;

   wobei ein Nachweis eines Amplifikationsprodukts das Vorliegen von *Escherichia coli* anzeigt.

7. Verfahren nach Anspruch 6, bei dem der Nachweisschritt umfasst, Hybridisieren des Amplifikationsprodukts mit einer Nukleinsäuresonde, welche eine Länge von 26 - 1000 Nukleotiden aufweist und enthält, eine Sequenz ausgewählt aus der Gruppe, bestehend aus SEQ ID NO: 5 - 8, oder eine hierzu komplementäre Sequenz.

8. Verfahren nach Anspruch 7, bei dem die Nukleinsäuresonde eine Länge von 26 - 50 Nukleotiden aufweist.

9. Verwendung einer Nukleinsäure nach einem der Ansprüche 1 bis 3 für den Nachweis von *E. coli.*

10. Kit zum Nachweisen des Vorliegens von *E. coli* in einer Probe, welcher umfasst einen Satz von Nukleinsäuren nach einem der Ansprüche 1 bis 3; und Puffer, Enzyme oder einen Träger, die (der) für den Nachweisschritt geeignet sind (ist).

Revendications

1. Jeu d'acides nucléiques comprenant:

   un premier acide nucléique comprenant une séquence choisie parmi SEQ ID NO: 1 ou 3, et
   un second acide nucléique comprenant une séquence choisie parmi SEQ ID NO: 2 ou 4,

chaque acide nucléique ayant une longueur de 18 - 40 nucléotides.

2. Jeu d'acides nucléiques selon la revendication 1, le premier acide nucléique comprenant SEQ ID NO: 1 et le second acide nucléique comprenant SEQ ID NO: 2, ou le premier acide nucléique comprenant SEQ ID NO: 3 et le second acide nucléique comprenant SEQ ID NO: 4.

3. Jeu d'acides nucléiques selon la revendication 1 ou 2, chaque acide nucléique ayant une longueur de 18 - 30 ou 24-32 nucléotides.

4. Acide nucléique obtenu d'une amplification d'une matrice d'acide nucléique d'*Escherichia coli* avec un jeu d'acides nucléiques selon l'une quelconque des revendications précédentes, ou un fragment de celui-ci,
ledit acide nucléique ou fragment de celui-ci ayant une longueur de 26 - 1000 nucléotides, comprenant une séquence choisie parmi le groupe constitué par SEQ ID NO: 5 - 8, ou une séquence complémentaire de celle-ci.

5. Acide nucléique selon la revendication 4, ladite acide nucléique ayant une longueur de 26 - 500 nucléotides, de préférence de 26 - 200 nucléotides, de façon davantage préférée de 26 - 50 nucléotides.

6. Procédé de décèlement d'*Escherichia coli* dans un échantillon, comprenant:

mise à disposition d'un échantillon;
amplification de l'acide nucléique contenu là-dedans avec un jeu d'acides nucléiques selon l'une quelconque des revendications 1 à 3; et
décèlement d'un produit d'amplification;
un décèlement d'un produit d'amplification indiquant la présence d'*Escherichia coli.*

7. Procédé selon la revendication 6, dans lequel l'étape de décèlement comprend l'hybridation du produit d'amplification avec une sonde d'acide nucléique ayant une longueur de 26 - 1000 nucléotides et comprenant une séquence choisie parmi le groupe constitué par SEQ ID NO: 5 - 8 ou une séquence complémentaire de celle-ci.

8. Procédé selon la revendication 7, dans lequel la sonde d'acide nucléique a une longueur de 26 - 50 nucléotides.

9. Utilisation d'un acide nucléique selon l'une quelconque des revendications 1 à 3 pour le décèlement d'*E. coli*.

10. Nécessaire pour le décèlement de la présence d'*E. coli* dans un échantillon, comprenant
un jeu d'acides nucléiques selon l'une quelconque des revendications 1 à 3; et des tampons, des enzymes ou un support approprié(s) pour l'étape de décèlement.