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International
Journal
Of Modern Engineering Research (IJMER)

Effect of Hybervariable Regions of 16S rRNA Gene on *In Silico* Microarray for Simultaneous Detection ofCommon Foodborne Pathogens

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ABSTRACT: *To ensure food safety, detected by microarray method is one of the most trending approaches in recent years. 16S ribosomal RNA gene is one of the most common genes using in identification bacteria and detection major foodborne pathogens. In this paper, we analyzed the effect of variable regions of 16S rRNA gene and the combination between them on simultaneous detect foodbone pathogens. We selected specific probes and design primers from the suitable regions for microarray simultaneous detection 15 major foodborne pathogens purpose. In the output, we confirm that 16S ribosomal RNA could distinguish 9 pathogens to species level (including Clostridium perfringens, Clostridium botulinum, Campylobacter jejuni, Escherichia coli, Vibrio cholerae, Vibrio vulnificus, Listeria monocytogenes, Staphylococcus aureus and Yersinia enterocolitica). The groups of pathogens which could be differentiated to below genus level but still upper species level were Bacillus cereus group, Campylobacter jejuni/Campylobacter coli group and Yersinia enterolitica/ Yersinia pseudotuberculosis group. Other pathogens which could not be distinguished to genus level or species level due to their high similarities with their related organisms are Salmonella, Shigella and Vibrio parahaemolyticus. This study also confrimed that the combination respectively between V1,V and V3 regions and between V6,V7 and V8 regions were the best options for foodborne pathogens detection purpose. 35 specific probes were chosen and 2 pairs of primers were designed in the purpose to apply to detect and identify foodborne pathogens in practical situations.*
Keywords: *food borne pathogens, microarray, insilico, 16S rRNA.*

I. Introduction

According to U.S Food and Drug Administration (FDA), regularity foodborne pathogens are *Escherichia coli*, *Salmonella* spp., *Shigella* spp., *Campylobacter* spp., *Yersinia enterocolitica*, *Yersinia pseudotuberculosis*, *Vibrio cholerae*, *Vibrio parahaemolyticus, Vibrio vulnificus*, *Listeria monocytogenes*, *Staphylococcus aureus*, *Bacillus cereus*, *Clostridium perfringens* and *Clostridium botulinum* [1].

Currently, pathogens in food are primary detected by culture-based isolations and biochemical identifications methods. These methods have relied on chemical and immunological characteristics of bacteria itself and needs 5-7 days to complete. In addition, conventional methods sometimes lack specificity and some bateria were known with weak toxigenicity in the past now have became foodborne pathogens which need to be removed out of food. Other bacteria are assumed to have a possibility to alter their biological characteristics [2, 3]. These reasons make foodborne pathogens discriminated by conventional methods may inefficient and advantages methods are required to ensure the quality of food product and eliminated threat of food poisoning caused by pathogenic bacteria.

The advents of genetic-based technologies make foodborne pathogens detection more specific and sensitive than ever before. One of most important steps is the occurrence and development of Polymerase chain reaction techniques (PCR). Some of these applied techniques have been accepted as standard methods for identification foodborne pathogens. However, one of the major drawbacks of PCR techniques is the limitation of quantity of bacteria contamination in food can be detected by single reaction [4]. Eventhought multiplex PCR have an abilitiy to amplify various targets, this technique is still limited due to the low resolution of agarose gels in traditional PCR or the limited choices of fluorescent detectors in real-time PCR. In overview, there’s still an inefficient ways in detection multiple foodborne pathogens potentially comminated in a large amount of foods [5].

In recent years, the development ofmany kinds of microarrray techniques provide opportunities to simultaneos detection foodborne pathogens with high accuracy and sensitively in just only two major steps: (1) Amplifying samples in PCR reaction with universal primers, specific primers or universal and specific primers mixed; (2) Hybridization PCR product with specific probes selected seperately for each targets. Consequently,

in theoretical, these techniques are one of the most trending approaches in multiple detection foodborne pathogens [3]. Selecting accordant gene sequences for primer design and specific probes selection is one of the most essentional factors which affect the success possibility of microarray techniques. 16S ribosomal gene sequence is considered as a new standard in detection and identification bacteria. Databases about this gene is relatively sufficient whereas other candidate regions like 16-23S spacer region have big number of mutations but this region is too short to identify some bacteria to species level or 23S rRNA has more mutation rates than 16S rRNA but information about this gene has not completed yet [6, 7].

Due to Soumitesh Chakravorty *et al.* (2007), 16S rRNA gene consists 9 hypervariable region (V) which can be used to distingush different bacteria [7]. Some researchs applied these regions into simultaneous detection foodborne pathogens. Chiang *et al*. (2006) designed universal primers to amplify from V1 to V3 regions and select 15 specififc probes to detect *Bacillus* spp., *Escherichia coli* spp., *Salmonella* spp., *Staphylococcus* spp. and *Vibrio* spp. 179/182 randomly selected strains was successfully identified with non-specific cross-reaction [8]. Eom *et al.* (2007) chose V1 regionsto multiply detect 8 pathogens includes: *Escherichia coli* K12, *Shigella dysenteriae*, *Salmonella typhimurium*, *Salmonella enteritidis*, *Vibrio cholerae*, *Vibrio vulnificus*, *Vibrio parahaemolyticus* and *Campylobacter jejuni*. The results shown that V1 was the most suitable region to differentate species of *Vibrio* genus [9].Wang *et al.* (2007) combinated between ampifly and select specific probes from V8 to V9 regions with invA and virA, whichrespectively represents for *Shigella* spp. and *Samonella* spp. to detect 22 common foodborne pathogens. In output, 112/115 strains (97,4 %) were correctly identified, the sensitive of the method archived 10^2 CFU/ml for *E. coli* contiminated samples [10]. Jiang Ming-Xing *et al.* (2009) used the combination of PCR with reverse dot blot techniques to test 540 fecal samples, 2 universal primer was designed to ampliflied variables regions of 16S and 23S rRNA of 12 pathogenic bacteria inclules *Salmonella* spp., *Brucella* spp., *Escherichia coli* O157:H7, *Clostridium botulinum*, *Bacillus cereus, Clostridium perfringens*, *Vibrio parahaemolyticus*, *Shigella* spp., *Yersinia enterocolitica*, *Vibrio cholerae, Listeria monocytogenes* and *Staphylococcus aureus*. In results, 12 above pathogenic bacteria can distingushed with the detection limit were 103 CFU and the consistent rate compared with the traditional culture method was up to 88.75% [11].

Goal of this study is building phylogenic tree, selecting specific probe and design universal primer to revaluated the possibility of variable regions of 16S rRNA and the combination between them to *in silico* microarray forstimultaneous detection of 15 common foodborne pathogens according to FDA.

II. Materials and Methods

2.1. Sequence retrieval and phylogenetic analysis

Sequences coding for 16S ribosomal RNA of 15 common foodborne pathogens was retrieved from Genbank database, the chosen sequences must have at least 1400bp and do not exist ambigous residues).

The Neighbour-joining method from Clustal W software (Conway Institute UCD Dublin)was used to multiple align and built dendogram in purpose to analysis all conserved and hypervariable regions of 16S ribosomal RNA gene [12,13]. 16S rRNA gene sequence of Chlamydia trachomatis was used as an outgroup [7]. Dendograms was created from full sequences of 16S rRNA and the combinations between V1, V2 and V3; betweenV4 and V5 and between V6, V7 and V8 were be compared to chose the best option for design universal primers and chose specific probes. The V9 region was not chosen bacause its lack of information [7]

2.2. Specific probes selection

From hypervariable regions which have most disimilarities rates, specific probes were chosen from Oligo 7 software (Molecular Biology Insights, Inc.) and also from related researchs [14]. The suitable length of probes was estimated from 20 to 40 bp [15]. Otherparameters including hairprin and self-dimer were analyted by IDT Oligo Analyzer 3.1 tool (https://sg.idtdna.com/calc/analyzer). Chosen specific probes of each target pathogens was tested with all anotherbacteria in Table 1by Annhyb (Bioinformatics Organization) and reconfirmed the specificity throught Blast (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and ProbeMatch (http://rdp.cme.msu.edu/probematch/search.jsp) tools [16, 17].

2.3. Universal primers design

16S ribosomal RNA sequence of*Escherichia* coli O157:H7 (Genbank Accession NR_074891.1) and Oligo 7 software. Forward and Reverse primer was designed in order to anneal with conserved regions which have position at before and after most suitable regions chosen from last experiment. Candidate primers is selected by the method of Kenji Onodera *et al*. (2004) to avoid 3’ end of primers dropping to one of triplets: GGG,, CGT, ATT, CGA, TAA and TTA [18]. Secondary structures of primers including hairprin, self-dimer and hetero dimer were analyzed by IDT Oligo Analyzer 3.1.

III. Results and Discussion

3.1. Phylogenetic analysis

40 sequences retrieved from Genbank Database were chosen to analyze (Table 1). Dendograms built from whole 16S ribosomal RNA gene sequence and from the combinations between V1, V2 and V3; betweenV4 and V5 and between V6, V7 and V8(available download from http://tinyurl.com/mrlqs5d) shown that 16S rRNA gene has abilities to separate among internal 15 common target pathogens, excepts between *Yersinia pseudotuberculosis* and *Y. peptis*; between *Campylpacter jejuni* and *C.coli*; and between species of *Bacillus cereus* group. (including*B. cereus*, *B. anthracis*, *B. mycoides* and *B. thuringiensis*). This results, while consistent with Chakravorty *et al.* (2007), also remarkedly illustrated that the combination of V1, V2 and V3; and the combination of V6, V7 andV8 were the most profitable choices while the combination of V4 and V5 was the less useful target in order to distinghush foodborne pathogens. We decided to choose 2 combinations hypervarible regions, one from V1 to V3 and another from V6 to V9 to select specific probes and compare each others to determine the best option for microarray experiments.

Table I.*List of suitable sequences retrived from GenBank Database*

No.	Species/Genus	GenBank Accession No.
1	*Escherichia coli*	NR_074891.1 (*Escherichia coli* O157:H7), AE014075 (*E. coli* O157:H7), AE005174 (*E. coli* O157:H7), CP001665 (*E. coli* BL21), NR_102804 (*E. coli* K12)
2	*Salmonella*	NR_074934 (*S. paratyphi*), AE014613 (*S. typhi*), CP007422 (*S. enteritidis*)
3	*Shigella*	NR_074882 (*Shigella fWalexneri*), AE014073 (*S. flexneri*), AB855731 (*S. dysenteriae*)
4	*Campylobacter*	L14630, GQ479821 (*Campylobacter jejuni*); NR_121751 (*C. coli*), NR_043603 (*C. curvus*), AF219233 (*C. fetus*)
5	*Yersinia enterocolitica*	CP007448
6	*Yersinia pseudotuberculosis*	NC_006155
7	*Yerisinia pestis*	AE017042
8	*Vibrio cholerae*	NR_074810, KF661544, KF661543
9	*Vibrio parahaemolyticus*	NR_074196, EU660364, EU624428
10	*Vibrio vulnificus*	NR_036888
11	*Listeria monocytogenes*	S55472, M58822
12	*Staphylococcus aureus*	NR_075000, BX571856
13	*Bacillus*	NR_074540 (*Bacillus cereus*), CP008846 (*B. anthracis*), AY373357 (*B. mycoides*), DQ993674 (*B. subtilis*), KJ698649 (*B. thuringiensis*)
14	*Clostridium perfringens*	NR_074482
15	*Clostridium botulinum*	CP002011, X68317, X68187
16	*Chlamydia trachomatis*	NR_025888

3.2. Specific probes selection

35 specific probes was selected (Table II, III), only 5 probes of them was carefully screened from other releated researchs, including Jian-Ming Xing *et al.* (2009), Ho-Seop Eom *et al.* (2007), Yu-Cheng Chiang *et al.* (2006) and Wang et al (2007); Rest of the group was selected from Oligo 7. Therefore, it's clearly seen that most of specific probes using in this study is the new choice, the reason of this trend may be primary caused by the frequently updates and rereviews of bioinfomatics databases, which lead to most of specific probes in related research were no longer suitable for microarray experiments.

There're 18 specific probes were selected from hypervariable regions V1 to V3 could be used to distingush 8 bacteria to species level, including *Clostridium botulinum*, *C. perfringens*, *Escherichia coli*, *Listeria monocytogenes*, *Staphylococcus aureus*, *Vibrio cholerae*, *Vibrio vulnificus* and *Yersinia enterocolitica*.

Other 17 specific probes chosen form from hybervariable regions V6 to V8 have a possibility to differentiate 8 bacteria to species level, consists of *Clostridium botulinum*, *Clostridium perfringens*, *Escherichia coli*, *Listeria monocytogenes*, *Staphylococcus aureus*, *Vibrio cholerae* and *Yersinia enterocolitica*..

Pathogens that could be differntiated to below genus levels but still upper species level were*Bacillus cereus* group, *Campylobacter coli/Campylobacter jejuni* group and *Yersinia pestis/ Yersinia pseudotuberculosis*.

Because of high similarities among closed related species or family group; *Salmonella* spp., *Shigella* spp. could not be detect not only genus but also species level while *Vibrio parahaemolyticus* could not be determined to species levels. This trend was mentioned on Bergey's Manual of Systematic Barteriology (19,20).

The effectof each hypervariable regions on foodborne pathogens microarray detection were decribed on Table IV.

Table II.*Probes selection from V1 to V3 hypervariable regions*

No.	Probe name	Sequence	Target	Length	Annealing position	Hybervariable region
1	BC5	AGTTGAATAAGCTGGCACCTTGAC	*Bacillus cereus group*	24	469	V3
2	BC7	CGTCGCATTAGCTAGTTGGTGAGG	*Bacillus cereus group*	24	245	V2
3	CB1	ACCGCATAATATAAGAGAATCGCA	*Clostridium botulinum*	24	162	V2
4	CJ4	AAGGTATAGTTAATCTGCCCTACACA	*Campylobacter jejuni/ Campylobacter coli*	26	115	V1
5	CJ8	GAGGATGACACTTTTCGGAGCGTA	*Campylobacter* sp.	24	403	V3
6	CP1	ACCGCATAACGTTGAAAGATGGCA	*Clostridium perfringens*	24	163	V2
7	CP5	AAGATAATGACGGTACCCAAGGAG	*Clostridium perfringens*	24	442	V3
8	CP7	GGGAAACGGATTAGCGGCGGACGG	*Clostridium perfringens*	24	77	V1
9	ECO1	CAGATGGGATTAGCTTGTTGGTGGG GTAACGG	*Escherichia coli*	32	234	V2
10	ECO2	AAGTACTTTCAGCGGGGAGGAAGG GAGTAAA	*Escherichia coli*	31	430	V3
11	ECO-Chiang	AGGGAGTAAAGTTAATACCTTTGCT	*Escherichia coli*	25	452	V3
12	LM1	ATACCGAATGATAAAGTGTGGCGC	*Listeria monocytogenes*	24	181	V2
13	STA1	TCACTTATAGATGGATCCGCGCTG	*Staphylococcus aureus*	24	226	V2
14	VC1	CGATGGCTAATACCGCATAACCTC	*Vibrio cholerae*	24	156	V2
15	VC5	GGGCCTTGCGCTACCGGATATG	*Vibrio cholerae*	22	204	V3
16	VIVU-Eom	AAACAAGTTTCTCTGTGCTGCCGC	*Vibrio vulnificus*	24	59	V1
17	YER-Jian	CATAAAGGTTAATAACCTTTGTGA TTGACGT	*Yersinia enterocolitica*	31	448	V3
18	YP2	AGCACTTTCAGCGAGGAGGAAGG GGTTGAGTTTAA	*Yersinia pestis/ Yersinia pseudotuberculosis*	35	432	V3

Table III.*Probes selection from V6 to V8 hypervariable regions*

No.	Probe name	Sequence	Target	Length	Annealing position	Hybervariable region
1	BC3	AGCTAATCTCATAAAACCGTTCTCAGT	*Bacillus cereus group*	27	1284	V8
2	BC9	ACAATGGACGGTACAAAGAGCTGC	*Bacillus cereus group*	24	1245	V8
3	CB2	CCGTGAGGTGGAGCAAAACTTATA	*Clostridium botulinum*	24	1227	V8
4	CB3	TCTAATGAGACTGCCTGGGTAAC	*Clostridium botulinum*	23	1112	V7
5	CB4	ACAATGGTAGGTACAATAAGACGC	*Clostridium botulinum*	24	1199	V7
6	CJ2	ATGGCATATACAATGAGACGCAA	*Campylobacter jejuni/ Campylobater coli*	23	1214	V8
7	CJ3	GCTAGAACTTAGAGACAGGTGC	*Campylobacter jejuni/ Campylobater coli*	22	1006	V6
8	CJ6	ACCTGGGCTTGATATCCTAAGAAC	*Campylobacter jejuni/ Campylobater coli*	24	955	V6
9	CJ7	ATAGAGATATGAGGGTGCTAGCTT	*Campylobacter jejuni*	24	982	V6
10	CP2	TCTAGCGAGACTGCCTGGGTTAA	*Clostridium perfringens*	23	1116	V7

11	ECO3	AGAGATGAGAATGTGCCTTCGGGA	*Escherichia coli*	24	1011	V6
12	LM-Wang	ACTGAGAATAGTTTTATGGGATTAGG	*Listeria monocytogenes*	26	1290	V8
13	STA2	TACAAAGGGCAGCGAAACCGTGAG	*Staphylococcus aureus*	24	1257	V8
14	VC3	ACATCCAGAGAATCTAGCGGAGAC	*Vibrio cholerae*	24	987	V6
15	VC4	AATCTCACAAAGTACGTCGTAGTC	*Vibrio cholerae*	24	1273	V8
16	YER-Wang	TACGACAGACTTTATGTGGTCCGCTTGC	*Yersinia enterocolitica*	28	1266	V8
17	YP1	AGAGATGCTAAAGTGCCTTCGGGA	*Yersinia pestis/ Yersinia pseudotuberculosis*	24	1012	V6

Table IV.*Effect of hybevariable region on discriminate foodborne pathogens; black squares respresented for the regions could used to discriminate target pathigens*

No.	Target	V1	V2	V3	V6	V7	V8
1	*Bacillus cereus group*		■	■			■
2	*Clostridium botulinum*		■			■	■
3	*Clostridium perfringens*	■	■	■		■	
4	*Campylobacter sp.*			■			
5	*Campylobacter jejuni/ Camplylobacter coli*	■			■		■
6	*Campylobacter jejuni*				■		
7	*Escherichia coli*		■	■	■		
8	*Listeria monocytogenes*		■				■
9	*Staphylococcus aureus*		■				■
10	*Vibrio cholerae*		■	■	■		■
11	*Vibrio vulnificus*	■					
12	*Yersinia enterocolitica*			■			■
13	*Yersinia pseudotuberculosis/ Yersinia pestis*			■	■		

Also depending on probes selection results, we estimated that SNPs (single-nucleotide polymorphism) positioning in 982 and 991 of *Camplylobacter jejuni*'s V6 segment (Accession no. L14630) could used to discriminate *C. jejuni* with *C. coli*, *C. curvus*and *C. fetus* whereas SNPs in position 73 and 83 of *Vibrio vulnificus'* V1 segment (Accesion no NR_036888) could be ultilized to distingush *V. vulnificus* with *V. cholerae* and *V. parahaemolyticus*(Figure 1).

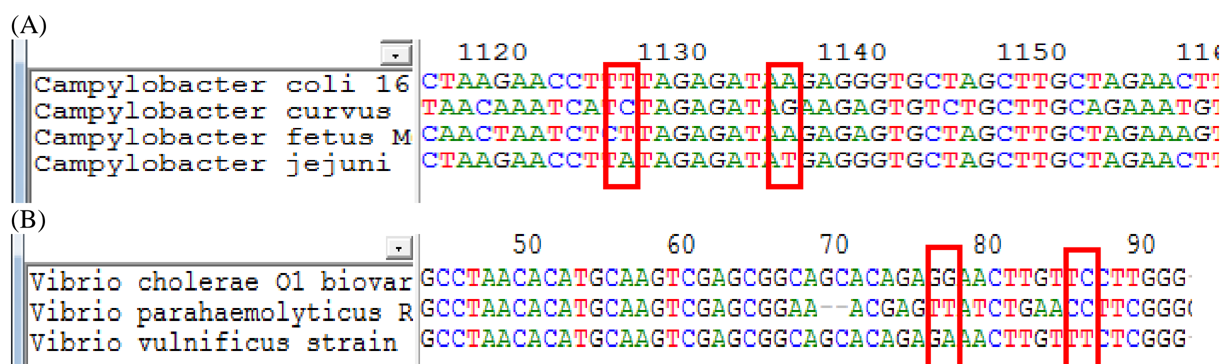

Figure 1. *SNPs differntiated Campylobacter jejuni with C. coli, C. curvus and C. fetus (A;) and Vibrio vulnificus with V. cholerae and V. parahaemoliticus (B).*

3.3. Universal primer design

2 pairs of universal primers was designed to amplify target hypervariable regions. The first universal primers (1F-1R) ampified hybervariable regions from V1 to V3, product length was 561bp and the annealing position of forward and reverse primer was 16 and 556, respectively.

The second universal primers (2F-2R) amplify hypervariable regions from V1 to V3, product length was 720bp and the annealing posiiton of forward and resverse primer was 801 and 1521, respectively.

None of 2 chosen pairs of universal primers have 3'end flop into one of 3 triplets: GGG, CGT, ATT, CGA, TAA and TTA and exsist secondary structure (hairpin, self-dimer and hetero dimer).

Table V. *Primer Design*

No.	Primer name	Sequence	Length	%GC	Tm	Amfly position	Hairpin	Self-dimer	Hetero-dimer	Product length	Ampify regions
1	1F	ATCATGGCTCAGATTGAACGC	21	47.6	60.1	16	0.63	-5.38	-6.21	562	V1-V3
	1R	CGCTTTACGCCCAGTAATTCCG	22	54.5	61.6	556	-0.68	-5.36			
2	2F	TAGTCCACGCCGTAAACGATG	21	52.4	60.5	801	-1.3	-4.95	-4.64	720	V6-V9
	2R	AGGAGGTGATCCAACCGCA	19	61.9	65.9	1521	-1.89	-4.64			

IV. Conclusion

Our study shown that, althought some target pathogens could not be dicriminated to genus or species level due to high similarities between them and closed related species or family groups (*Salmonella*, spp.*Shigella* spp. and *Vibrio parahaemolyticus*), 16S ribosomal RNA gene sequence was suitable enough to differenteriate 9/15 target pathogens to species level (*Clostridium perfringens*, *Clostridium botulinum*, *Campylobacter jejuni*, *Escherichia coli*, *Vibrio cholerae*, *Vibrio vulnificus, Listeria monocytogenes*, *Staphylococcus aureus* and *Yersinia enterocolitica*) and some target pathogens to below genus level but not to species level (*Bacillus cereus* group, *Campylobater jejuni/ Campylobacter coli*group and *Yersinia pestis/ Yersinia pseudotuberculosis*group). The results of this study also support previous researchs in choosing the combination of hybervariable regions respectively from V1 to V3 and from V6 to V8 for identified by sequencing or microarray techniques.

Bioinformatic databases play an important role in specific probes selection. The regularly changes or updates of information in common databases such as GeneBank or Ribosomal Database Project lead to many specific probes from previous researchs is no longer useful. We re-selected 38 specific probes which comfortable with present status of databases; we also re-designed 2 universal probes in order to apply to simultaneous detection of common foodborne pathogens in reality situations.

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