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# The 16S rDNA Gene Sequencing and Specific Probes Designing for the Identification of *Edwardsiella tarda*

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DNA probes for the 16S rRNA have been designed for the detection of *Edwardsiella tarda*. In order to accomplish this purpose, the 16S rRNA gene from *E. tarda* has been cloned and sequenced. Two highly feasible oligonucleotide probe sites have been determined by the database analysis programs presented by PCGENE and BLAST. These two probes have been evaluated by slot blot hybridization analysis. Hetero- and homo-trimeric templates have been synthesized using these two probe sites. The templates have been further multimerized by PCR to generate between 150 and 300 bp long DIG-11-dUTP labeled probes. Unlike 3' end labeled oligonucleotide probes or templates, multimerized probes showed no cross-hybridization in the given experimental condition. Furthermore, a significant increase in sensitivity has been observed with these probes. This method, we presented here, may be useful for the designing of probes for the detection of other fish pathogenic microorganisms also.

Key words: nucleotide sequence, 16S rDNA, specific probes, *Edwardsiella tarda*

## Introduction

Microbial diseases are a serious impediment to commercial fish production, and the research related to these pathogens, especially the diagnostic method, is advancing rapids oligonucleotide probes designed from the rRNA sequences are playing an important role in different areas of detection, identification and quantification of microorganisms (Lane et al., 1985; Mattew and Kricka, 1988; Sayler and Layton, 1990; Amann et al., 1995). Specificity of 16S rRNA probes can generally be freely adjusted by using selected regions within the larger rRNA molecules as hybridization target for synthetic oligonucleotides. Microbial species or subspecies can be distinguished by oligonucleotides complementary to the most variable regions of the molecules, and by targeting regions of increasing conservation, probes can be made to encompass specific genera or higher taxons (Amann et al., 19 95).

For the detection of specific rRNA on the single cell level, various reporter molecules such an radioisotope, non-radioactive system and fluorescent dyes have been utilized (Langer et al., 1981; Amann et al., 1990a; Mansifeld et al., 1995). Subsequently, to improve the sensitivity and applications of detection, oligonucleotide probes covalently bound to multiple fluorescent dye molecules both in hybridizing sequence and non-complementary tail have been synthesized (Wallner et al., 1993). It has also been found that simultaneous application of

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several fluorescently mono-labelled oligonucleotide probes directed at different target sites of one rRNA molecule increased signal intensity additively (Amann et al., 1990b).

*Edwardsiella tarda* is an opportunistic pathogen of many species of animals and it causes serious diseases in at least 15 different species of fish (Bullock and Herman, 1985). And, in the efficient management of *E. tarda* infection in fish under culture condition, rapid and specific identification of the pathogen is inevitable. In this background, the present study has been undertaken to design an effective oligonucleotide probe from 16S rRNA that could be used to distinguish *E. tarda* from other pathogenic bacteria of fish. The present paper deals with the identification of sequence idiosyncrasies, the synthesis and labeling of probes and the experimental evaluation of the specificity and sensitivity of the probes.

## Materials and Methods

### *Bacterial strains*

Four bacterial strains *E. tarda, Vibrio anguillarum, Staphylococcus epidermidis* and *Streptococcus* sp. (National Fisheries Research & Development Agency, Pusan, Korea) were grown in a brain heart infusion (BHI, Difco) supplemented with 0.5% NaCl and 1.5% agar (for solid medium) or tryptic soy broth (TSB, Difco) medium. *E. tarda, S. epidermidis* and *Streptococcus* sp. were grown in BHI medium at 37℃ while *V. anguillarum* was grown in TSB medium at 30℃.

### *Isolation, extraction, and analysis of 16S rRNA gene*

Chromosomal DNA of each bacterial strain was isolated by the method described by Marmur (1961). Cell pellets, grown until reaching $OD_{600}=4.0$, were recovered by centrifugation from the cultures, and resuspended in SET buffer (75 mM NaCl, 25 mM EDTA, 20 mM Tris, pH 7.5). Bacterial cell wall was removed by incubation with 2.0 mg/ml lysozyme at 37℃ for 5 min. The cell lysates were gently mixed with 1/10 volume of 10% SDS, 1/3 volume of 5 M NaCl, one volume of chloroform and supernatant was recovered immediately after centrifugation. After ethanol precipitation, pellet was dissolved in TE buffer and incubated in 1/10 volume of Proteinase K (0.5 mg/ml) (Boehringer Mannheim. USA) and RNase A (Sigma, USA) mixture for 15 min at 37℃. Pure chromosomal DNA was recovered by the phenol-chloroform treatment followed by ethanol precipitation.

Two oligonucleotide primers 16A and 16GX (Table 1) were synthesized for the cloning of 16S rDNA of *E. tarda* by PCR (FINE PCR, Korea) amplification. Amplification was conducted in a total volume of 50 $\mu\ell$ containing primer (100 pmole), chromosomal DNA (100 ng), 5 $\mu\ell$ of 10 X *Taq* DNA polymerase reaction buffer (100 mM Tris pH 8.3, 400 mM KCl, 15 mM $MgCl_2$, 10 mM DTT, 500 $\mu g/m\ell$ BSA) and 4 $\mu\ell$ of 2.5 mM dNTP. After heat denaturation at 94℃ for 10 min, 1 $\mu\ell$ (1 unit) of *Taq* DNA polymerase was added to the reaction mixture overlaid with 30 $\mu\ell$ of mineral oil (Sigma, USA) and amplified for 30 cycles at 94℃ for 2 min and annealing at 72℃ for 2 min.

The final product was confirmed by 1.0% agarose gel electrophoresis and subcloned into pUC 18 *PstI-EcoRI* and *EcoRI-EcoRI* sites after digestion with *EcoRI*. Sequencing of each subclone was performed by single strand DNA sequencing method with Pharmacia Automatic DNA Sequencer (ABI PRISM 373 DNA sequencer, Perkin Elmer, USA).

### *Multimerization of oligonucleotide probes*

Amplification and multimerization were conducted in separate PCR reactions. The first PCR reaction was conducted by mixing primers and templates at a ratio of 500 to 1 ratio and then amplifying for 30 cycles with one minute denaturation at 94℃ and 2 min annealing at 54℃ without DIG-dUTP. The second PCR reaction was conducted with the following changes: Template was substituted by 1 $\mu\ell$ of the first PCR reaction mixture; DIG-dUTP and primers were added in the reaction was carried out at the selected annealing temperatures. The multimerized product was analyzed by 1.5% agarose gel electrophoresis.

### *Labeling and slot blot hybridization*

Oligonucleotides and templates were labeled at the 3′ end with DIG-11-ddUTP by DIG oligonucleotide 3′-end labeling kit (Boehringer

Table 1. Primers and oligonucleotide probes used for the studies

| Name | Sequence (5′~3′) | purpose | Location |
|---|---|---|---|
| 16 A | GGCTGCAGAACACATGCAAGTCGAACGGT | 16S rDNA amplification | 50~70 |
| 16 GX | GGCTTAAGTGTTCCGGGCCCTTGCATAAG | 16S rDNA amplification | 1374~1394 |
| Bact.[1)] | GCTGCCTCCCGTAGGAGT | Universal 16S rDNA probe | 338~356 |
| E-1 | CAGCGGAGAAAGCAAGCTTTCTCCCT | *E. tarda* detection | 54~81 |
| E-2 | ATTGTGAGCGCTATTAACGT | *E. tarda* detection | 414~438 |

[1)] Bact. is the universial probe for the detection of eubactria (Martinez et al., 1994)

Mannheim, Germany). Labeling mixture contains 100 pmole of oligonucleotide or template DNA, 4 $\mu\ell$ of 5X reaction buffer (1 M potassium cacodylate, 125 mM Tris-HCl, 1.25 mg/ml bovine serum albumin, pH 6.6), 4 $\mu\ell$ of 25 mM $CoCl_2$, 1 $\mu\ell$ of 1 mM DIG-ddUTP and 2.5 unit of terminal transferase in a 25 $\mu\ell$ reaction mixture. Reaction was conducted at 37℃ for 15 min and stopted by adding 1 $\mu\ell$ of 200 mM EDTA. PCR DIG Probe Synthesis Kit (Boehringer Mannheim, USA) was used for the labelling of multimerization during PCR. For the labeling PCR DIG probe synthesis mix (2 mM dATP, 2 mM dCTP, 2 mM dGTP, 1.3 mM dTTP, 0.7 mM-alkali-labile DIG-11-dUTP, pH 7.0) was used instead of 2.5 mM dNTP mix for the amplification.

Slot blot hybridigation was carried out as per the procedure outlined by Dyson (1991). 0.5 $\mu$g of chromosomal DNA (10 $\mu\ell$) was loaded to each slot on the positive charged membrane (Hybond N. Amersham International. Little Chalfont, UK) using slot blot hybridization apparatus (Hoefer Scientific Instruments. San Francisco. Calif, USA). DIG Easy Hyb (20 ml/100 $cm^2$ of filter, Boehringer Mannheim, Germany) was used for 30 min of pre-hybridization at 42℃ followed by 3 h hybridization in the hybridization chamber (HB-1D Hybridiser, Techne, USA) at the same temperature. About 5 pmole (1 $\mu\ell$) of labeled probes were added to the 2 ml of hybridization solution. After hybridization, membrane was equilibrated in the washing buffer (10mM maleic acid, 15 mM NaCl, 0.03% (v/v) Tween 20, pH 7.5) for 1 min. Membrane was further incubated at room temperature for 60 min in blocking solution with Anti-Dig-Ap (1 : 10,000 dilution) and washed twice for 15 min each. After washing, membrane was placed in 1 : 100 diluted CSPD (Boehringer Mannheim, Germany) in detection buffer (10mM Tris-HCl, 10 mM NaCl, pH 9.5) for 5 min and exposed to x-ray film (Amersham, USA) for 30 min.

## Results and Discussion

### *Sequence analysis and oligonucleotide probe design for E. tarda 16S rDNA*

An amplified product was observed after electrophoresis. This amplified band was further confirmed by *EcoRI* digestion because most of eubacterial 16S rDNAs had conserved *EcoRI* digestion site in the middle of its sequences. As expected, about 600 bp and 700 bp of *EcoRI* digested fragments were observed, which had *PstI-EcoRI* and *EcoRI-EcoRI* restriction endonuclease sites for the further subcloning. The partial DNA sequence (1346 bp) of *E. tarda* 16S rRNA (#AF 053975) was determined from these subclones (Fig. 1). Out of the 1,295 bp sequenced, nine mismatched base pairs were found by comparing these two strains.

Two most unique oligonucleotides probe region E-1 and E-2 were determined using computer database (PCGENE and BLAST)(Table 1). Probe E-1 showed similarity with the rDNA of *Hameophilas* sp., *Pasteurella aerogenes, Pasteurella dagmatis, Pasteurella multocida* but E-2 did not show any similarity in the these bacteria.

Six out of the eight mismatched base pairs between two strains of *E. tarda* were located in the 410~430 bp region. This region was overlapped with the oligonucleotide probe E-2 (Fig. 1) which included the 6 bp mismatches between two strains out of a total 26 base synthesized. Two biotypes of *E. tarda* exist in nature, which is known as the wild-type and biogroup I (Janda and Abbott, 1993).

```
                10                  20                  30                  40                  50                  60
                :                   :                   :                   :                   :                   :
1   AACACATGCA  AGTCGAACGG  TAGCAGGGAG  AAAGCTTGCT  TTCTCCGCTG  ACGAGCGGCG
                                                        E-1
61  GACGGGTGAG  TAATGTCTGG  GGATCTGCCT  GATGGAGGGG  GATAACTACT  GGAAACGGTA
121 GCTAATACCG  CATAACGTCG  CAAGACCAAA  GTGGGGGACC  TTCGGGCCTC  ATGCCATCAG
181 ATGAACCCAG  ATGGGATTAG  CTAGTAGGTG  GGGTAATGGC  TCACCTAGGC  GACGATCCCT
                                        A
241 AGCTGGTCTG  AGAGGATGAC  CAGCCACACT  GGAACTGAGA  CACGGTCCAN  ACTCCTACGG
301 GAGGCAGCAG  TGGGGAATAT  TGCACAATGG  GCGCAAGCCT  GATGCAGCCA  TGCCGCGTGT
361 ATGAAGAAGG  CCTTCGGGTT  GTAAAGTACT  TTCAGTAGGG  AGGAAGGTGT  GAACGTTAAT
                                                                CGT E-2
421 AGCGCTCACA  ATTGACGTTA  CCTACAGAAG  AAGCACCGGC  TAACTCCGTG  CCAGCAGCCG
      A GT
481 CGGTAATACG  GAGGGTGCAA  GCGTTAATCG  GAATTACTGG  GCGTAAAGCG  CACGCAGGCG
541 GTTTGTTAAG  TTGGATGTGA  AATCCCCGGG  CTTAACCTGG  GAACTGCATC  CAAGACTGGC
601 AAGCTAGAGT  CTCGTAGAGG  GAGGTAGAAT  TCCAGGTGTA  GCGGTGAAAT  GCGTAGAGAT
661 CTGGAGGAAT  ACCGGTGGCG  AAGGCGGCCT  CCTGGACGAA  GACTGACGCT  CAGGTGCGAA
721 AGCGTGGGGA  GCAAACAGGA  TTAGATACCC  TGGTAGTCCA  CGCTGTAAAC  GATGTCGATT
781 TGGAGGTTGT  GCCCTTGAGG  CGTGGCTTCC  GAAGCTAACG  CGTTAAATCG  ACCGCCTGGG
841 GAGTACGGCC  GCAAGGTTAA  AACTCAAATG  AATTGACGGG  GGCCCGCACA  AGCGGTGGAG
901 CATGTGGTTT  AATTCGATGC  AACGCGAAGA  ACCTTACCTA  CTCTTGACAT  CCAGCGAATC
961 CTGTAGAGAT  ACGGGAGTGC  CTTCGGGAAC  GCTGAGACAG  GTGCTGCATG  GCTGTCGTCA
1021 GCTCGTGTTG  TGAAATGTTG  GGTTAAGTCC  CGCAACGAGC  GCAACCCTTA  TCCTTTGTTG
1081 CCAGCGGTTC  GGCCGGGAAC  TCAAAGGAGA  CTGCCAGTGA  TAAACTGGAG  GAAGGTGGGG
1141 ATGACGTCAA  GTCATCATGG  CCCTTACGAG  TAGGGCTACA  CACGTGCTAC  AATGGCGTAT
1201 ACAAAGAGAA  GCGAACTCGC  GAGAGCAAGC  GGACCTCATA  AAGTACGTCG  TAGTCCGGAT
                    C
1261 TGGAGTCTGC  AACTCGACTC  CATGAAGTCG  GAATCGCTAG  TAATCGTGGA  TCAGAATGCC
1321 ACGGTGAATA  CGTTCCCGGG  CCTTGT
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Fig. 1. Partial nucleotide sequences (1346bp) of E. tarda 16S rDNA (#AF053975). Italic characters are selected probe sites for E-1 and E-2. The sequence differences between two strains (GeneBank Acession #AF053975 and #AF015759) are indicated at the bottom of each bases.

However, we do not have information whether these two strains have any morphological or pathological differences. If they possess any difference, probe E-2 could be used as a strain specific as well as a species specific oligonucleotide probe.

### *Evaluation of oligonucleotide and multimeric probes*

The selected 3'-end DIG labeled oligonucleotide probes E-1 and E-2 were evaluated by slot blot hybridization (Fig. 2). Chromosomal DNA of five bacterial strains including *E. tarda* were loaded in equal volume on the slot and specificity of the probes was tested by hybridization analysis. The probe E-2 showed better specificity compared to probe E-1 which had cross-hybridized with *V. anguillarum*, and *Streptococcus* sp.. For the multimerization of oligonucleotides probe E1 and

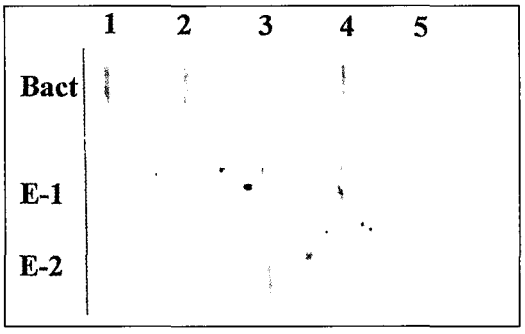


Fig. 2. Slot blot hybridization with 3' end ddUTP-DIG labelled oligonucleotide probes E-1 and E-2. 1. *E. coli*, 2. *V. anguillarum*, 3. *E. tarda*, 4. *Streptococcus* sp., and 5. *S. epidermidis*. (Universial oligonucleotide probe (Bact) was used as control).

E2, hetero-trimeric template E1-E2-E1 (A), and homo-trimeric template E1-E1-E1 (B) and E2-E2-E2 (C) were synthesized (Fig. 3). These templates

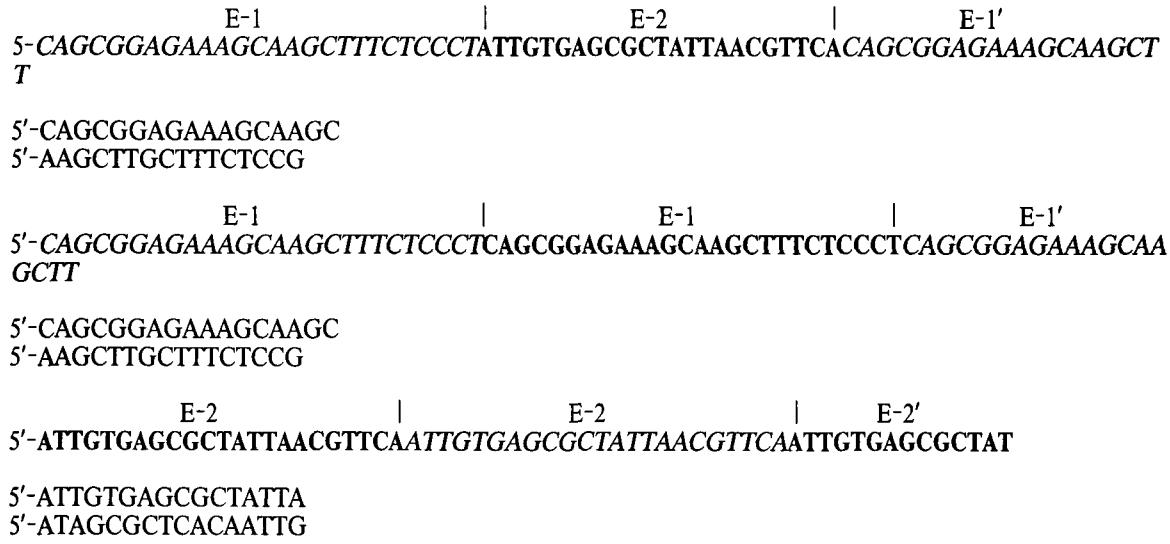


Fig. 3. Templates A, B, and C designed from oligonucleotide probes and their primers for multimerization by PCR. E-1′ and E-2′ indicate the partial sequences of E-1 and E-2.

were labeled with DIG at the 3′-end and hybridized with chromosomal DNA isolated from five bacterial strains including *E. tarda* (Fig. 4B). The sensitivities of all these 3′ end-labeled trimeric template probes, A, B and C were found to increase in comparison to the oligonucleotide probes under the same hybridization condition. However, the improvement in specificity of template probes was not significant.

### *Multimerization by PCR*

The templates were further amplified and multimerized by PCR with proper primers. The PCR reactions were performed twice; in the first PCR, templates were amplified with higher primer to template ratio without label in the reaction mixture to amplify double strands trimeric templates. In the second PCR, reaction was performed with a lower primer to template ratio to amplify and multimerize the templates at the same time. Multimerization of template could be accelerated by self annealing between sense and antisense strands with limited primer concentration in the reaction mixture. Optimal annealing temperatures for multimerization and amplification of template, A, B and C were observed around 54℃ in the second PCR. At this annealing temperature, the templates were multimerized to an average size of 400 bp (Fig. 4A). It was reported that about 300 base polynucleotide probes could penetrate efficiently into the fixed gram negative bacterial cell by *in situ* hybridization (Trebesius, 1994) or by colony hybridization (Baez and Juneja, 1995). Therefore, the prepared probes could be used for rapid identification by *in situ* single cell detection as well as for slot blot hybridization.

Multimeric probes A′, B′ and C′ generated by PCR were evaluated by comparing their corresponding 3′ end labelled template probes, A, B and C (Fig. 4B). It was observed that multimeric probes showed improved specificity and sensitivity. The sensitivity of oligonucleotide probes depended on its size and the number of label attached. It was reported that the sensitivity of oligonucleotides probes could be improved up to 30 fold by generating repeated unit through ligation (Longmire and Ratliff, 1994). Also, dramatic increase in sensitivity of probes labeled by random priming could be achieved through amplification of the tandem repeated regions of mucin gene by PCR (Trendellenburg and Hanski, 1997). For a DIG-UTP/UTP ratio of 6.5 : 3.5, the labeling efficiency is one DIG-UTP per every 25 base in the reaction mixture of *in vitro* transcription (Trebessius et al., 1994). Therefore, if it is assumed that the labelling efficiency is same during the PCR reaction, and about 15 labels could be conferred to each average size multimerized probes (400 bp). Hetero-multimeric probe A′ and homo-multimeric probe B′

A)


B)

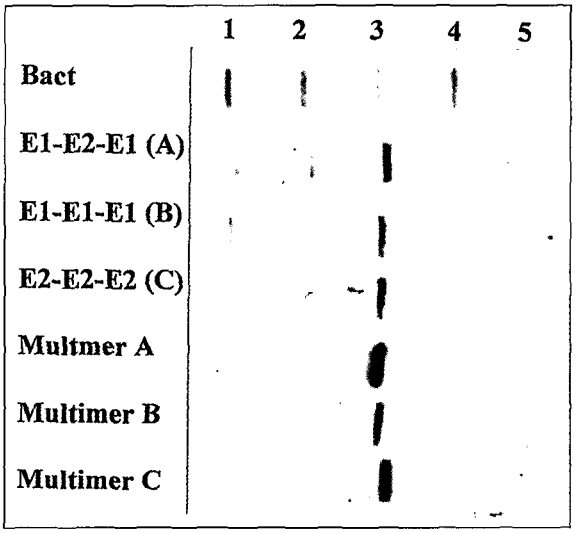


Fig. 4. A) Gel electrophoresis (1.5%) of multimerized products (A', B' and C') after second PCR with templates A, B and C. Optimal multimerization during polymerization were determined at various annealing temperatures. Template A and C were multimerized at 54℃ while template B was multimerized at 55℃.
B) Slot blot hybridization of chromosomal DNA of 1. *E. coli*, 2. *V. anguillarum*, 3. *E. tarda*, 4. *Streptococcus* sp., and 5. *S. epidermidis* with 3' end ddUTP-DIG labelled templates (A, B, and C) or dUTP-DIG labelled multimeric probes A', B' and C'.

showed dramatic improvement in specificity and sensitivity compared to template probe A or oligonucleotide probe E-1. Similar improvement in specificity was observed with homo-multimeric probe B' compared to its corresponding templates and oligonucleotide probes.

However, multi-labelling of probes does not explain the improvement of specificity which could especially be observed in probes A' and B'. Recently, it was reported that hybridization results depend not only in the binding of a probe, but also in the formation of extended polymeric network by multiple oligonucleotide sequences (Elghanian and Storhoff, 1997). Also, mismatched bases in a longer multimerized probes give more destabilizing effect than a mismatch in a oligonucleotide under same hybridization condition. Therefore, it is more likely that multimer A' and B' will have more destabilizing effect during hybridization with mismatched sites than template probe templates or oligonucleotide probes. In addition, the multimerized probes could be stabilized after hybridization by network formation between repeated sequence of negative and positive strands. If this is the case, rRNA which ranges from thousands to millions of molecules per cell is the ideal target for hybridization using the multimerized probes.

The technique developed in this study might be useful in designing rRNA based probe design where the species specific sites for the probe design are limited because of highly conserved sequence between species. Also, the method presented here allows the mass production of multiple non-radioisotope labeled multimeric probes. Therefore, this probe design technique might be useful for commercial application as a kit for the detection of other pathogenic microorganism also.

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